HORMONES AND REPRODUCTION OF VERTEBRATES

Volume 2

Amphibians

Editors
David O. Norris and Kristin H. Lopez

Hormones and Reproduction of Vertebrates

Hormones and Reproduction of Vertebrates, Volume 1 — Fishes
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Volume 2: Amphibians

David O. Norris
Department of Integrative Physiology
University of Colorado
Boulder, Colorado

Kristin H. Lopez
Department of Integrative Physiology
University of Colorado
Boulder, Colorado

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Dedication

Richard Evan Jones

This series of five volumes on the hormones and reproduction of vertebrates is appropriately dedicated to our friend and colleague of many years, Professor Emeritus Richard Evan Jones, who inspired us to undertake this project. Dick spent his professional life as a truly comparative reproductive endocrinologist who published many papers on hormones and reproduction in fishes, amphibians, reptiles, birds, and mammals. Additionally, he published a number of important books including *The Ovary* (Jones, 1975, Plenum Press), *Hormones and Reproduction in Fishes, Amphibians, and Reptiles* (Norris and Jones, 1987, Plenum Press), and a textbook, *Human Reproductive Biology* (Jones & Lopez, 3rd edition 2006, Academic Press). Throughout his productive career he consistently stressed the importance of an evolutionary perspective to understanding reproduction and reproductive endocrinology. His enthusiasm for these subjects inspired all with whom he interacted, especially the many graduate students he fostered, including a number of those who have contributed to these volumes.

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Preface

Hormones and Reproduction of Vertebrates Preface to the Series

Every aspect of our physiology and behavior is either regulated directly by hormones or modified by their actions, as exemplified by the essential and diverse roles of hormones in reproductive processes. Central to the evolutionary success of all vertebrates are the regulatory chemicals secreted by cells that control sexual determination, sexual differentiation, sexual maturation, reproductive physiology, and reproductive behavior. To understand these processes and their evolution in vertebrates, it is necessary to employ an integrated approach that combines our knowledge of endocrine systems, genetics and evolution, and environmental factors in a comparative manner. In addition to providing insight into the evolution and physiology of vertebrates, the study of comparative vertebrate reproduction has had a considerable impact on the biomedical sciences and has provided a useful array of model systems for investigations that are of fundamental importance to human health. The purpose of this series on the hormones and reproduction of vertebrates is to bring together our current knowledge of comparative reproductive endocrinology in one place as a resource for scientists involved in reproductive endocrinology and for students who are just becoming interested in this field.

In this series of five volumes, we have selected authors with broad perspectives on reproductive endocrinology from a dozen countries. These authors are especially knowledgeable in their specific areas of interest and are familiar with both the historical aspects of their fields and the cutting edge of today's research. We have intentionally included many younger scientists in an effort to bring in fresh viewpoints. Topics in each volume include sex determination, neuroendocrine regulation of the hypothalamus–pituitary–gonadal (HPG) axis, separate discussions of testicular and ovarian functions and control, stress and reproductive function, hormones and reproductive behaviors, and comparisons of reproductive patterns. Emphasis on the use of model species is balanced throughout the series with comparative treatments of reproductive cycles in major taxa.

Chemical pollution and climate change pose serious challenges to the conservation and reproductive health of wildlife populations and humans in the twenty-first century, and these issues must be part of our modern perspective on reproduction. Consequently, we have included chapters that specifically deal with the accumulation of endocrine-disrupting chemicals (EDCs) in the environment at very low concentrations that mimic or block the critical functions of our reproductive hormones. Many authors throughout the series also have provided information connecting reproductive endocrinology to species conservation.

The series consists of five volumes, each of which deals with a major traditional grouping of vertebrates: in volume order, fishes, amphibians, reptiles, birds, and mammals. Each volume is organized in a similar manner so that themes can be easily followed across volumes. Terminology and abbreviations have been standardized by the editors to reflect the more common usage by scientists working with this diverse assembly of organisms we identify as vertebrates. Additionally, we have provided indices that allow readers to locate terms of interest, chemicals of interest, and particular species. A glossary of abbreviations used is provided with each chapter.

Finally, we must thank the many contributors to this work for their willingness to share their expertise, for their timely and thoughtful submissions, and for their patience with our interventions and requests for revisions. Their chapters cite the work of innumerable reproductive biologists and endocrinologists whose efforts have contributed to this rich and rewarding literature. And, of course, our special thanks go to our editor, Patricia Gonzalez of Academic Press, for her help with keeping us all on track and overseeing the incorporation of these valuable contributions into the work.

David O. Norris

Kristin H. Lopez

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Preface to Volume 2 Amphibians

This volume provides a background in the development and function of the reproductive system of amphibian vertebrates with an emphasis on the roles of bioregulators (hormones, pheromones, etc.) in directing the formation and activities of the hypothalamus–pituitary–gonadal (HPG) axis. Where possible, the topics have been arranged similarly to the other volumes in this series to facilitate the efforts of readers looking for comparative data on reproductive processes. A discussion of the diversity of mechanisms involved in amphibian sex differentiation is followed by chapters describing the morphology, physiology, and hormonal regulation of the gonads and sex accessory structures. Maternal adaptation has been selected as a special topic for amphibians due to its evolutionary importance and its roles in the diverse reproductive modes exhibited by this vertebrate group. The interaction of the hypothalamus–pituitary–adrenal (HPA) axis with the HPG axis is explored in the impacts of stress on reproduction. The role of hormones and pheromones in reproductive behavior is another special focus. The functions of hormones as well as environmental cues in reproductive cycles are described for the major amphibian groups (Anura: frogs and toads; Urodela: newts and salamanders; Gymnophiona: caecilians). Finally, there is a chapter dealing with the impacts of environmental chemicals that function as endocrine disruptors of reproduction in amphibians, which should be of interest to conservationists, ecologists, and toxicologists as well as reproductive physiologists.

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Contributors

Stéphane Flament, Dominique Chardard, Amand Chesnel, and Hélène Dumond, Nancy-Université, Université Henri Poincaré, Vandoeuvre-lès-Nancy, France

Pei-San Tsai, University of Colorado at Boulder, Boulder, CO, USA

Catherine R. Propper, Northern Arizona University, Flagstaff, AZ, USA

Mari Carmen Uribe Aranzábal, Universidad Nacional Autońoma de México, Ciudad de México, Mexico

David M. Sever, Southeastern Louisiana University, Hammond, LA, USA

Nancy L. Staub, Gonzaga University, Spokane, WA, USA

James A. Carr, Texas Tech University, Lubbock, TX, USA

Hartmut Greven, Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany

Sarah K. Woodley, Duquesne University, Pittsburgh, PA, USA

Rakesh K. Rastogi, Gianluca Polese, and Biagio D'Aniello, University of Naples Federico II, Napoli, Italy

Claudia Pinelli, and Gabriella Chieffi-Baccari, Second University of Naples, Caserta, Italy

David O. Norris, University of Colorado at Boulder, Boulder, CO, USA

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Chapter 1

Sex Determination and Sexual Differentiation in Amphibians

Stéphane Flament, Dominique Chardard, Amand Chesnel, and Hélène Dumond

Nancy-Université, Université Henri Poincaré, Vandoeuvre-lès-Nancy, France

SUMMARY

We review here sex determination and differentiation in amphibians. Many of the observations and experimental results are available for both the Anura and Caudata orders whereas the literature is very poor regarding Gymnophiona. We describe genetic sex determination that is associated with sex chromosomes that display various patterns, from homomorphism to heteromorphism. Gonadal differentiation is also discussed. The general features leading the undifferentiated gonad to differentiate as an ovary or a testis appear to be similar in anurans and caudates, but the origin of germ cells is very different. We also focus on sensitivity to temperature, a factor that can induce sex reversal in several species by modulating steroid hormone synthesis. These molecules are indeed major players in the sex differentiation process. The recent discovery of a sex-determining gene (DM-W) in *Xenopus laevis* could increase our knowledge of the sex determination/differentiation process in the near future.

1. INTRODUCTION

The class Amphibia is comprised of three orders: Anura (Salientia), Caudata (Urodela), and Gymnophiona (Apoda).

The order Anura (more than 4500 species) includes frogs and toads grouped into approximately thirty families, of which Leptodactylidae, Hylidae, and Ranidae are the largest. Salamanders, newts, sirens, amphiuma, waterdogs, and mudpuppies comprise the order Caudata. There are ten living families grouped into three suborders: Cryptobranchoidea, Salamandroidea, and Sirenoidea. The largest family is the Plethodontidae (lungless salamanders), found almost exclusively in the Americas, which comprises more than half of all known caudates (500 species). The order Gymnophiona is the least studied and consists of caecilians that resemble giant earthworms rather than typical amphibians.

Although there are several exceptions, most amphibians are biphasic: they go through an aquatic stage and a terrestrial stage at some point in life. Although a few species are viviparous, most species produce eggs surrounded by jelly layers that are deposited in water. Larvae hatch from these protective translucent envelopes and, after a period of aquatic life, they metamorphose into terrestrial semi-aquatic adults. Some species are neotenic: they do not metamorphose or do so incompletely, they retain larval characteristics into adulthood, and reproduce in a larval or semi-larval state.

Due to their development being realized autonomously in water, amphibians have been used extensively to study developmental biology, with the well-known African clawed frog *Xenopus laevis* as an experimental model. The present chapter reviews recent data concerning an important event occurring during development: sex determination and differentiation. We focus on the sensitivity of these processes to environmental factors, especially temperature, a factor that can induce sex reversal. Finally, we describe the effects of steroid hormones that appear as major players, as well as the genes involved in these mechanisms.

2. SEX DETERMINATION

In amphibians, sex can be genetically determined. Among species using genetic sex determination, some are male heterogametic (the male is heterozygous at a sex-determining locus) while others are female heterogametic. Some species have reinforced this allelic difference by differentiating sex chromosomes. However, only approximately 4% of the amphibians karyotyped have cytologically differentiated sex chromosomes (Hillis & Green, 1990; Hayes, 1998; Schmid & Steinlein, 2001). Indeed, in most cases, sex chromosomes are homomorphic; i.e., they are morphologically undifferentiated when studied with classical cytogenetic methods. Hence, the identification of the heterogametic sex is often deduced from other approaches.

2.1. Sex Chromosomes

Embryonic gonad transplantations were used in classical experiments on the American salamanders *Ambystoma mexicanum* and *Ambystoma tigrinum* (Humphrey, 1942). In tail-bud embryos, the primordium of the ovary from one side of the body was replaced by the primordium of a testis taken from a genetically male embryo. Because at this early stage the sex of the individual cannot be recognized, the right combination occurred in 25% of transplantations. During the further development of the embryo, the transplanted testis hormonally modified the ovary into a functional testis. As soon as sex reversal was achieved, the transplanted testis was removed. Thus, the sex-reversed female possessed a testis producing spermatozoa from genetically female germ cells. At adulthood, when these sex-reversed animals were mated with normal females, their progeny contained 25% males. This result indicates that the *Ambystoma* female is the heterogametic sex (ZW) (Figure 1.1(a)).

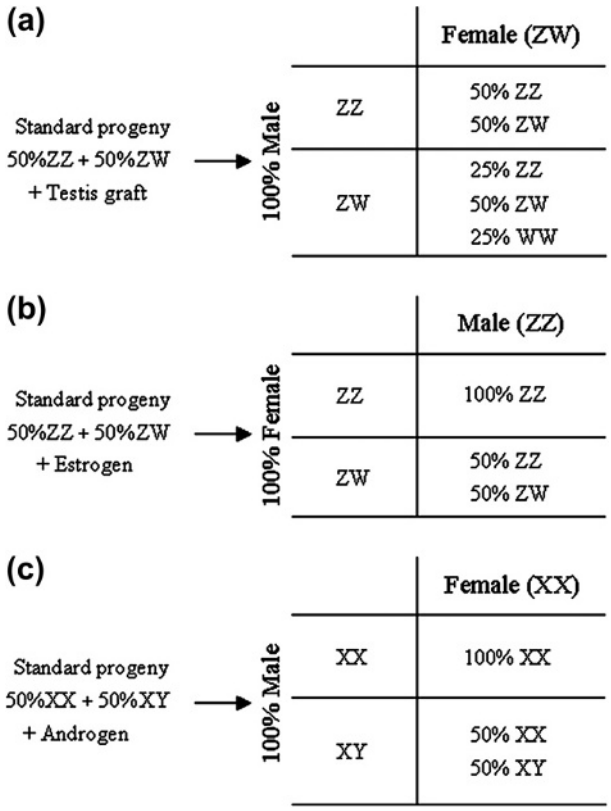

FIGURE 1.1 Theoretical percentages of genotypes in progenies from crosses between standard and sex-reversed individuals. (a) In case of female heterogamety, with female to male sex reversal obtained after masculinization of host gonad with testis graft. (b) In case of female heterogamety, with male to female sex reversal obtained after estrogen treatment. (c) In case of male heterogamety, with female to male sex reversal obtained after androgen treatment.

In other amphibians such as the urodele *Pleurodeles waltl* (Gallien, 1951) or the anuran *X. laevis* (Chang & Witchi, 1955), sex reversal was obtained by treating larvae with estradiol (E_2). The larvae developed as functional females whatever their sexual genotype. Half of them, when crossed with normal males, produced all-male progenies, demonstrating female heterogamety (ZW) (Figure 1.1(b)).

Breeding experiments and analysis of the progeny of sex-reversed females have shown a male heterogamety (XY) in some species (e.g., *Rana japonica*, *Rana rugosa*, *Hyla arborea japonica*, *Bombina orientalis*) (Schmid & Steinlein, 2001) (Figure 1.1(c)).

The microscopic observation of lampbrush chromosomes from prophase I-arrested oocytes could be used to confirm the presence of ZZ/ZW sex chromosomes (bivalent IV) in *P. waltl* (Lacroix, 1968a; 1968b). In that species, at ambient temperature, heteromorphic loops in the ZW bivalent are not visible under a phase contrast microscope, but are revealed by *in-situ* protein immunodetection with antibodies (Lacroix et al., 1985) or *in-situ* hybridization with RNA probes (Penrad-Mobayed, Moreau, & Angelier, 1998). These heteromorphic RNA-labeled loops constitute a specific marker of the differential segment of the sex chromosomes. The RNA sequence interacts with lampbrush loop-associated proteins. This interaction is thermosensitive: treatment of adult animals for 18 hours at 34°C reduces the labeling signals, whereas treatment for seven days at 8°C enhances the labeling signals. The identity of these sex-specific RNA-binding proteins is still unknown. Using such induced loops as markers, one trisomic female for bivalent IV (ZZW) was identified, confirming the major role of the W chromosome in the mechanism of female sex determination in this species (Lacroix et al., 1990).

A similar situation was described in *Pleurodeles poireti*; at ambient temperature, the W chromosome of the bivalent IV has also been distinguished by a set of lampbrush loops displaying distinct morphology under a phase contrast microscope (Lacroix, 1970).

The European treefrog *Hyla arborea* has homomorphic sex chromosomes, but male heterogamety was recently revealed by the sex-specific pattern of a microsatellite-like marker (Berset-Brandly, Jaquiery, & Perrin, 2006). At locus Ha5-22, all females investigated were homozygous for allele 235 and all males were heterozygous for alleles 235 and 241, pointing to a location within the non-recombining region of nascent sex chromosomes, with allele 235 fixed on the proto X and allele 241 on the proto Y. The successful amplification of Ha5-22 in several hylid species as well as the nature of its tandem repeat suggested a location within the coding region of a conserved gene. It was recently identified as the homolog of Med15, a key component of the mediator coactivator complex (Niculita-Hirzel,

Stöck, & Perrin, 2008). The X and Y alleles differ by three frame-preserving indels (eight amino acids in total) within their glutamine-rich central part. These differences have the potential to affect the conformation of the mediator complex and to activate genes in a sex-specific way. Thus, they might represent the first steps toward the acquisition of a male-specific function.

The cell membrane-associated HY antigen or a cross-reactive antigen is present in the gonad of the heterogametic sex (XY or ZW) in all vertebrates so far studied, including amphibians. It was used to determine the heterogametic sex in several species with homomorphic sex chromosomes, including *X. laevis*, *Bufo bufo*, *Pyxicephalus adspersus*, *Rana ridibunda*, *Pelodytes punctatus*, *P. waltl*, *A. mexicanum*, and *Triturus vulgaris* (for reviews see Schmid & Steinlein, 2001; Eggert, 2004).

Incorporation of the thymidine analog bromodeoxyuridine into the replication banding was used in the European water frog *Rana esculenta* to identify the XY chromosomes (pair number four) that are still in a primitive stage of morphological differentiation (Schempp & Schmid, 1981). Males have a late replicating band in the long arm of the Y chromosome that is lacking in the X. It has been proposed that this late replicating band consists of repetitive DNA since its replication begins later than other regions.

In some cases, sex chromosomes can only be identified by specific staining of their heterochromatin. For instance, heterochromatic telomeres are present in the long arms of only one of the homologs (Y) of the pair number four in *Triturus alpestris* and *T. vulgaris* (Schmid, Olert, & Klett, 1979). The mitotic karyotypes of *Triturus cristatus carnifex* and *Triturus cristatus cristatus* are very similar and display distinct sex chromosomes (fourth pair). Both the X and Y chromosomes are submetacentric. The X chromosome carries a faint C-band in the middle of the short arm and a subterminal C-band on the long arm. The Y chromosome carries these two C-bands and an additional one on the long arm.

In other salamanders of the genus *Hydromantes*, the X chromosome is acrocentric while the Y is submetacentric. In the X chromosome, heterochromatin is present in the long arm and at the centromeric region, whereas in the Y it is found in the centromeric region and in the short arm (Nardi, Andronico, De Lucchini, & Batistoni, 1986).

Highly heteromorphic sex chromosomes are present in some amphibians. The first example was found in the South African bullfrog *P. adspersus* (Schmid, 1980). Females are heterogametic and the W chromosome is smaller than the Z and its short arm is completely heterochromatic. The American salamanders of the genus *Necturus* have the most highly differentiated XY chromosomes yet discovered in the caudate amphibians (Sessions & Wiley, 1985). The Y chromosomes are about one quarter the size of the X chromosomes and almost completely constituted of heterochromatin. In contrast, the Y chromosome of the South American marsupial frog *Gastrotheca riobambae* is larger than the X; this is a very rare situation among vertebrates (Schmid, Haaf, Giele, & Sims, 1983).

Amphibians provide a unique example of population variation in male and female heterogamety: the Japanese frog *R. rugosa* (Miura, 2008). The Japanese populations of this species are divided into four genetic forms that inhabit four different geographical regions. These forms differ in heterogametic sex determination and sex chromosome content. The central Japan form has differentiated XY sex chromosomes, the northwest Japan form has differentiated ZW chromosomes, and the two other forms (Kanto and west Japan) display male heterogamety (as deduced from hormonally induced sex reversal and breeding experiments) but they have homomorphic chromosomes. Recently, populations located west of the XY form were shown to display female heterogamety (Ogata, Ohtani, Hasegawa, & Miura, 2008). Since they belong genetically to the XY form, their origin is probably very recent and they were designated as the Neo-ZW form.

Finally, some amphibians display exceptional types of sex chromosomes. In an endemic New Zealand frog, *Leiopelma hochstetteri*, a Z chromosome is absent from both males and females and a univalent W chromosome is present only in females: males are OO ($2n = 22$) and females are OW ($2n = 22 + W$) (Green, 1988). This W chromosome is neither a fragment of another chromosome nor a member of a multiple ZW1W2/ZZ system.

In a population of the neotropical frog *Eleutherodactylus maussi* (leaf litter frog) from northern Venezuela, homomorphic XY chromosomes and a derived Y-autosome translocation coexist (Schmid et al., 2002). Indeed, females have $2n = 36$ chromosomes (16 pairs + XXAA) whereas 95% of males have $2n = 35$ due to a centric fusion (Robertsonian) between Y and an autosome (16 pairs + XAA^{y}). The remaining males (5%) exhibit the same $2n = 36$ chromosomes as do all the female specimens. They represent the ancestral status (XYAA) before the Y-autosome fusion.

Thus, in amphibians, both male and female heterogamety are represented, often in the same family, genus, species, or even population. However, sex chromosomes are not often evidently heteromorphic and special situations or methods must be used to identify sexual genotype.

2.2. Evolution of Sex Chromosomes

The best-studied vertebrate sex chromosomes are the XY system of mammals and the ZW systems of birds and snakes (Ezaz, Stiglec, Veyrunes, & Marshall Graves, 2006). In the general context of genetic sex determination, a gene should be at the top of a sex-determining cascade. This master sex-determining gene is generally thought to be an

autosomal gene for which two different alleles have developed and for which homozygosity leads to the development of one sex and heterozygosity to the other. The evolution of an XY or ZW system occurs when (1) suppression of recombination maintains the region of the chromosome containing this gene in a constant heterozygous state and (2) the sex-determining allele on this chromosome is dominant in determining male (Y) or female (W) sex. Suppression of recombination is the first and crucial step in sex chromosome evolution. Then, the suppression of recombination between X and Y or Z and W spreads out from the sex determining gene and encompasses larger portions of the Y or W. Mutations that destroy genes could accumulate in the non-recombining region of the Y or W and deletions, insertions, accumulation of transposable elements, and expansion of repetitive sequences are the hallmarks of this 'asexual decay.' Such changes accumulate to a threshold, at which time they become observable at the cytological level as an accumulation of heterochromatin and chromosomal shrinkage owing to loss of genetic material.

A number of observations about sex chromosomes in amphibians support the hypothesis that the initial steps in the evolution of sex chromosomes were accumulations of repetitive DNA in the W and Y chromosomes. The presence of repetitive sequences could bring about asynchrony in the DNA replication pattern, thus reducing the frequency of crossing over between the chromosomes. For instance, in *Triturus helveticus*, no heteromorphism has been demonstrated in the male karyotype. However, during meiosis of male germ cells, the long arms of the pair number 5 show a decreased frequency of chiasmata formation. This suggests that, in the Y long arm, repetitive DNA sequences are already concentrated although no constitutive heterochromatin can be observed (Schmid et al., 1979). In more advanced situations, inversions are already present (*Hydromantes*). Finally, most of the highly evolved Y and W chromosomes are reduced to small and almost completely heteromorphic structures (*Necturus*, *Pyxicephalus*).

In *R. rugosa*, the Z and Y chromosomes on the one hand and the W and X chromosomes on the other are morphologically almost identical, based on shape and replication banding pattern. This suggests that Z and W share origins with Y and X, respectively. Sequences from the ADP/ATP translocase gene prove that Z and Y share an origin with chromosome 7 of the west Japan form and that W and X share a different origin to chromosome 7 of the Kanto form. A pericentromeric inversion is the structural change responsible for the morphology of the sex chromosomes. This is assumed to have occurred once on chromosome 7 during differentiation of the west Japan form and once on chromosome 7 of the Kanto form during hybridization (Miura, 2008).

2.3. Markers of Sex Chromosomes

Several genes have been mapped on sex chromosomes and different methods allow their analysis in order to identify sexual genotype: enzyme activity measurement, cytology, and fluorescent *in-situ* hybridization. These analyses are very useful when they can be performed before sex differentiation (from tail biopsy, for example). Such markers also provide a useful tool to demonstrate sex reversal.

Peptidase-1 is a dimeric enzyme encoded by two genes, peptidase-1A and peptidase-1B, located respectively on chromosomes Z and W in *P. waltl* (Ferrier, Jaylet, Cayrol, Gasser, & Buisan, 1980). The polymorphism of its electrophoretic pattern on starch gel plates has been used to identify the ZZ, ZW, and WW sexual genotypes in this species (Dournon, Collenot, & Lauthier, 1988). This method also discriminates ZW females and ZZ females in *P. poireti*, but with different patterns from those obtained in *P. waltl* (Dournon & Houillon, 1984). So far, no other genes have been localized on the sex chromosomes of the Caudata. In contrast, several enzymes encoding genes have been mapped on the sex chromosomes in anurans of the genus *Rana*: for example, acotinase-1 in *Rana clamitans* and peptidase C and superoxyde dismutase-1 in *Rana pipiens* (Schmid & Steinlein, 2001).

In the racophorid frog *Buergeria buergeri*, the nucleolar organizer is on the Z chromosome and, as a result, males have two nucleoli per nucleus whereas females have only one (Schmid, Ohta, Steinlein, & Guttenbach, 1993).

In *R. rugosa*, several genes have been mapped on sex chromosomes: androgen receptor (AR), SF-1/Ad4BP, and Sox 3 (Ohtani, Miura, & Ichikawa, 2003; Uno et al., 2008). Nevertheless, these genes are not located on the differential region between the heterochromosomes. The AR gene displays a very low expression rate of the W allele and is thus expressed about half as much in ZW females as in ZZ males (Ohtani et al., 2003).

There is no evidence of dosage compensation in amphibians.

In *X. laevis*, the DM-W gene has been recently mapped on the W chromosome (Yoshimoto et al., 2008).

3. SEX DIFFERENTIATION

The gonads originate from an outpocketing of cells localized on the ventral part of the anterior moiety of the mesonephros. At the beginning, they constitute bipotential organs identical in male and female, containing a cortex and a medulla. These gonads contain both germ cells and somatic cells. Germ cells have an extragonadal origin and the mechanisms leading to their specification are detailed hereafter. There is still debate regarding the origin of the somatic cells (Hayes, 1998). It was primarily reported from

studies of Ranidae that the cortex was derived from the coelomic epithelium whereas the medulla originated from the mesonephric blastema. Some later studies reported the contribution of the interrenal blastema rather than the mesonephric blastema to the medulla. Other studies in *X. laevis*, *R. pipiens*, and *Racophorus arboreus* suggested that the coelomic epithelium contributed not only to the cortex but also to the medulla, giving the two compartments a common origin. In other vertebrates, especially mammals, the contributions of the coelomic epithelium and the mesonephros have been more clearly determined by the use of cell microinjections and co-cultures between wild-type gonads and transgenic mesonephroi. Such studies would be very helpful in Amphibia to elucidate the origin of somatic gonadal cells.

3.1. Origin of Germ Cells

From a single cell, the fertilized egg, two lineages will appear during development: the soma and the germline. Germ cell specification does not occur by the same mechanisms in Anuran and Caudata: primordial germ cells (PGCs) are predetermined by the inheritance of germ cell determinants (germ plasm) in the first group while these cells are specified later during embryonic development through cell−cell interactions in the second group. This main difference could be a result of developmental constraint during gastrulation (Johnson et al., 2003).

In the anuran *X. laevis*, we have known for three decades that either exposure of the vegetal egg surface to UV irradiation or removal of the vegetal cytoplasm causes a reduction in the number of germ cells formed. The effects of UV irradiation in *Rana* can be reversed by the transfer of vegetal cytoplasm from an unirradiated fertilized egg. From these observations, it was deduced that the vegetal cytoplasm contains maternal determinants required to specify the germ cell lineage. The germ cell determinants are thought to be among a group of vegetally localized RNAs. Most of these RNAs encode RNA-binding proteins, leading to the hypothesis that PGC development is dependent upon post-transcriptional regulation.

This subject has been studied mainly in *X. laevis*, in which more than a dozen maternal RNAs that are tightly localized to the vegetal cortex of the fully grown oocyte have been identified (King, Zhou, & Bubunenko, 1999). Many of these vegetal RNAs are associated with islands of 'germ plasm,' which contain clusters of mitochondria and electron-dense organelles called germinal granules (Heasman, Quarmby, & Wylie, 1984). The germ plasm segregates unequally during cleavage, into only a few blastomeres, and contains factors that specify these cells to become PGCs (Houston & King, 2000a).

Xcat2 is one of these vegetal RNAs and encodes an RNA-binding protein related to nanos (Mosquera, Forristall, Zhou, & King, 1993), the product of an essential gene in Drosophila and the mouse that is required for the acquisition of germline fate and for germ cell migration. The DAZ (deleted in azoospermia) gene encodes an RNA-binding protein located on the Y chromosome of human males, and deletion of this locus correlates with azoospermia. In *X. laevis*, Xdazl (a DAZ homolog) RNA is localized to the germ plasm in oocytes and embryos (Houston, Zhang, Maines, Wasserman, & King, 1998). From depletion studies, it has been suggested that its product is required for germ cells to migrate toward the gonad (Houston & King, 2000b). Dazl has also been cloned in *Xenopus tropicalis*, in which the localization pattern is similar to that in *X. laevis* (Sekizaki et al., 2004). Xpat mRNA contains sequences in its 3′UTR that direct it to the vegetal pole during oogenesis (Hudson & Woodland, 1998; Machado et al., 2005). The protein encoded by Xpat also localizes to germ plasm and its mislocalization leads to ectopic assembly of structures resembling germ plasm, demonstrating a possible role for Xpat in organization and positioning of germ plasm (Machado et al., 2005). Xpat interacts via its C-terminal domain with XPix1, a WD40 microtubule-associated protein (Hames et al., 2008). DEADSouth is another germ plasm localized mRNA (MacArthur, Houston, Bubunenko, Mosquera, & King, 2000). It encodes a member of a small sub-family of the DEAD-box RNA-dependent helicases related to eIF4A.

During very early stages of oogenesis, the germ plasm RNAs use the early or METRO (for 'message transport organizer') pathway to localize vegetally into a macroscopic structure called the mitochondrial cloud, which lies close to the nucleus. These RNAs occupy a more restricted area of the vegetal cortex than do non-germ plasm-localized vegetal RNAs (including the transforming growth factor β family member Vg1 and the T-box transcription factor VegT), which are localized by the late pathway. Xcat2 RNA is incorporated into the germinal granules within the germ plasm (Heasman et al., 1984; Kloc & Etkin, 1995; Kloc et al., 2002). Other germline RNAs adopt distinct distributions within the germ plasm: Xpat RNA associates with the periphery of the granules, whereas Xdazl RNA is more generally distributed in the matrix (Kloc et al., 2002). In stage 2 oocytes, the mitochondrial cloud detaches from the nucleus and merges into a nearby cortical region, thereby bringing the germ plasm RNAs to the cortex. During subsequent stages of oogenesis, the mitochondrial cloud fragments, leading to subcortical germ plasm islands (Heasman et al., 1984).

In the four-cell embryo, germ plasm is found as approximately 80 yolk-free islands in the vegetal cortical area. In later stages, the germ plasm coalesces into larger pools that are asymmetrically distributed to only four blastomeres. At these stages (cleavage and blastula), the germ plasm remains near the plasma membrane of the

PGCs. At gastrulation, the germ plasm moves to a perinuclear location. The PGCs then migrate from the posterior endoderm to the dorsal endoderm and through the mesentery into the genital ridges, multiplying as they go, so that by the swimming tadpole stage they number about 20−50. A portion of the descendants of germline founder cells cannot migrate correctly to the genital ridges, and a few ectopic PGCs are eliminated by apoptosis or necrosis (Ikenishi, Ohno, & Komiya, 2007).

In caudate amphibians, the fate of PGCs is determined by inductive signaling, as in mammals (Wakahara, 1996). Here, the segregation of the germ cell lineage occurs relatively late in embryonic development. This aspect has been studied in detail in *P. waltl* by fine surgical manipulations of embryos. In that species, when the ventral or ventro−lateral parts of the marginal zone from a blastula are cultured into the blastocoele of a young gastrula, PGCs appear in this tissue, demonstrating that PGC precursors are located in the presumptive mesoderm (Maufroid & Capuron, 1973). The mesodermal origin of PGC precursors has been also described in two *Triturus* species (Nieuwkoop, 1947) and in *A. mexicanum* (Smith, 1964).

When the ectoderm and the endoderm of young *P. waltl* gastrulae are associated and cultured *in vitro*, dorsal endoderm mainly induces notochord, somites, and pronephric tubules while ventral endoderm promotes pronephric tubules, mesenchyme, and PGC differentiation (Maufroid & Capuron, 1977a; 1977b). Therefore, PGCs arise from totipotent ectodermal cells induced by an unknown signal from ventral endoderm. The chordomesoderm also exerts an inhibitory role on PGC specification (Capuron & Maufroid, 1981; Maufroid & Capuron, 1985). Since differentiating notochord secretes several BMP antagonists (chordin, noggin, follistatin), one can hypothesize that PGC specification in urodeles is a BMP-dependent process like in mammals.

When ventral endoderm from different stages is associated with ectoderm, the maximal inductive capability on PGC formation is observed at the beginning of gastrulation (stage 8a, 95%), then this activity decreases progressively (stage 9, 44%; stage 10, 9.33%) and finally disappears at neurulation (stage 13) (Maufroid & Capuron, 1977b). Moreover, grafts of mesodermal explants extirpated from young gastrula to mid neurula (stages 8a, 9, 13, 15, and 18) on dorsal or ventral regions from tailbud embryos (stage 22) showed that inhibition of PGC formation by dorsal structures of host embryos disappears by stage 13 (Capuron & Maufroid, 1981). These results suggest that PGCs arise from a pool of somatic cells from stage 8a (probably through BMP signalling) but also indicate that germline fate is definitely specified at stage 13.

In *P. waltl*, PGCs initially are located in the posterior region of the larvae near the Wolffian ducts and close to the future cloaca; the migration towards the genital ridges takes place between stages 35 and 41 (Dournon, Demassieux, Durand, & Lesimple, 1989).

Our knowledge about germ cell specification in caudate amphibians is still parcellar because of a lack of PGC markers. Recently, isolation of the germline-specific molecular marker VASA in *A. mexicanum* did not allow the identification of PGC precursors at the time of their specification since maternal VASA transcript accumulates in the oocyte and persists until hatching (Bachvarova et al., 2004). Similar results were obtained in *P. waltl*, in which zygotic expression of VASA ortholog was detected from stage 33 (Dumond et al., 2008a). A DAZL homolog has been cloned in *A. mexicanum* (Johnson, Bachvarova, Drum, & Masi, 2001). In oocytes and embryos, maternal Axdazl RNA is not localized and the earliest cell-specific expression is found in PGCs after they become located in the vicinity of the genital ridges. In *Cynops pyrrhogaster*, Cydazl transcript and protein are first detected in germ cells that have arrived at the genital ridges and have been surrounded by gonadal somatic cells (late stage 59), and they are also detected in proliferating germ cells in developing gonads (stage 60) (Tamori, Iwai, Mita, & Wakahara, 2004). These findings support the conclusion of Johnson et al. (2003) that caudate amphibians have no germ plasm and that the DAZL gene is induced by patterning signals in the embryo.

3.2. The Undifferentiated Gonad

The differentiation of the gonads seems to follow common mechanisms at least in anurans and caudates. To describe the steps, it is interesting to refer to *P. waltl* because in this species genotypic sex can be determined very early (cf. sex chromosome markers). From developmental stage 42 to stage 53, there is no histological difference between male and female, and the gonad at this time is considered bipotential or undifferentiated. At the periphery of the undifferentiated gonad, the cortex contains both somatic cells and gonia while in the central part the medulla contains only somatic cells (Figure 1.2). It should be noticed that, during the undifferentiated stage in *P. waltl* (from stage 50) and some other species, the gonadal anlage separates into two parts: the fat body devoid of gonia and the gonad itself (Figure 1.2).

The proliferation of germ cells has been studied in *P. waltl* larvae by mitotic index determination and germ cell counts (Dournon et al., 1989; Dournon, Houillon, & Pieau, 1990). P0 has been determined as a period during which there is no proliferation, P1 is characterized by a moderate proliferation, and P2 by a higher proliferation rate. Since the P0 period is longer in female larvae, this leads to a lower number of germ cells at the end of the P1 period when compared to males. During P2 (after stage 50), the proliferation rate increases more quickly in female larvae

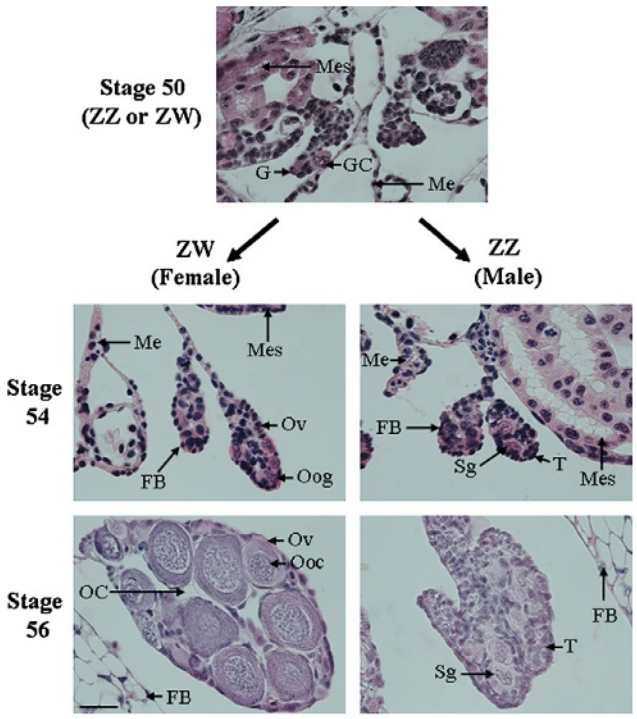

FIGURE 1.2 Development of the gonads during larval life in *Pleurodeles waltl*. At stage 50, the gonad (G) is undifferentiated and has the same aspect in both sexes: it is a small longitudinal organ on each side of the gut mesentary (Me) near the mesonephros (Mes) and it contains a few cortical germ cells (GCs). At stage 54, the ovary (Ov) is characterized by oogonia (Oog) located in the cortex of the gonad and a nascent ovarian cavity (OC), whereas the testis contains spermatogonia (Sg) in its medulla. In both cases, the differentiation of the fat body (FB) has begun. At stage 56 (end of metamorphosis), the ovary contains numerous oocytes (Ooc) that have entered prophase I of meiosis and has begun to accumulate yolk; the ovarian cavity is well developed. In contrast, the testis still contains spermatogonia (Sg) (not yet entered meiosis); in the medulla, these cells are found in lobules and will constitute cysts. The fat body is well differentiated in both ZZ and ZW larvae. Bar = 50 µm; all pictures were obtained at the same magnification. See color plate section at the end of the book.

than in males. Female larvae possess a higher number of germ cells than male larvae up to stage 54 + 60 days, at which point meiosis entry occurs in female larvae (Dumond et al., 2008b).

3.3. From the Bipotential Gonad to the Testis or the Ovary

Gonadal differentiation in gonochoristic species follows a similar process in anuran and caudate amphibians, but, again, our knowledge of Gymnophiona is very poor.

The earliest sign of histological differentiation of gonads is a modification of the localization of germ cells. In the ovary, oogonia stay in the cortex where they proliferate. Then, these cells associate with somatic cells and constitute follicles. Most of the oogonia enter meiosis and this process is arrested at prophase I. Recently, retinoic acid has been shown to trigger meiosis onset in *P. waltl* (Wallacides, Chesnel, Chardard, Flament, & Dumond, 2009). The ovary differentiates as an ovisac since medullary regression leaves a cavity (Figure 1.2). In the testis, germ cells migrate from the cortex towards the medulla where they mix with differentiating Sertoli cells in units termed cysts that are themselves included in lobules (Figure 1.2). Spermatogonia will enter meiosis after metamorphosis. The cortex devoid of germ cells becomes albuginea, the testis envelope. However, the phenotype of recently differentiated gonads can be ascertained only by histological examination. Morphological diagnosis by observation under a dissecting microscope can be performed later, around metamorphosis.

3.4. Tractus Differentiation and Post-metamorphosis Events

During differentiation, gonads produce hormones, mainly steroids, that influence reproductive duct differentiation. In female larvae, Wolffian ducts will be maintained since they will be required for urine elimination (there is no metanephros and hence no secondary ureter, as is the case in amniotes) and Müllerian ducts will differentiate into oviducts. In male larvae, Wolffian ducts will differentiate as an uro-spermiduct but the situation of Müllerian ducts is more complex. In anurans they usually will disappear, whereas in caudate amphibians they often will be maintained without differentiation. The persistence of Müllerian ducts in males of *P. waltl* can be demonstrated by E_2 treatment, which triggers oviduct differentiation (Figure 1.3) (Tiffoche, Chesnel, Jego, & Le Pennec, 1993). Estradiol, testosterone (T), and dihydrotestosterone are equally effective in stimulating oviduct differentiation in *A. tigrinum* (Norris, Carr, Summers, & Featherston, 1997). The existence of an anti-Müllerian hormone (AMH) (also known as Müllerian-inhibiting substance (MIS)) in amphibians is still an open question.

When compared to that in other vertebrates, gonadal differentiation is a slow process in amphibians: it occurs during larval life around metamorphosis except in some neotenic forms. Some events also occur after metamorphosis, especially in males: meiosis entry occurs in juvenile animals while old adults display multiple testes. This means that, in each male gonad, several lobes develop successively during adult life. The formation of these lobes has been studied, for instance in *Salamandra salamandra* (Joly, 1971) and recently in *P. waltl* (Flament, Dumond, Chardard, & Chesnel, 2009). Gymnophiona could also possess multiple testes (Smita, Oommen, Jancy, & Akbarsha, 2004).

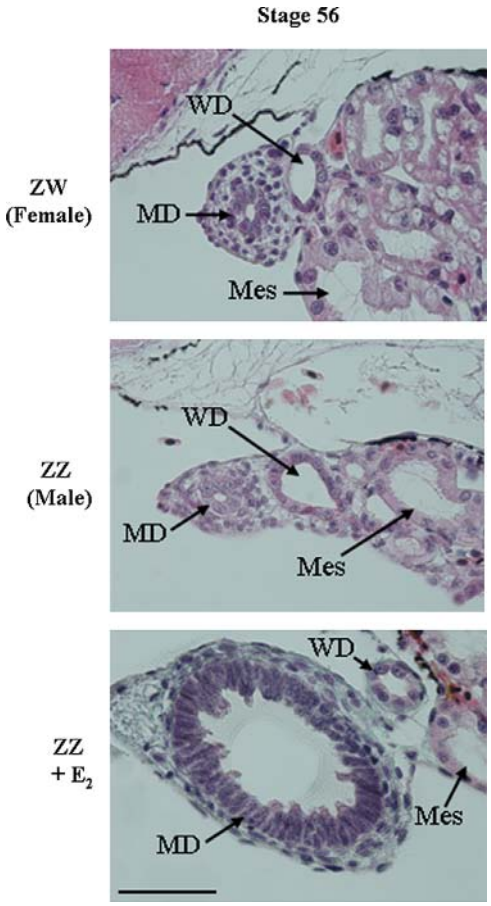

FIGURE 1.3 Urogenital ducts of *Pleurodeles waltl*. At stage 56, male (ZZ) and female (ZW) possess both Wolffian and Müllerian ducts located in the vicinity of the mesonephros (Mes). The Wolffian duct (WD) is used for urine elimination in both sexes, and in males it is also used for sperm transport. The Müllerian duct (MD) will differentiate into the oviduct in females, whereas in males it will be maintained without differentiation but has the capacity to differentiate into oviduct upon estradiol stimulation. Bar = 100 µm; all pictures were obtained at the same magnification. See color plate section at the end of the book.

4. SENSITIVITY OF SEX DETERMINATION/ DIFFERENTIATION TO EPIGENETIC FACTORS

Environmental factors can influence sex determination/ differentiation in some vertebrates. Reptiles are the best examples since developmental temperatures of embryos influence gonadal sex in numerous species. A similar phenomenon exists in some amphibian species. However, although sex reversal can be induced experimentally and has been studied extensively in the laboratory, its occurrence in wild populations is difficult to appreciate. *Rana temporaria* is the first species in which changes in gonadal differentiation under the effects of the rearing temperature of tadpoles was described (Witschi, 1914). Later, the effects of abnormally high or low temperatures during larval development were extended to some other anuran and urodelan species.

4.1. Effect of Rearing Temperature

The effect of temperature on gonadal sex is exerted during a limited window of time during development called the thermosensitive period (TSP). The TSP is defined by the two stages of development between which a thermal (cold or hot) treatment can induce male or female sex reversal. If thermal treatment is performed before or after this period, it does not modify the sex ratio so extensively.

In anurans in which the genetic sex was unknown, sex reversal has been deduced from biased sex ratios and from both macroscopic and microscopic examination of gonads around metamorphosis. Observations on *R. temporaria* indicate that high temperatures ($\geq 25°C$) are masculinizing, whereas low temperatures ($\leq 12°C$) are feminizing (Witschi, 1914; Piquet, 1930). However, the sexual race of the populations used in these studies is unknown and these results must be taken with prudence (in 'differentiated races,' 50% males and 50% females are observed at metamorphosis whereas, in 'undifferentiated races,' all individuals show ovaries at metamorphosis with 50% of them becoming testes during the three next months). The experiments performed in *Rana sylvatica* are more convincing (Witschi, 1929a; 1929b). When gonads had begun to differentiate, larvae from this sexually differentiated species were placed at $32 \pm 2°C$ for 15 to 33 days. At the end of this treatment, 53.9% of the individuals were phenotypic males while 46.1% were intersexes displaying ovotestes at different steps of ovarian masculinization.

Heat treatment administered during larval life also had a masculinizing effect in other anuran species, including *Rana. japonica*, *R. catesbeiana* and *B. bufo*, (for a review see Chardard, Penrad-Mobayed, Chesnel, Pieau, & Dournon, 2004). In addition, a possible feminizing effect of cold treatment (at temperatures $\leq 12°C$), similar to that observed in *R. temporaria*, was suggested in *B. bufo* (Piquet, 1930). In the subspecies *B. b. formosus*, high temperatures (25 or 30°C) applied all through the larval stage had no significant effect on sex ratio, although some animals had rudimentary or underdeveloped gonads. When high temperatures were applied through the larval and juvenile stages or only at the juvenile stages, a preponderance of males was obtained. The occurrence of two hermaphrodites among the heat-treated animals was also in agreement with a sex reversal process

In caudate amphibians, the first study indicating a sex-reversing effect of temperature was performed in a sexually semi-differentiated race of *Hynobius retardatus*. It was initially reported that, as in the Anura, heat treatment was masculinizing (Uchida, 1937a; 1937b). However, more recent studies on this species convincingly demonstrated

that, when larvae were reared at 28°C, there was a male to female sex reversal (Sakata, Tamori, & Wakahara, 2005; Sakata, Miyazaki, & Wakahara, 2006). In this species, gonadal differentiation takes place from 20 to 30 days after hatching and the TSP seems to extend from 15 to 30 days after hatching, although this has not been studied in detail. Temperatures of 20, 22, 24, or 26°C did not modify the sex ratio and, unfortunately, larvae reared at temperatures higher than 28°C did not survive (Sakata et al., 2006).

Studies were also carried out in sexually differentiated species: two closely related species of *Pleurodeles*, *P. waltl* and *P. poireti* (Dournon & Houillon, 1984; 1985; Dournon et al., 1990) and two subspecies of *T. cristatus*, *T. c. cristatus* and *T. c. carnifex* (Wallace, H., Badawy, & Wallace, B., 1999; Wallace, H., and Wallace, B., 2000). Since the genetic sex of all or some individuals was identified (peptidase-1 analysis in *P. waltl* and C-banded chromosome analysis in *T. c. carnifex* and *T. c. cristatus*), it was clear that gonadal differentiation of heat-treated individuals did not agree with their sexual genotype.

In *P. waltl*, the period during which heat treatment induces sex reversal of ZW genotypic females has been identified by shifting larvae from 20 ± 2°C to 30, 31 or 32°C, at different stages and for various times (Dournon & Houillon, 1985; Chardard et al., 2004). All ZW larvae (genotypic females) were sex reversed when they were reared at 32°C between stages 43 and 54. When rearing at 32°C began later than stage 43 or was stopped earlier than stage 54, it did not yield 100% sex reversal. Thus, TSP extends between stages 43 and 54 and is approximately two months long. As mentioned above, at stage 43, i.e., at the beginning of the TSP, the gonads of *P. waltl* are undifferentiated and at stage 54, i.e., at the end of the TSP, they are just beginning to differentiate into testes or ovaries. Thus, in *P. waltl*, gonads appear histologically undifferentiated during the major part of the TSP. A temperature of 30°C applied during the whole TSP was less efficient than 32°C since it did not induce sex reversal of all genotypic females. In addition, exposure at 32°C at stage 43 followed by exposure at 30°C at different stages before the end of the TSP and until metamorphosis did not yield 100% sex reversal.

In *P. poireti*, when larvae from a standard progeny were reared at 30°C from stage 42 to 54, some ZZ genotypic males were sex-reversed: 65.7% females, 22.9% males, and 11.4% intersexes were observed at metamorphosis. Thus, high temperature has opposite effects in *P. waltl* and *P. poireti*, with complete or partial sex reversal affecting ZW genotypic females in *P. waltl* and ZZ genotypic males in *P. poireti* (Dournon & Houillon, 1984; Dournon et al., 1990; Dorazi, Chesnel, & Dournon, 1995).

In *T. c. cristatus* and *T. c. carnifex* that display XX/XY sex chromosomes, high temperatures have masculinizing effects whereas low temperatures induce feminization. In *T. c. cristatus*, when the rearing temperature was between 18 and 24°C, progenies showed a 1 : 1 sex ratio. Treatment of larvae at 28°C resulted in a majority of males (61%), some of which were diagnosed as XX males. Trials at and below 16°C resulted in a significant excess of females (68% to 100%), among which XY females were diagnosed (Wallace, H., & Wallace, B., 2000). The cold treatment started from the egg stage. It could be delayed until first feeding stage and still cause feminization but should be prolonged for most of larval life.

Larvae of *T. c. carnifex* reared at temperatures ranging from 16 to 26°C show a 1 : 1 sex ratio at metamorphosis. Cold treatment (13°C) induced male to female sex reversal (78%) when applied from the uncleaved egg to metamorphosis (9−10 months), whereas no sex reversal could be detected when cold treatment was limited to feeding stages (7−8 months) (Wallace et al., 1999). However, in the case of a thermal treatment starting at the 'feeding larvae' stage, temperatures of 28, 30, and 31°C biased the sex ratio significantly in favor of males (respectively 67, 74, and 67%) and XX males were diagnosed in the 30°C trial. Here, the duration of heat exposure was only three months (at 28°C), suggesting that TSP in *T. c. cristatus* is similar to that in *P. waltl*, although experiments never induced 100% sex reversal (Wallace, H., & Wallace, B., 2000).

4.2. Breeding Experiments

The analysis of the progeny of heat- or cold-treated individuals was also performed to confirm or even demonstrate sex reversal. In addition, such analysis includes data on mating behavior and fertility, which are the ultimate stages of sex differentiation.

The ZW males of *P. waltl* and the ZZ females of *P. poireti* are fertile, indicating that the factors controlling spermatogenesis and oogenesis are autosomal or are not located on the sex-determining region of the W chromosome. When ZW males of *P. waltl* were bred with ZW standard females, progenies with 25% ZZ males and 75% ZW and WW females were produced at room temperature, as expected. The females with the novel WW genotype were also fertile: when they were crossed with ZZ standard males, the progenies reared at room temperature were 100% female, as expected (Dournon & Houillon 1984; Dournon et al. 1990). In *P. poireti*, crossing ZZ females with ZZ standard males produced 100% males, as expected (Dournon & Houillon, 1984).

XX males of *T. c. carnifex* were also reared until maturity. They had miniature testes with premeiotic spermatogonia and spaces presumably left by degenerated spermatocytes. These animals failed to fertilize eggs, suggesting that a fertility factor on the Y chromosome is required for entry into the meiotic stages of spermatogenesis in this species (Wallace et al., 1999). No data were

reported about the fertility of XY females of *T. c. carnifex* (Wallace et al. 1999; Wallace, H., & Wallace, B., 2000).

Thus, it is clear that temperature has various sex-reversing effects in a limited number of amphibian species. Depending on the species, high temperature can be masculinizing or feminizing. These antagonistic effects are even observed in two species of *Pleurodeles*. In all cases, the TSP starts before gonadal differentiation, but the molecular target of temperature is still unknown. However, we will see now that steroidogenesis and/or steroid hormone signaling pathways are indirectly affected by thermal treatment.

5. THE ROLE OF STEROID HORMONES

5.1. Treatment with Steroids

A great variety of studies have shown that treatments of the embryos or larvae of vertebrates with steroids lead to sex reversal. Due to the aquatic development of larvae, steroids are often simply diluted in the rearing water. The first studies in vertebrates were conducted in birds and amphibians in the 1930s: for example, treatment of tadpoles of *R. esculenta* with estrogens induced differentiation of males (Padoa, 1936). Numerous subsequent studies have shown that many steroids could induce sex reversal in many species of Anura and Caudata (for reviews see Gallien, 1962; Hayes, 1998; Wallace et al., 1999). Many conflicting results have been obtained. Indeed, the same effect can be observed with different steroids: for example, masculinization can be obtained with progesterone, corticoids, androgens, and even estrogens (Hayes, 1998). The same steroid can also have different effects depending on species: similar doses of E_2 induce masculinization in *X. laevis* (Gallien, 1953), have no effect in *R. japonica* (Yoshikura, 1962), and induce feminization in *R. pipiens* (Richards & Nace, 1978). The same steroid can also exert antagonistic effects in the same species depending on dosage: for example, in Ranidae, E_2 induces feminization at low dosage and masculinization at high dosage (Gallien, 1962). It has been suggested that steroid effects on sex differentiation are related to male or female heterogamety (Gallien, 1962), but exhaustive comparison of results obtained in anurans revealed no relation of steroid effects to the genetic sex-determining system, nor with phylogeny (Hayes, 1998). Time and duration of treatment, and particularly steroid uptake and metabolism, are factors that may explain the variety of results of steroid treatments in amphibians. Depending on the species, exogenous steroids may be metabolized into inactive or more potent compounds. For example, in *P. waltl*, while T has no effect on ZW larvae sex differentiation, it induces sex reversal of ZZ larvae. This paradoxical effect may be explained by conversion of exogenous T to E_2 by aromatase since non-aromatizable androgens such as dihydrotestosterone or 11β-hydroxyandrostenedione have no effect on ZZ larvae and induce male differentiation of ZW larvae (Chardard, Kuntz, Chesnel, & Flament, 2003).

5.2. Synthesizing Enzymes

In order to prove that steroids are natural inducers of sex differentiation, it has to be shown that steroids are produced before or at the time of gonadal differentiation. Steroid levels at the time of sex differentiation have not been measured in amphibian gonads due to the size of tadpoles and larvae, and the fact that levels of total steroids in whole tadpoles do not necessarily reflect differences in gonadal content between sexes. However, amphibian larvae possess steroidogenic enzymes in their gonads at the time of sex differentiation. There are very few studies examining endogenous steroid production: studies have essentially been focused on 3β-hydroxy-Δ5-steroid dehydrogenase (3β-HSD) for demonstration of steroid production potentialities. For example, this enzyme has been detected by histochemistry in gonads of *R. catesbeiana* (Hsu, Chiang, & Liang, 1977), *P. waltl* (Collenot, G., & Collenot, A., 1977) and *X. laevis* (Kang, Marin, & Kelley, 1995). Recently, the expression of sex steroid-synthesizing enzymes P450 side chain cleavage ($P450_{scc}$), 3β-HSD, P450 17α hydroxylase (CYP17), 17β-hydroxysteroid dehydrogenase (17β-HSD), and 5α-reductase (5α-red) has been reported in *R. rugosa*. No difference was found in the expression of $P450_{scc}$, 3β-HSD, 17β-HSD, and 5α-red between males and females. CYP17 expression is upregulated in gonads of male tadpoles before the onset of sex differentiation (Iwade, Maruo, Okada, & Nakamura, 2008; Maruo, Suda, Yokoyama, Oshima, & Nakamura, 2008). Moreover, CYP17 protein is detected in somatic cells of indifferent gonads and the conversion rate of [^{3}H] progesterone into [^{3}H] androstenedione is higher in males than in females (Sakurai et al., 2008). Finally, upregulation of CYP17 transcription is observed in sex-reversing ovaries following T treatment of female tadpoles (Iwade et al., 2008). These studies suggest that androgen production is higher in males than in females and that these androgens could play a role in testis differentiation of *R. rugosa*. Conversely, several studies have reported higher expression of aromatase, the enzyme that converts androgens to estrogens, during female differentiation. In *P. waltl*, aromatase activity is higher in differentiating ovaries than in differentiating testes; this difference occurs just before histological differentiation of the gonads and is abolished during sex reversal of ZW larvae under the thermal treatment effect (Chardard, Desvages, Pieau, & Dournon, 1995). In the same way, in *H. retardatus*, aromatase mRNA is much more expressed (at least 100 times more) in female gonads than in male gonads. Interestingly, in this species, rearing

larvae at 28°C leads to sex reversal of males into females with an upregulation of aromatase expression (Sakata et al., 2006). In *R. rugosa*, higher expression of aromatase during ovarian differentiation has also been reported (Kato, Matsui, Takase, Kobayashi, & Nakamura, 2004; Maruo et al., 2008). All these studies point to the regulation of aromatase as a key factor during female differentiation in amphibians, as observed in other oviparous vertebrates.

5.3. Steroid Hormone Receptors

Gonads can also respond to steroids. Sex steroid receptors have been characterized in several amphibian species but few studies have focused on detailed expression of these receptors during sex differentiation. In *X. laevis*, androgen receptor (AR) and estrogen receptor (ER) expression have been found in tadpoles but no difference between sexes was reported (Bogi, Levy, Lutz, & Kloas, 2002). In *R. rugosa*, it has been suggested that AR expression directly or indirectly suppresses aromatase expression during gonadal differentiation (Ohtani et al., 2003). In *P. waltl*, the expression of ERα is higher in female than in male gonads after the commitment of ovarian development, which is in accordance with the role of estrogens in ovary differentiation (Ko, Chesnel, Mazerbourg, Flament, & Chardard, 2008).

5.4. Inhibition of Steroid Action or Synthesis

With the lack of genetic tools in amphibians, the removal of steroid action has been performed by chemical treatments with various molecules known as antiandrogens or antiestrogens, or with inhibitors of certain steroidogenic enzymes. Indeed, it is not clear whether steroids are the cause or an early consequence of gonadal differentiation. Here again, various effects have been observed depending on the species. In *R. esculenta*, the anti-androgen cyproterone acetate induced masculinization of gonads, which led to the conclusion that the inducers of gonadal differentiation were not gonadal steroids (Chieffi, Iela, & Rastogi, 1974). The same observation has been reported in *R. catesbeiana*, but it was shown that masculinization occurs following decreased 3β-HSD activity by cyproterone acetate (Hsu, C., Hsu, L., & Liang, 1979). Indeed, the 3β-HSD inhibitor cyanoketone also induced masculinization in this species (Hsu, Yu, Ku, Chang, & Liu, 1991). Moreover, in another anuran species, *X. laevis*, cyproterone acetate acts as a real antiandrogen and feminizes male tadpoles (Bogi et al., 2002). In oviparous vertebrates, aromatase inhibitor treatments leads to various degrees of female to male sex reversal. This is also the case in amphibians. In *R. catesbeiana*, the intraperitoneal implantation of capsules containing 4-hydroxyandrostenedione in tadpoles induced sex reversal, with 79% of ovaries being transformed into testes (Yu, Hsu, Ku, Chang, & Liu, 1993). In *P. waltl*, treatment of ZW larvae with the aromatase inhibitor fadrozole led to the development of males, and sex reversal was correlated to the degree of aromatase activity inhibition (Chardard & Dournon, 1999; Kuntz, Chardard, Chesnel, Grillier-Vuissoz, & Flament, 2003). In the same way, cultured gonads of *X. laevis* in the presence of 20µg/l of fadrozole all showed characteristics of male phenotype (Myata & Kubo, 2000).

5.5. Steroids and Temperature

It seems that there is a link between the sex-reversing effect of temperature and steroidogenesis. Several studies have been performed in *P. waltl*. In that species, the masculinizing effect of temperature is inhibited when E_2 is added to the rearing water (Zaborski, 1986). The increase in aromatase activity usually observed in ZW larvae does not occur in the case of heat treatment (Chardard et al., 1995). Besides, the female-enriched expression of aromatase is not observed in heat-treated ZW larvae (Kuntz et al., 2003b; Ko et al., 2008). These results suggest that in *P. waltl*, in the case of heat treatment, E_2 synthesis is impaired. Moreover, real-time PCR analyses revealed that ERα expression in gonads of ZW larvae reared at 32°C was lower than in control ZW larvae, demonstrating, in addition, an inhibitory effect of temperature on ERα expression (Ko et al., 2008).

In *H. retardatus*, when larvae were reared at the female-producing temperature (28°C), an upregulation of aromatase expression was observed (Sakata et al., 2005; 2006).

In conclusion regarding steroids, these hormones can sex-reverse amphibians, steroids are produced in the gonads at the time of sex differentiation, and inhibition of steroid action leads to sex reversal. All these results show that, if steroids are not primary inducers, they are at least key regulators of gonadal differentiation. This has been clearly demonstrated for estrogens but not for androgens. However, experimental situations, such as parabiosis in *P. waltl*, suggest that testicular hormones play a role in gonadal differentiation as they can counteract ovarian development. Indeed, association between ZZ and ZW individuals induces a freemartin-like effect where differentiation of ovaries of the ZW parabiont is inhibited (Figure 1.4) (Dumond et al., 2008b). The chemical nature of the involved hormones, steroids, or AMH has yet to be determined.

6. GENES INVOLVED IN GONADAL SEX DIFFERENTIATION

The understanding of sex determination and differentiation in amphibians will require the identification of sex-determining genes, of their downstream genetic cascade, and of

Treatment	ZZ gonad	ZW gonad	References
None	Testis	Ovary	Dumond et al., 2008a
E_2	Ovary	Ovary	Chardard et al., 2003
AI	NS*	Testis	Chardard and Dournon, 1999
T	Ovary	Ovary	Chardard et al., 2003
DHT	Testis	Testis	Chardard et al., 2003
ZW	Testis	Ovary	Dumond et al., 2008b
ZZ	Testis	Inhibition	Dumond et al., 2008b

* NS = not studied

FIGURE 1.4 Effect of various treatments on gonadal differentiation of *Pleurodeles waltl*. When added to the rearing water, steroids can induce male to female sex reversal (estradiol, E_2) or female to male sex reversal (dihydrotestosterone, DHT). Treatment with testosterone (T) induces a paradoxical effect: sex reversal of the ZZ gonad (due to aromatization) and no effect on the ZW gonad. Inhibitors of steroidogenic enzymes, especially the aromatase inhibitor (AI) fadrozole, also induce female to male sex reversal. However, in case of parabiosis between ZZ and ZW embryos, the effects of endogenous hormones on the gonads are different from those observed with steroids: the ZZ gonads differentiate as a testis whereas the differentiation of the ZW gonads is inhibited. This could suggest the production by the *Pleurodeles waltl* testis of AMH in addition to androgens.

the link with steroidogenesis. Knowledge of the genes involved in sex determination/differentiation pathways is more extensive in mammals than in other vertebrates, and most of them have been found in nonmammalian vertebrates, in which it seems they have conserved roles. Many of these genes also have been cloned in amphibians; however, as yet it has not been possible to consider any of them as a sex-determining gene due to the lack of a clearly dimorphic expression during development.

WT1 is a zinc finger transcription factor encoded by the Wilms' tumor suppressor gene, which is involved in early gonadal development in mammals. In *R. rugosa*, WT1 is expressed early during development and no sexually dimorphic expression can be observed in the developing gonad (Yamamura et al., 2005).

SF-1/Ad4BP is a transcriptional factor that was originally found to be a mammalian homolog of the Drosophila Ftz-F1 (fushi tarazu factor 1). It is essential for the transcription of genes functionally related to steroidogenesis, such as P450(CYP) steroid hydroxylases, 3β-hydroxysteroid dehydrogenase, and steroidogenic acute regulatory protein (StAR). SF-1 was isolated in *R. rugosa* from a testis cDNA library (Kawano, Miura, Morohashi, Takase, & Nakamura, 1998). The SF-1 gene was located on the short arms of W and X and the long arms of Z and Y (Uno et al., 2008). SF-1 mRNA was found in brain, spleen, adrenal–kidney, and testis but not in the ovary. The same team reported that another type of mRNA (β) was produced by alternative splicing (Nakajima, Takase, Miura, & Nakamura, 2000). They found that the expression of both isoforms became stronger in the testis of frogs at stage 25, at which point metamorphosis is complete, with a higher expression level for α than β. By *in-situ* hybridization, SF-1α expression was found in spermatogonia and spermatocytes in the testis and in stage A oocytes in the ovary (Takase, Nakajima, & Nakamura, 2000). Using an immune serum directed against *R. rugosa* SF-1 produced as a fusion protein with glutathione S-transferase, SF-1 expression was detected in extragonadal tissues in the embryo at very early stages and in the somatic cells of the adult testis (Kawano, Furusawa, Matsuda, Takase, & Nakamura, 2001).

Using an anti-mouse SF-1 antibody, a sexual dimorphism in the SF-1 protein was reported in *R. catesbeiana*: the females had an increased level of SF-1 expression following ovarian differentiation, whereas the expression in males declined as the testis developed (Mayer, Overstreet, Dyer, & Propper, 2002).

A transient female-enriched expression of SF-1 was also described in the caudate *P. waltl* (Kuntz et al., 2006). In this model, a regulation of SF-1 expression by estrogens has been proposed, since (1) the upregulation occurs after the onset of the ovarian-specific increase of aromatase mRNA expression and (2) SF-1 expression appears to be thermosensitive.

FGF9, WNT4, and FOXL2 are involved in the differentiation of the ovary in mammals (see Volume 5, Chapter 1). They have recently been cloned from *R. rugosa*. FGF9 belongs to the fibroblast growth factor (FGF) family, the members of which act through membrane receptors. Like WT1, it is expressed early during development and the developing gonad does not show sexually dimorphic expression (Yamamura et al., 2005). WNT4 is a member of the WNT family of cell-signal molecules acting through frizzled receptors. A WNT4 cDNA has been isolated from the testis (Oshima, Hayashi, Tokunaga, & Nakamura, 2005). In adults, WNT4 mRNA was found in all studied organs. In tadpoles, RT-PCR studies indicated that WNT4 mRNA was expressed in the differentiating gonads with no difference between males and females. FOXL2 belongs to the forkhead family of transcription factors. RT-PCR analyses revealed an expression in the brain, the testis, and the ovary of *R. rugosa*, with the highest level of mRNAs found in the ovary (Oshima, Uno, Matsuda, Kobayashi, & Nakamura, 2008). During gonad differentiation, its expression was dimorphic (female-enriched). In the ovary it was immunochemically detected in the somatic cells surrounding oocytes. In co-transfection assays in *X. laevis* A6 cells, it was shown that FOXL2 stimulated *R. rugosa* aromatase transcription. These authors also isolated a *X. laevis* FOXL2 cDNA but they did not analyze the expression pattern in that species.

DAX-1 is a member of a group of nuclear receptors originally isolated in humans from the region called dosage-sensitive sex reversal (DSS) that is located on the X chromosome. DAX-1 has been cloned in the frog *R. rugosa*, where it was transcribed in the testis more strongly than in the ovary at stage 25—i.e., after completion of metamorphosis (Sugita, Takase, & Nakamura, 2001). Its expression in the ovary decreased gradually as ovarian development proceeded. Two months after metamorphosis, DAX-1 was found only in male gonads. When female tadpoles were sex-reversed following administration of T, DAX-1 expression was rapidly upregulated.

SOX9 (SRY-related high mobility group box 9) is a transcription factor involved in testis differentiation in mammals; its transcription is activated just after the expression of SRY. Two mRNA isoforms have been isolated from the adult testis of *R. rugosa* (Takase, Noguchi, & Nakamura, 2000). SOX9α encodes a 482-amino-acid protein containing the HMG box, whereas SOX9β lacks the HMG box and is a truncated form of SOX9α (265 amino acids). In adult frogs, SOX9α mRNA was expressed mainly in the brain and testis and its expression was higher than SOX9β. The adult ovary did not produce SOX9. During tadpole development, both isoforms were found not only in the testis but also in the ovary, and testis-specific expression appeared only in juvenile animals two months post-metamorphosis.

In *X. laevis*, a similar situation was observed: in adults, SOX9 was expressed in many organs including the testis but no expression was found in the ovary, whereas, in tadpoles, SOX9 mRNA was detected in gonad−mesonephros complexes from stages 53 to 56 in both males and females (Osawa, Oshima, & Nakamura, 2005). More recently, SOX9 expression was studied in *X. tropicalis* (El Jamil, Kanhoush, Magre, Boizet-Bonhoure, & Penrad-Mobayed, 2008). In that species, SOX9 mRNA and protein were first detectable after metamorphosis in both sexes. In the testis, the expression was restricted to the nucleus of Sertoli-like cells. In the ovary, in contrast to what is described in other vertebrates, SOX9 mRNA and protein were present and detected in germ cells: SOX9 localization was cytoplasmic in previtellogenic oocytes and nuclear in vitellogenic oocytes.

DMRT1 (doublesex- and mab-3-related transcription factor 1) encodes a protein that contains a zinc finger-like DNA binding motif called the DM domain, which is shared between the fruit fly *Drosophila* doublesex and the nematode worm *Caenorhabditis elegans* mab-3. Both doublesex and mab-3 play key roles in sex determination in these species, and DMRT1 has been shown either to have a male-specific or a male-enriched expression in several vertebrates. This gene is located on the Z chromosome in the chicken. Moreover, a DMRT1 coortholog dmY/dmrt1bY causes testis formation as a testis-determining gene in the medaka fish (Matsuda et al., 2002) (see Volume 1, Chapter 1).

In the frog *R. rugosa*, DMRT1 is autosomal and has been mapped on the long arm of chromosome 1 in both ZZ/ZW and XX/XY individuals (Uno et al., 2008). This result indicates that DMRT1 may not be related to sex determination in that species. In frogs having the XY system for sex determination, DMRT1 is expressed male-specifically after sex determination (from stage 25) (Shibata, Takase, & Nakamura, 2002; Aoyama et al., 2003). In the testis of tadpoles and in frogs one month after metamorphosis, immunohistochemical studies revealed that DMRT1 protein was expressed in interstitial cells and Sertoli cells. In adult frogs, germ cells also expressed DMRT1 (Aoyama et al., 2003). In that species, when female to male sex reversal is induced by T injection, DMRT1 expression occurs in the sex-reversed gonads (16 days after the injection) (Shibata et al., 2002; Aoyama et al., 2003). In *X. laevis* that has a ZW determining system, DMRT1 is transcribed early but transiently during embryo development (from stages 13 to 38) (Yoshimoto et al., 2006). At stage 52, when gonads are not yet differentiated, it is re-expressed and its expression becomes gonad-specific (Yoshimoto et al., 2006). In juvenile animals, the expression was higher in the testis than in the ovary, whereas in adults, the DMRT1 transcript was observed exclusively in the testis (Osawa et al., 2005; Yoshimoto et al., 2006).

In the salamander *H. retardatus*, a partial cDNA fragment of DMRT1 has been cloned (Sakata et al., 2006). DMRT1 transcripts were found in gonad−mesonephros complexes of male larvae during the sexual differentiation period but they were absent from the complex in female larvae. When larvae were reared at the female-producing temperature (28°C) during the TSP, a complete suppression of DMRT1 expression was induced (Sakata et al., 2006).

In *P. waltl*, although DMRT1 expression has not been described in detail during sex differentiation, a testis-specific expression has been reported after metamorphosis in juvenile animals (Dumond et al., 2008b).

In *X. laevis*, a W-linked DM-domain gene has been identified recently as crucial for ovary development, probably acting as a female-determining gene (Yoshimoto et al., 2008). The authors studied DMRT1, which had been cloned previously in *X. laevis* (Osawa et al., 2005). By Southern blot analysis, they observed that the full-length cDNA probe, containing the DM domain sequence, hybridized with an 8 kb DNA fragment only in samples from females, in addition to the 4 kb bands in both sexes. The 8 kb fragment was another DM domain gene and was named DM-W. Fluorescence *in-situ* hybridization revealed that DM-W was located on one chromosome in females and no signals were found in males. The DM-W protein possessed 194 amino acids and both the DM domain and its flanking regions had a high identity with the corresponding

regions of DMRT1. In contrast, the C-terminal region (AA 124-194) of DM-W showed no significant similarity with DMRT1. Although DMRT1 was expressed continuously in the gonads after sex differentiation in both sexes, DM-W was expressed transiently during sex differentiation (stages 48 to 50). Transgenic ZZ tadpoles expressing DM-W showed either ovotestes or ovaries.

Thus, *X. laevis* DM-W constitutes the first evidence of a sex-determining gene in amphibians. There is no doubt that its discovery should lead to advances in the understanding of the sex determination/differentiation process in amphibians in the near future.

7. UNDIFFERENTIATED RACES, HERMAPHRODITISM, AND UNISEXUALS

In addition to the mechanisms described above, which were deduced from the study of gonochoristic species, a few amphibians display unusual sex differentiation processes.

Undifferentiated races have been reported in several ranid species (including *R. temporaria*, *R. catesbeiana*, *R. esculenta*, and *Rana ornativentris*), in a single bufonid (*B. bufo*), and in *R. arboreus*. Undifferentiated races also exist in salamanders (*H. retardatus* and *Ambystoma maculatum*). In some populations of *R. temporaria*, all of the animals display ovaries at metamorphosis. Six to nine months after metamorphosis, some of these individuals transform into males, going through an intersexual stage. Similar reports have been made for *Racophorus schlegelii* (Amanuma, 1963a; 1963b).

Sequential hermaphroditism exists in some species. In the reed frog *Hyperolius viridiflavus ommatosticus* captive females, which laid at least one time, several (7 out of 24) began calling, developed gular pouches, and apparently fertilized eggs (Grafe & Linsenmair, 1989).

Bufonids are not truly hermaphroditic. However, both males and females possess two rudimentary ovaries that develop at the cephalic end of the gonads (Tanimura & Iwasawa, 1986) and are known as the Bidder's organs. If the testes are surgically removed, these organs develop into fully functional ovaries that can produce fertilizable eggs (Witschi, 1933). Although they exhibit seasonal growth along with the testes and respond to exogenous gonadotropins, under normal conditions the Bidder's organs do not function as ovaries in males (see Pancak-Roessler & Norris, 1991).

Although classical sexual reproduction is observed in the majority of amphibians, an atypical form of reproduction also exists in the North American salamander genus, *Ambystoma*. This genus displays unisexuals that are thelytokous and automictic. They have been referred to as parthenogenetic (Uzzel, 1969; Downs, 1978), gynogenetic (Elinson, Bogart, Licht, & Lowcock, 1992), hybridogenetic (Normark, Judson, & Moran, 2003), and both gynogenetic and hybridogenetic (Bogart, Elinson, & Licht, 1989). Parthenogenesis and gynogenesis are genetically equivalent. Sperm is required to stimulate the development of the eggs of gynogens but is not incorporated so the offspring are genetically identical to their mothers. Hybridogenesis is hemiclonal; one genome is transmitted clonally and vertically but the other genome is removed and replaced each generation.

So far, 22 distinct diploid, triploid, tetraploid, and pentaploid unisexual *Ambystoma* are known to be syntopically associated with one or more of four morphologically distinctive bisexual species (*Ambystoma jeffersonianum*, *Ambystoma tigrinum*, *Ambystoma laterale*, and *Ambystoma texanum*). Diploid and polyploid unisexuals have nuclear genomes that combine the haploid genomes of two to four of these species, but the mitochondrial DNA is unlike any of those four species and is similar to a fifth species, which could be the common maternal ancestor, *Ambystoma barbouri* (Hedges, Bogart, & Maxson, 1992; Bogart, 2003). It appears that nuclear genomes are taken from sympatric males within populations and are incorporated into the diploid or polyploid nuclei of unisexual individuals. But, unlike hybridogenesis, the male-derived nuclear genomes are not kept intact nor are they eliminated at the ensuing meiosis event. The authors believe that this exemplifies a new unisexual reproductive mode, kleptogenesis (Bogart, Bi, Fu, Noble, & Niedzwiecki, 2007).

8. CONCLUSION

A wide variety of mechanisms regulate sex determination/differentiation in amphibians. Despite advances in the isolation of the genes involved in sex determination/differentiation in other vertebrates, the roles of these genes in amphibians are still poorly understood. However, the recent discovery of DM-W in *X. laevis* is very promising. What is clear is the important role played by steroid hormones in the differentiation process, with a link between steroids, especially steroid-producing enzymes, and temperature-induced sex reversal. This role of steroids in gonad differentiation is recognized in other groups but is more discussed in mammals, depending on the model. Finally, the major implication of steroids in sex differentiation is one of the reasons why particular attention should be paid to amphibians. Indeed, due to the fact that their development is performed in aquatic environments, they are sensitive to a wide variety of pollutants. Endocrine disruptors have the possibility of disturbing reproduction at different levels, including those of sex differentiation (see Chapter 11, this volume), and such mechanisms could be involved in the worldwide decline of amphibian species (Stuart et al., 2004).

ABBREVIATIONS

17β-HSD	17β-Hydroxysteroid dehydrogenase
3β-HSD	3β-Hydroxysteroid dehydrogenase
5α-red	5α-Reductase
AMH	Anti-Müllerian hormone
AR	Androgen receptor
CYP11A1	P450 side chain cleaving enzyme
CYP17	17α-Hydroxylase
DMRT1	Doublesex- and mab-3-related transcription factor 1
DSS	Dosage-sensitive sex reversal
ER	Estrogen receptor
FGF	Fibroblast growth factor
GC	Germ cell
METRO	Message transport organizer
MIS	Müllerian-inhibiting substance
PGC	Primordial germ cell
SOX9	SRY-related high mobility group box 9
StAR	Steroidogenic acute regulatory protein
TSP	Thermosensitive period

REFERENCES

Amanuma, A. (1963a). Comparative study of the effect of androgen on the gonadal development in amphibia. *J. Biol. Osaka Univ., 14*, 25−46.

Amanuma, A. (1963b). Modification of gonadal development in *Rhacophorus schelgelii arborea* to the treatment with methyltestosterone. *J. Biol. Osaka Univ., 14*, 15−24.

Aoyama, S., Shibata, K., Tokunaga, S., Takase, M., Matsui, K., & Nakamura, M. (2003). Expression of Dmrt1 protein in developing and in sex-reversed gonads of amphibians. *Cytogenet. Genome Res., 101*, 295−301.

Bachvarova, R. F., Masi, T., Drum, M., Parker, N., Mason, K., Patient, R., et al. (2004). Gene expression in the axolotl germ line: Axdazl, Axvh, Axoct-4, and Axkit. Dev. *Dyn., 231*(4), 871−880.

Berset-Brandly, L., Jaquiery, J., & Perrin, N. (2006). A sex-specific marker reveals male heterogamety in European tree frogs. *Mol. Biol. Evol., 23*, 1104−1106.

Bogart, J. P. (2003). Genetics and systematics of hybrid species. In D. M. Sever (Ed.), *"Reproductive biology and phylogeny of Urodela" Vol. 1* (pp. 109−134). Enfield, New hampshire: M/s Science.

Bogart, J. P., Bi, K., Fu, J., Noble, D. W., & Niedzwiecki, J. (2007). Unisexual salamanders (genus *Ambystoma*) present a new reproductive mode for eukaryotes. *Genome, 50*(2), 119−136.

Bogart, J. P., Elinson, R. P., & Licht, L. E. (1989). Temperature and sperm incorporation in polyploid salamanders. *Science, 246*, 1032−1034.

Bogi, C., Levy, G., Lutz, I., & Kloas, W. (2002). Functional genomics and sexual differentiation in amphibians. *Comp. Biochem. Physiol. B, 133*, 559−570.

Capuron, A., & Maufroid, J. P. (1981). Role of the endoderm in the ventro−lateral mesoderm and primordial germ cells determination in *Pleurodeles waltlii*. *Arch. Anat. Microsc. Morphol. Exp., 70*(4), 219−226.

Chang, C. Y., & Witchi, E. (1955). Breeding of sex reversed males of *Xenopus laevis* Daudin. *Proc. Soc. Exp. Zool. Med., 93*, 140−144.

Chardard, D., & Dournon, C. (1999). Sex reversal by aromatase inhibitor treatment in the newt *Pleurodeles waltl*. *J. Exp. Zool., 283*, 43−50.

Chardard, D., Desvages, G., Pieau, C., & Dournon, C. (1995). Aromatase activity in larval gonads of *Pleurodeles waltl* during normal sex differentiation and sex reversal under thermal treatment effect. *Gen. Comp. Endocrinol., 99*, 100−107.

Chardard, D., Kuntz, S., Chesnel, A., & Flament, S. (2003). Effects of androgens on sex differentiation of the urodele *Pleurodeles waltl*. *J. Exp. Zool., 296*, 46−55.

Chardard, D., Penrad-Mobayed, M., Chesnel, A., Pieau, C., & Dournon, C. (2004). Thermal sex reversals in amphibians. In N. Valenzuela, & V. L. Lance (Eds.), *"Temperature-dependent sex determination in Vertebrates"* (pp. 59−67). Washington: Smithsonian Books.

Chieffi, G., Iela, L., & Rastogi, R. K. (1974). Effect of cyproterone, cyproterone acetate and ICI 46,474 on gonadal sex differentiation in *Rana esculenta*. *Gen. Comp. Endocrinol., 22*, 532−535.

Collenot, G., & Collenot, A. (1977). 3β-Hydroxysteroid dehydrogenase activity in the larval gonads of *Pleurodeles waltlii* (amphibian, urodele); a new histochemical technique for serial sections. *J. Embryol. Exp. Morphol., 42*, 29−42.

Dorazi, R., Chesnel, A., & Dournon, C. (1995). Opposite sex determination of gonads in two *Pleurodeles* species may be due to a temperature-dependent inactivation of sex chromosomes. *J. Heredity, 86*, 28−31.

Dournon, C., & Houillon, C. (1984). Démonstration génétique de l'inversion fonctionnelle du phénotype sexuel femelle sous l'action de la température d'élevage chez l'Amphibien Urodèle *Pleurodeles waltlii* Michah. *Reprod. Nutr. Dev., 24*, 361−378.

Dournon, C., & Houillon, C. (1985). Thermosensibilité de la différenciation sexuelle chez l'amphibien urodèle *Pleurodeles waltlii* Michah. Conditions pour obtenir l'inversion du phénotype sexuel de toutes les femelles génétiques sous l'action de la température d'élevage. *Reprod. Nutr. Dev., 25*, 671−688.

Dournon, C., Collenot, A., & Lauthier, M. (1988). Sex-linked peptidase-1 patterns in *Pleurodeles waltlii* Michah. (Urodele Amphibian): genetic evidence for a new codominant allele on the W sex chromosome and identification of ZZ, ZW and WW sexual genotypes. *Reprod. Nutr. Dev., 28*, 979−987.

Dournon, C., Houillon, C., & Pieau, C. (1990). Temperature sex-reversal in amphibians and reptiles. *Int. J. Dev. Biol., 34*, 81−92.

Dournon, C., Demassieux, C., Durand, D., & Lesimple, M. (1989). Primordial germ cell proliferation in the salamander *Pleurodeles waltl*: genetic control before gonadal differentiation. *Int. J. Dev. Biol., 33*(4), 477−485.

Downs, F. L. (1978). Unisexual *Ambystoma* from the Bass islands of Lake Erie. *Occ. Pap. Mus. Zool. Univ. Mich., 685*, 1−36.

Dumond, H., Kuntz, S., Chesnel, A., Ko, C. I., Wallacides, A., Chardard, D., et al. (2008a). Sexual development of the urodele amphibian. *Pleurodeles waltl Sex. Dev., 2*(2), 104−114.

Dumond, H., Maufroid, J. P., Ko, C. I., Chardard, D., Chesnel, A., & Flament, S. (2008b). Freemartin in the amphibian *Pleurodeles waltl*: parabiosis between individuals from opposite sex triggers both germ and somatic cells alterations during female gonad development. *Mol. Reprod. Dev., 75*, 439−449.

Eggert, C. (2004). Sex determination: the amphibian models. *Reprod. Nutr. Dev., 44*(6), 539−549.

El Jamil, A., Kanhoush, R., Magre, S., Boizet-Bonhoure, B., & Penrad-Mobayed, M. (2008). Sex-specific expression of SOX9 during gonadogenesis in the amphibian *Xenopus tropicalis*. *Dev. Dyn., 237*, 2996−3005.

Elinson, R. P., Bogart, J. P., Licht, L. E., & Lowcock, L. A. (1992). Gynogenetic mechanisms in polyploid hybrid salamanders. *J. Exp. Zool., 264*, 93−99.

Ezaz, T., Stiglec, R., Veyrunes, F., & Marshall Graves, J. (2006). Relationships between vertebrate ZW and XY sex chromosome systems. *Curr. Biol., 16*, 736−743.

Ferrier, V., Jaylet, A., Cayrol, C., Gasser, F., & Buisan, J. J. (1980). Etude électrophorétique des peptidases érythrocytaires chez *Pleurodeles waltlii* (Amphibien, Urodèle): mise en évidence d'une liaison avec le sexe. *C.R. Acad. Sci. Série D, 290*, 571−574.

Flament, S., Dumond, H., Chardard, D., & Chesnel, A. (2009). Lifelong testicular differentiation in *Pleurodeles waltl* (Amphibia, Caudata). *Reprod. Biol. Endocrinol., 7*(1), 21.

Gallien, L. (1951). Sur la descendance unisexuée d'une femelle de *Pleurodeles waltlii* Michah ayant subi pendant sa phase larvaire l'action gynogène du benzoate d'oestradiol. *C.R. Acad. Sci., 233*, 828−830.

Gallien, L. (1953). Inversion totale du sexe chez *Xenopus laevis* à la suite d'un traitement gynogène par le benzoate d'oestradiol, administré pendant la vie larvaire. *C.R. Acad. Sci. Paris, 240*, 1565−1566.

Gallien, L. (1962). Comparative activity of sexual steroids and genetic constitution in sexual differentiation of amphibian embryos. *Gen. Comp. Endocrinol. Supp1*. 346−355.

Grafe, T. U., & Linsenmair, K. E. (1989). Protogynous sex change in the reed frog *Hyperolius viridiflavus*. *Copeia, 1989*, 1024−1029.

Green, D. M. (1988). Cytogenetics of the endemic New Zealand frog, *Leiopelma hochstetteri*: extraordinary supernumerary chromosome variation and a unique sex-chromosome system. *Chromosoma, 97*, 55−77.

Hames, R. S., Hames, R., Prosser, S. L., Euteneuer, U., Lopes, C. A., Moore, W., Woodland, H. R., & Fry, A. M. (2008). Pix1 and Pix2 are novel WD40 microtubule-associated proteins that colocalize with mitochondria in *Xenopus* germ plasm and centrosomes in human cells. *Exp. Cell Res., 314*(3), 574−589.

Hayes, T. B. (1998). Sex determination and primary sex differentiation in amphibians: genetic and developmental mechanisms. *J. Exp. Zool., 281*, 373−399.

Heasman, J., Quarmby, J., & Wylie, C. C. (1984). The mitochondrial cloud of *Xenopus* oocytes: the source of germinal granule material. *Dev. Biol., 105*, 458−469.

Hedges, S. B., Bogart, J. P., & Maxson, L. R. (1992). Ancestry of unisexual salamanders. *Nature, 356*(6371), 708−710.

Hillis, D. M., & Green, D. M. (1990). Evolutionary changes of heterogametic sex in the phylogenetic history of amphibians. *J. Evol. Biol., 3*, 49−64.

Houston, D. W., & King, M. L. (2000a). Germ plasm and molecular determinants of germ cell fate. *Curr. Top. Dev. Biol., 50*, 155−181.

Houston, D. W., & King, M. L. (2000b). A critical role for Xdazl, a germ plasm-localized RNA, in the differentiation of primordial germ cells in Xenopus. *Development, 127*(3), 447−456.

Houston, D. W., Zhang, J., Maines, J. Z., Wasserman, S. A., & King, M. L. (1998). A Xenopus DAZ-like gene encodes an RNA component of germ plasm and is a functional homologue of Drosophila boule. *Development, 125*(2), 171−180.

Hsu, C. Y., Chiang, C. H., & Liang, H. M. (1977). A histochemical study on the development of hydroxysteroid dehydrogenases in tadpoles ovaries. *Gen. Comp. Endocrinol., 32*, 272−278.

Hsu, C. Y., Hsu, L. H., & Liang, H. M. (1979). The effect of cyproterone acetate on the activity of Δ5-3ß-hydroxysteroid dehydrogenase in tadpole sex transformation. *Gen. Comp. Endocrino., 39*, 404−410.

Hsu, C. Y., Yu, N. W., Ku, H. H., Chang, L. T., & Liu, H. W. (1991). Estradiol secretion by tadpole ovaries masculinized by implanted capsules of cyanoketone. *Develop. Growth. Differ., 33,* 210−208.

Hudson, C., & Woodland, H. R. (1998). Xpat, a gene expressed specifically in germ plasm and primordial germ cells of *Xenopus laevis*. *Mech. Dev., 73*(2), 159−168.

Humphrey, R. R. (1942). Sex of the offspring fathered by two *Amblystoma* females experimentally converted into males. *Anat. Rec., 82*, 469.

Ikenishi, K., Ohno, T., & Komiya, T. (2007). Ectopic germline cells in embryos of *Xenopus laevis*. *Develop. Growth Differ., 49*(7), 561−570.

Iwade, R., Maruo, K., Okada, G., & Nakamura, M. (2008). Elevated expression of P450c17 (CYP17) during testicular formation in the frog. *Gen. Comp. Endocrinol., 155*, 79−87.

Johnson, A. D., Bachvarova, R. F., Drum, M., & Masi, T. (2001). Expression of axolotl DAZL RNA, a marker of germ plasm: widespread maternal RNA and onset of expression in germ cells approaching the gonad. *Dev. Biol., 234*(2), 402−415.

Johnson, A. D., Drum, M., Bachvarova, R. F., Masi, T., White, M. E., & Crother, B. I. (2003). Evolution of predetermined germ cells in vertebrate embryos: implications for macroevolution. *Evol. Dev., 5* (4), 414−431.

Joly, J. (1971). Les cycles sexuels de *Salamandra* (L.). I—Cycle sexuel des males. *Ann. Sci. Nat., 13*, 451−504.

Kang, L., Marin, M., & Kelley, D. B. (1995). Androgen biosynthesis and secretion in developing *Xenopus laevis*. *Gen. Comp. Endocrinol., 100*, 293−307.

Kato, T., Matsui, K., Takase, M., Kobayashi, M., & Nakamura, M. (2004). Expression of P450 aromatase protein in developing and in sex-reversed gonads of the XX/XY type of the frog *Rana rugosa*. *Gen. Comp. Endocrinol., 137*, 227−236.

Kawano, K., Miura, I., Morohashi, K., Takase, M., & Nakamura, M. (1998). Molecular cloning and expression of the SF-1/Ad4BP gene in the frog *Rana rugosa*. *Gene, 222*(2), 169−176.

Kawano, K.-I., Furusawa, S., Matsuda, H., Takase, M., & Nakamura, M. (2001). Expression of steroidogenic factor-1 in frog embryo and developing gonad. *Gen. Comp. Endocrinol., 123*, 13−22.

King, M. L., Zhou, Y., & Bubunenko, M. (1999). Polarizing genetic information in the egg: RNA localization in the frog oocyte. *Bioessays, 21*, 546−557.

Kloc, M., & Etkin, L. D. (1995). Two distinct pathways for the localization of RNAs at the vegetal cortex in *Xenopus* oocytes. *Development, 121*(2), 287−297.

Kloc, M., Dougherty, M. T., Bilinski, S., Chan, A. P., Brey, E., King, M. L., et al. (2002). Three-dimensional ultrastructural analysis of RNA distribution within germinal granules of Xenopus. *Dev. Biol., 241*(1), 79−93.

Ko, C., Chesnel, A., Mazerbourg, S., Flament, S., & Chardard, D. (2008). Female-enriched expression of ERα during gonad differentiation of the urodele amphibian *Pleurodeles waltl*. *Gen. Comp. Endocrinol., 156*, 234−245.

Kuntz, S., Chardard, D., Chesnel, A., Grillier-Vuissoz, I., & Flament, S. (2003a). Steroids, aromatase and sex differentiation of the newt *Pleurodeles waltl*. *Cytogenet. Genome Res., 101*(3−4), 283−288.

Kuntz, S., Chardard, D., Ko, C. I., Dumond, H., Ducatez, M., Callier, M., et al. (2006). Female-enriched and thermosensitive expression of steroidogenic factor-1 during gonadal differentiation in *Pleurodeles waltl*. *J. Mol. Endocrinol., 36*(1), 175−186.

Kuntz, S., Chesnel, A., Duterque-Coquillaud, M., Grillier-Vuissoz, I., Callier, M., Dournon, C., et al. (2003b). Differential expression of P450 aromatase during gonadal sex differentiation and sex reversal of the newt *Pleurodeles waltl*. *J. Steroid Biochem. Mol. Biol., 84*(1), 89−100.

Lacroix, J. C. (1968a). Etude descriptive des chromosomes en écouvillon dans le genre *Pleurodeles* (Amphibien, Urodèle). *Ann. Embryo. Morph., 1*, 179−202.

Lacroix, J. C. (1968b). Variations expérimentales ou spontanées de la morphologie et de l'organisation des chromosomes en écouvillon dans le genre *Pleurodeles* (Amphibien, Urodèle). *Ann. Embryo. Morph., 1*, 205−248.

Lacroix, J. C. (1970). Mise en évidence sur les chromosomes en écouvillon de *Pleurodeles poireti* Gervais, Amphibien Urodèle, d'une structure liée au sexe, identifiant le bivalent sexuel et marquant le chromosome W. *C. R. Acad. Sci. Paris (D), 271*, 102−104.

Lacroix, J. C., Azzouz, R., Boucher, D., Abbadie, C., Pyne, C. K., & Charlemagne, J. (1985). Monoclonal antibodies to lampbrush chromosome antigens of *Pleurodeles waltlii*. *Chromosoma, 92*, 69−80.

Lacroix, J. C., Azzouz, R., Simon, F., Bellini, M., Charlemagne, J., & Dournon, C. (1990). Characterization of the W and Z lampbrush heterochromosomes in the newt *Pleurodeles waltl*. W chromosome plays a role in sex female determination. *Chromosoma, 99*, 307−414.

MacArthur, H., Houston, D. W., Bubunenko, M., Mosquera, L., & King, M. L. (2000). DEADSouth is a germ plasm specific DEAD-box RNA helicase in *Xenopus* related to eIF4A. *Mech Dev., 95*(1-2), 291−295.

Machado, R. J., Moore, W., Hames, R., Houliston, E., Chang, P., King, M. L., & Woodland, H. R. (2005). *Xenopus* Xpat protein is a major component of germ plasm and may function in its organisation and positioning. *Dev. Biol., 287*(2), 289−300.

Maruo, K., Suda, M., Yokoyama, S., Oshima, Y., & Nakamura, M. (2008). Steroidogenic gene expression during sex determination in the frog *Rana rugosa*. *Gen. Comp. Endocrinol., 158*, 87−94.

Matsuda, M., Nagahama, Y., Shinomiya, A., Sato, T., Matsuda, C., Kobayashi, T., Morrey, C. E., Shibata, N., Asakawa, S., Shimizu, N., Hori, H., Hamaguchi, S., & Sakaizumi, M. (2002). DMY is a Y-specific DM-domain gene required for male development in the medaka fish. *Nature, 417*(6888), 559−563.

Maufroid, J. P., & Capuron, A. (1973). Experimental demonstration of germinal cells in the presumptive lateral mesoderm of the young gastrula of *Pleurodeles waltii* (urodele amphibian). *C. R. Acad. Sci., 676*(5), 821−824.

Maufroid, J. P., & Capuron, A. (1977a). Induction of the mesoderm and primordial germ cells by the endoderm of *Pleurodeles waltlii* (Amphibia, Urodele): development during gastrulation. *C. R. Acad. Sci., 284*(17), 1713−1716.

Maufroid, J. P., & Capuron, A. (1977b). Recent researches on primordial germ cells of *Pleurodeles waltlii* (Amphibia, Urodele). *Mem. Soc. Zool. Fr., 41*, 43−60.

Maufroid, J. P., & Capuron, A. P. (1985). A demonstration of cellular interactions during the formation of mesoderm and primordial germ cells in *Pleurodeles waltlii*. *Differentiation, 29*(1), 20−24.

Mayer, L., Overstreet, S., Dyer, C., & Propper, C. (2002). Sexually dimorphic expression of steroidogenic factor 1 (SF-1) in developing gonads of the American bullfrog, *Rana catesbeiana*. *Gen. Comp. Endocrinol., 127*, 40−47.

Miura, I. (2008). An evolutionary witness: the frog *Rana rugosa* underwent change of heterogametic sex from XY male to ZW female. *Sex. Dev., 1*, 323−331.

Mosquera, L., Forristall, C., Zhou, Y., & King, M. L. (1993). A mRNA localized to the vegetal cortex of *Xenopus* oocytes encodes a protein with a nanos-like zinc finger domain. *Development, 117*(1), 377−386.

Myata, S., & Kubo, T. (2000). *In vitro* effects of estradiol and aromatase inhibitor treatment on sex differentiation in *Xenopus laevis* gonads. *Gen. Comp. Endocrinol., 119*, 105−110.

Nakajima, T., Takase, M., Miura, I., & Nakamura, M. (2000). Two isoforms of FTZ-F1 messenger RNA: molecular cloning and their expression in the frog testis. *Gene, 248*, 203−212.

Nardi, I., Andronico, F., De Lucchini, S., & Batistoni, R. (1986). Cytogenetics of the European plethodontid salamanders of the genus *Hydromantes* (Amphibia, Urodela). *Chromosoma, 94*, 377−388.

Niculita-Hirzel, H., Stöck, M., & Perrin, N. (2008). A key transcription factor on the nascent sex chromosome of European tree frogs (Hylea arborea). *Genetics, 179*, 1721−1723.

Nieuwkoop, P. O. (1947). Experimental investigations on the origin and determination of the germ cells, and on the development of the lateral plates and germ ridges in Urodeles. *Arch. Neerl. Zool., 8*, 1−205.

Normark, B. B., Judson, O. P., & Moran, N. A. (2003). Genomic signatures of ancient asexual lineages. *Biol. J. Linn. Soc., 79*, 69−84.

Norris, D. O., Carr., J. A., Summers, C. H., & Featherston, R. (1997). Interactions of androgens and estradiol on sex accessory ducts of larval tiger salamanders, *Ambystoma tigrinum*. *Gen. Comp. Endocrinol., 106*, 348−355.

Ogata, M., Ohtani, H., Hasegawa, Y., & Miura, I. (2008). The ZZ/ZW sex-determining mechanism originated twice and independently during evolution of the frog *Rana rugosa*. *Heredity, 100*, 92−99.

Ohtani, H., Miura, I., & Ichikawa, Y. (2003). Role of aromatase and androgen receptor expression in gonadal sex differentiation of ZW/ZZ frogs *Rana rugosa*. *Comp. Biochem. Physiol. C, 134*, 215−225.

Osawa, N., Oshima, Y., & Nakamura, M. (2005). Molecular cloning of Dmrt1 and its expression in the gonad of *Xenopus*. *Zool. Sci., 22*, 681−687.

Oshima, Y., Hayashi, T., Tokunaga, S., & Nakamura, M. (2005). Wnt4 expression in the differentiating gonad of the frog *Rana rugosa*. *Zoolog. Sci., 22*(6), 689−693.

Oshima, Y., Uno, Y., Matsuda, Y., Kobayashi, T., & Nakamura, M. (2008). Molecular cloning and gene expression of Foxl2 in the frog *Rana rugosa*. *Gen. Comp. Endocrinol., 159*(2-3), 170−177.

Padoa, E. (1936). Effetto paradosale (mascolinizzazione) sulla differenziazione sessuale di girini *Rana esculenta* trattati con ormone follicolare. *Mon. Zool. Ital., 47*, 285−289.

Pancak-Roessler, M. K., & Norris, D. O. (1991). The effects of orchidectomy and gonadotropins on steroidogenesis and oogenesis in Bidder's organs of the toad, *Bufo woodhousii*. *J. Exp. Zool., 260*, 323−336.

Penrad-Mobayed, M., Moreau, N., & Angelier, N. (1998). Evidence for specific RNA/protein interactions in the differential segment of the W chromosome in the amphibian *Pleurodeles waltl*. *Dev. Growth Differ., 40*, 147−156.

Piquet, J. (1930). Détermination du sexe chez les batraciens en fonction de la température. *Rev. Suisse Zool., 37*, 173−281.

Richards, C. M., & Nace, G. W. (1978). Gynogenetic and hormonal sex reversal used in tests of the XX-XY hypothesis of sex determination in *Rana pipiens*. *Growth, 42*, 319−331.

Sakata, N., Miyazaki, K., & Wakahara, M. (2006). Up-regulation of P450arom and down-regulation of Dmrt1 genes in the temperature-dependent sex reversal from genetic males to phenotypic females in a salamander. *Dev. Genes Evol., 216*, 224−228.

Sakata, N., Tamori, Y., & Wakahara, M. (2005). P450 aromatase expression in the temperature-sensitive sexual differentiation of salamander (*Hynobius retardatus*) gonads. *Int. J. Dev. Biol., 49*, 417−425.

Sakurai, N., Maruo, K., Haraguchi, S., Uno, Y., Oshima, Y., Tsutsui, K., Matsuda, Y., Do Rego, J. L., Pelletier, G., Vaudry, H., & Nakamura, M. (2008). Immunohistochemical detection and biological activities of CYP17 (P450c17) in the indifferent gonad of the frog *Rana rugosa*. *J. Steroid Biochem. Mol. Biol., 112*, 5−12.

Schempp, W., & Schmid, M. (1981). Chromosome banding in Amphibia. VI. BrdU-replication patterns in Anura and demonstration of XX/XY sex chromosomes in *Rana esculenta*. *Chromosoma, 83*, 697−710.

Schmid, M. (1980). Chromosome banding in Amphibia. V. Highly differentiated ZW/ZZ sex chromosomes and exceptional genome size in *Pyxicephalus adspersus*. *Chromosoma, 80*, 69−96.

Schmid, M., Haaf, T., Geile, B., & Sims, S. (1983). Chromosome banding in Amphibia. VIII. An unusual XY/XX-sex chromosome system in *Gastrotheca riobambae* (Anura, Hylidae). *Chromosoma, 88*(1), 69−82.

Schmid, M., Ohta, S., Steinlein, C., & Guttenbach, M. (1993). Chromosome banding in Amphibia. XIX. Primitive ZW/ZZ sex chromosomes in *Buergeria buergeri* (Anura, Rhacophoridae). *Cytogenet. Cell Genet., 62*(4), 238−246.

Schmid, M., Olert, J., & Klett, C. (1979). Chromosome banding in Amphibia. III. Sex chromosomes in *Triturus*. *Chromosoma, 71*, 29−55.

Schmid, M., Feichtinger, W., Steinlein, C., Haaf, T., Schartl, M., Visbal García, R., et al. (2002). Chromosome banding in Amphibia. XXVI. Coexistence of homomorphic XY sex chromosomes and a derived Y-autosome translocation in *Eleutherodactylus maussi* (Anura, Leptodactylidae). *Cytogenet. Genome Res., 99*(1-4), 330−343.

Schmid, M., & Steinlein, C. (2001). Sex chromosomes, sex-linked genes, and sex determination in the vertebrate class Amphibia. In G. Scherer, & M. Schmid (Eds.), *"Genes and mechanisms in vertebrate sex determination"* (pp. 143−176). Basel: Birkhäuser Verlag.

Sekizaki, H., Takahashi, S., Tanegashima, K., Onuma, Y., Haramoto, Y., & Asashima, M. (2004). Tracing of *Xenopus tropicalis* germ plasm and presumptive primordial germ cells with the *Xenopus tropicalis* DAZ-like gene. *Dev. Dyn., 229*(2), 367−372.

Sessions, S. K., & Wiley, J. E. (1985). Chromosome evolution in salamanders of the genus *Necturus*. *Brimleyana, 10*, 37−52.

Shibata, K., Takase, M., & Nakamura, M. (2002). The Dmrt1 expression in sex-reversed gonads of amphibians. *Gen. Comp. Endocrinol., 127*, 232−241.

Smita, M., Oommen, O. V., Jancy, M. G., & Akbarsha, M. A. (2004). Stages in spermatogenesis of two species of caecilians, *Ichthyophis tricolor* and *Uraeotyphlus cf. narayani* (Amphibia: Gymnophiona): Light and electron microscopic study. *J. Morphol., 261*(1), 92−104.

Smith, L. D. (1964). A test of the capacity of presumptive somatic cells to transform into primordial germ cells in the Mexican axolotl. *J. Exp. Zool., 156*, 229−242.

Stuart, S. N., Chanson, J. S., Cox, N. A., Young, B. E., Rodrigues, A. S., Fischman, D. L., & Waller, R. W. (2004). Status and trends of amphibian declines and extinctions worldwide. *Science, 306*(5702), 1783−1786.

Sugita, J., Takase, M., & Nakamura, M. (2001). Expression of Dax-1 during gonadal development of the frog. *Gene, 280*, 67−74.

Takase, M., Nakajima, T., & Nakamura, M. (2000a). FTZ-F1α is expressed in the developing gonad of frogs. *Biochim. Biophys. Acta, 1494*, 195−200.

Takase, M., Noguchi, S., & Nakamura, M. (2000b). Two Sox9 messenger RNA isoforms: isolation of cDNAs and their expression during gonadal development in the frog *Rana rugosa*. *FEBS Lett., 466*(2-3), 249−254.

Tamori, Y., Iwai, T., Mita, K., & Wakahara, M. (2004). Spatio−temporal expression of a DAZ-like gene in the Japanese newt *Cynops pyrrhogaster* that has no germ plasm. *Dev. Genes Evol., 214*(12), 615−627.

Tanimura, A., & Iwasawa, H. (1986). Development of the gonad and Bidder's organ in *Bufo japonicus formosus*: Histological observation. *Sci. Rep. Niigata Univ. Ser. D (Biology), 23*, 11−21.

Tiffoche, C., Chesnel, A., Jego, P., & Le Pennec, J. P. (1993). Isolation and characterization of a cDNA clone encoding a *Pleurodeles* lectin. *Eur. J. Biochem., 213*(3), 901−907.

Uchida, T. (1937a). Studies on the sexuality of amphibians. II. Sexual induction in a sexually semi-differentiated salamander. *J. Fac. Sci. Hokkaido Imp. Univ. (Zool.), 6*, 35−58.

Uchida, T. (1937b). Studies on the sexuality of amphibia. III. Sex transformation in *Hynobius retardatus* by the function of high temperature. *J. Fac. Sci. Hokkaido Imp. Univ. (Zool.), 6*, 59−70.

Uno, Y., Nishida, C., Oshima, Y., Yokoyama, S., Miura, I., Matsuda, Y., & Nakamura, M. (2008). Comparative chromosome mapping of sex-linked genes and identification of sex chromosomal rearrangements in the Japanese wrinkled frog (*Rana rugosa*, ranidae) with ZW and XY sex chromosome systems. *Chromosome Res., 16*, 637−647.

Uzzell, T. M. (1969). Notes on spermatophore production by salamanders of the *Ambystoma jeffersonianum* complex. *Copeia, 1969*, 602−612.

Wakahara, M. (1996). Primordial germ cell development: is the urodele pattern closer to mammals than to anurans? *Int. J. Dev. Biol., 40*, 653−659.

Wallace, H., Badawy, G. M. I., & Wallace, B. M. N. (1999). Amphibian sex determination and sex reversal. *Cell. Mol. Life Sciences, 55*, 901−909.

Wallace, H., & Wallace, B. M. N. (2000). Sex reversal of the newt *Triturus cristatus* reared at extreme temperatures. *Int. J. Dev. Biol., 44*, 807−810.

Wallacides, A., Chesnel, A., Chardard, D., Flament, S., & Dumond, H. (2009). Evidence for a conserved role of retinoic acid in urodele amphibian meiosis onset. *Dev. Dyn.* in press.

Witschi, E. (1914). Experimentelle Untersuchungen über die Entwicklungsgeschichte der Keimdrusen von *Rana temporaria*. *Arch. Mikr. Anat. Entw., 85*, 9−113.

Witschi, E. (1929a). Studies on sex differentiation and sex determination in amphibians. II. Sex reversal in female tadpoles of *Rana sylvatica* following the application of high temperature. *J. Exp. Zool., 52*, 267−291.

Witschi, E. (1929b). Studies on sex differentiation and sex determination in amphibians. III. Rudimentary hermaphroditism and Y chromosome in *Rana temporaria*. *J. Exp. Zool., 54*, 157−223.

Witschi, E. (1933). Studies in sex differentiation and sex determination in amphibians. VI. The nature of Bidder's organ in the toad. *Am. J. Anat., 52*, 461−515.

Yamamura, Y., Aoyama, S., Oshima, Y., Kato, T., Osawa, N., & Nakamura, M. (2005). Molecular cloning and expression in gonad of *Rana rugosa* WT1 and Fgf9. *Zool. Sci., 22*, 1045−1050.

Yoshikura, M. (1962). Effects of estrogens on the gonads of hypophysectomized frog larvae, with special references to the time factor in the responses of sex primordial. *Kunamoto J. Sci., 6*, 13−51.

Yoshimoto, S., Okada, E., Oishi, T., Numagami, R., Umemoto, H., Tamura, K., Kanda, H., Shiba, T., Takamatsu, N., & Ito, M. (2006). Expression and promoter analysis of *Xenopus* DMRT1 and functional characterization of the transactivation property of its protein. *Develop. Growth Differ., 48*, 597−603.

Yoshimoto, S., Okada, E., Umemoto, H., Tamura, K., Uno, Y., Nishida-Umehara, C., Matsuda, Y., Takamatsu, N., & Ito, M. (2008). A W-linked DM-domain gene, DM-W, participates in primary ovary development in *Xenopus laevis*. *Proc. Natl. Acad. Sci., 105*(7), 2469−2474.

Yu, N. W., Hsu, C. Y., Ku, H. H., Chang, L. T., & Liu, H. W. (1993). Gonadal differentiation and secretions of estradiol and testosterone of the ovaries of *Rana catesbeiana* tadpoles treated with 4-hydroxyandrostenedione. *J. Exp. Zool., 265*, 252−257.

Zaborski, P. (1986). Temperature and estrogen dependent changes of sex phenotype and HY antigen expression in gonads of a newt. In H. C. Slavkin (Ed.), *"Progress in Developmental Biology, Part A"* (pp. 163−169). New York: Alan R. Liss Inc.

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Chapter 2

Neuroendocrine Control of Reproduction in Amphibians

Pei-San Tsai
University of Colorado at Boulder, Boulder, CO, USA

SUMMARY
Neurohormones secreted by the brain serve as important intermediaries that transduce environmental signals to peripheral tissues for reproductive regulation. There are significant gaps in our knowledge regarding the identity and function of amphibian neurohormones that control reproduction. However, evidence is emerging that, like mammals, neuroendocrine regulation of amphibian reproduction is achieved principally through the actions of the gonadotropin-releasing hormone (GnRH) system. This chapter discusses the ontogeny, function, and regulation of the amphibian GnRH systems. Additional roles of GnRH in peripheral reproductive regulation will also be discussed. Examining the neuroendocrine basis of amphibian reproduction provides an important means for understanding how disruption of the reproductive brain could occur under perturbed environmental conditions, leading to impaired fertility and declining population.

1. INTRODUCTION

Studies from the past few decades have confirmed that amphibians possess a hypothalamo–pituitary–gonadal (HPG) axis that functions in a way highly comparable to homeothermic vertebrates (Licht & Porter, 1987). A class with more than 6400 extant species (Frost, 2009), amphibians occupy diverse habitats, undergo patterns of seasonal reproduction unique to individual species, and respond to a large number of environmental stimuli to coordinate reproductive activities (Rastogi et al., 2002). However, a common theme among the species examined is the ability of neurohormones produced by the brain to potently alter downstream reproductive events. As in mammals, amphibian neurohormones serve as critical intermediaries that transduce environmental signals to peripheral tissues for the activation or suppression of reproduction.

Because appropriate tools such as homologous radioimmunoassays (RIAs) and transgenic models were lacking, our understanding of how neurohormones influence amphibian reproduction has lagged behind the mammalian field. However, recent advances in proteomics and genomics have facilitated the identification of neuropeptide candidates that may serve as upstream reproductive regulators in amphibians. Many of these are orthologs of known mammalian neuroendocrine regulators. Although the picture is far from complete, we anticipate that progress in this field will soon occur at a pace faster than that seen historically.

Of the amphibians in which reproductive neuroendocrine regulation has been examined, studies on anurans predominate, and those on caudates lag somewhat behind. Unfortunately, very little is known about the caecilians. Because of this taxonomic bias, the majority of this chapter will focus on anurans. However, the other two orders (Caudata and Gymnophiona) will be represented when information is available. The goal of this chapter is to provide an overview of the development, anatomy, and function of several reproductively relevant neuroendocrine systems. Further, their influences on the reproductive brain, pituitary gonadotropin (GTH) secretion, and peripheral organs such as the gonad will be discussed.

2. GONADOTROPIN-RELEASING HORMONE (GnRH) SYSTEMS

2.1. An Overview of the Amphibian Hypothalamic–Pituitary System

All amphibians examined to date, including anurans and caudates, have two forms of GTH that are structurally and functionally homologous to mammalian luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Licht & McCreery, 1985). As in mammals, the stimulation of amphibian GTH release is achieved principally through gonadotropin-releasing hormone (GnRH) (Licht & McCreery, 1985). Luteinizing hormone and FSH then

stimulate downstream gonadal effects comparable to those seen in mammals (Muller & Licht, 1980). The only RIAs available to date for the measurement of amphibian GTHs were developed in the bullfrog (Daniels, Licht, Farmer, & Papkoff, 1977; Oguchi, Tanaka, Aida, Yamamoto, & Kikuyama, 1997). Thus, the only direct evidence on the secretory dynamics of GTHs in response to GnRH has come from ranid frogs.

An early demonstration that the amphibian brain governs GTH secretion (and thus reproduction) was conducted on the bullfrog, *Rana catesbeiana* (McCreery, 1984). In this study, electrical stimulation of the preoptic area (POA) of the brain significantly increased circulating LH and FSH. It is now known that the POA is where the majority of GnRH neurons reside in the amphibian brain. These GnRH axons terminate in the median eminence, from where GnRH is released into the hypophysial portal system and travels in the blood to the anterior pituitary gland. Electrical stimulation likely depolarizes these neurons, which respond by releasing GnRH from the axon terminals. That GnRH is the primary secretogogue of amphibian pituitary GTH secretion was confirmed by a series of experiments on ranid frogs in the 1980s. These early experiments (Daniels & Licht, 1980; McCreery, Licht, Barnes, Rivier, & Vale, 1982; McCreery & Licht, 1983; Licht et al., 1984; Porter & Licht, 1985) unambiguously demonstrated stimulatory effects of the mammalian form GnRH on GTH release. Later experiments on ranid frogs further established that GnRH stimulates not only GTH release but also synthesis (Stamper & Licht, 1993a; 1993b; 1994). It is now well accepted that GnRH is the principal neurohormone that activates amphibian reproduction. Thus, neuroendocrine control of amphibian reproduction is most likely achieved through the modulation of the GnRH system, as in mammals (Richter & Terasawa, 2001).

2.2. Ontogeny, Distribution, and Function of the GnRH Systems

Amphibians, like the majority of tetrapods, possess two anatomically and functionally distinct GnRH systems in the brain. According to the nomenclature proposed by White and Fernald (Fernald & White, 1999), these two systems are termed GnRH-I and GnRH-II based on their ontogeny, anatomical distribution, and function. The GnRH-I system is located in the POA–hypothalamic area and is thought to be hypophysiotropic (Fernald & White, 1999). The GnRH-II system is located in the more caudal brain regions, predominantly the midbrain, where GnRH-II serves as a neurotransmitter/neuromodulator (Fernald & White, 1999). Its presence in the spinal cord has also been reported (Muske & Moore, 1988). For interest, GnRH-II is also the neurotransmitter released from the presynaptic fibers to excite the bullfrog sympathetic ganglia (Troskie et al., 1997). The GnRH-I and -II systems have different developmental origins and serve different functions. The dominant projection of GnRH fibers to the median eminence comes from the GnRH-I system (Rastogi, Meyer, Pinelli, Fiorentino, & D'Aniello, 1998), supporting a primary role of the GnRH-I system in the regulation of GTH release. However, the projection of some GnRH-II fibers to the median eminence (Rastogi et al., 1998) and the presence of radioimmunoassayable GnRH-II in the anuran pituitary (Li & Lin, 2000) suggest GnRH-II may also be hypophysiotropic.

Thus far, two molecular forms of GnRH in the amphibian GnRH-I system have been definitively identified via molecular cloning or amino acid sequencing. These are the mammalian form of GnRH (mGnRH) reported for *Xenopus laevis* (Hayes, Wray, & Battey, 1994), *Rana ridibunda* (Conlon, Tonon, & Vaudry, 1993; Collin et al., 1995), and *R. catesbeiana* (Wang et al., 2001), and the ranid frog form of GnRH (rfGnRH) reported for the Korean frog, *Rana dybowskii* (Yoo et al., 2000) (Figure 2.1). Additional studies using high performance liquid chromatography (HPLC) and RIAs corroborated that the molecular form of GnRH-I in most amphibian brains is in fact mGnRH (King & Millar, 1986; Sherwood, Zoeller, & Moore, 1986; King, Steneveld, & Millar, 1994; Licht, Tsai, & Sotowska-Brochocka, 1994; Somoza, Paz, Stefano, & Affanni, 1996). A study has shown that *Rana esculenta* contains salmon GnRH (sGnRH) in its GnRH-I system (Fasano, Goos, Janssen, & Pierantoni, 1993). However, this finding cannot be verified (Licht et al., 1994). The consensus to date is that each amphibian species possesses one form of GnRH in its GnRH-I system and that this form is predominantly mGnRH.

The GnRH-I system in jawed vertebrates has an unusual developmental origin. Although the GnRH-I neurons reside within the POA–hypothalamic region of the adult brain, they do not originate within the brain. In fact, they come from the ectodermal thickening of the developing nose called the olfactory placode (Wray, 2002). Upon fate specification, GnRH-I neurons migrate along the pathways established by the olfactory and vomeronasal nerves to reach their final destinations in the forebrain. To the author's knowledge, the only other forebrain neurons to have a peripheral origin are the mesencephalic neurons in the trigeminal nucleus (Narayanan, C., & Narayanan, Y., 1978).

The olfactory origin of the vertebrate GnRH-I system has been widely accepted (Kah et al., 2007), but the first suggestion of such an unusual origin actually came from studies on amphibians (Muske & Moore, 1988; 1990). In an early hallmark paper, these authors observed the presence of GnRH immunoreactive (ir) neurons along the terminal nerve (TN), a component of the olfactory system, in the

Mammal	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2	GnRH-I
Ranid Frog	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Trp-Pro-Gly-NH2	GnRH-I
Chicken II	pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH2	GnRH-II

FIGURE 2.1 Amino acid sequences of three forms of GnRH found in amphibian brains. The GnRH-I system consists of mGnRH (mammalian GnRH) and rfGnRH (ranid frog GnRH), whereas only cGnRH-II (chicken II GnRH) is found in the GnRH-II system. Shaded amino acids are conserved in all forms.

rough-skinned newt (*Taricha granulosa*), the leopard frog (*Rana pipiens*), and the Pacific treefrog (*Psuedacris regilla*) (Muske & Moore, 1988). In all three species, especially the newt, the GnRH-ir neurons form a single anatomically continuous network that encompasses the anterior TN to the septal–hypothalamic area. From this architecture, the authors deduced that 'the most plausible hypothesis for the origin of these LHRH-containing neurons is that they are derived embryonically from the TN' (Muske & Moore, 1988, p.149). This was the first report to boldly suggest an olfactory origin of the GnRH-I system. A year later, two studies independently demonstrated the emergence of GnRH-I neurons from the mouse olfactory placode (Schwanzel-Fukuda & Pfaff, 1989; Wray, Grant, & Gainer, 1989), supporting the universality of this observation. Later amphibian studies that ablated the olfactory placode of the urodeles, *Cynops pyrrhogaster* and *Ambystoma mexicanum*, obliterated the GnRH-I system (Murakami, Kikuyama, & Arai, 1992; Northcutt & Muske, 1994) without affecting the GnRH-II system (Northcutt & Muske, 1994), further confirming the olfactory placode as the source of GnRH-I neurons in amphibians.

The anatomical distribution of the GnRH-I system shows considerable variations among adult amphibian species, but several generalizations can be made. In all amphibians surveyed (anurans, urodeles, and caecilians), GnRH-I neurons are found in the TN, the medial septum, and the POA (Muske & Moore, 1990; Conlon et al., 1993; Muske & Moore, 1994; Northcutt & Muske, 1994; D'Aniello et al., 1995; Iela et al., 1996; Pinelli et al., 1997; Rastogi et al., 1998). However, anurans have the most caudally located GnRH-I neurons that extend as far as the infundibular hypothalamus in the diencephalon (Rastogi et al., 1998). This caudal distribution was not seen in urodeles and caecilians, whose GnRH-I system fails to extend beyond the POA. Another unexpected finding is that, in *X. laevis*, GnRH-I neurons are present in the habenulae and habenular commissure, suggesting extra-hypothalamic roles not directly related to their hypophysiotropic function (Rastogi et al., 1998). Representative distributions of GnRH-I in three amphibian orders are shown in Figure 2.2.

For the GnRH-II system, less is known about its ontogenetic origin and reproductive involvement. In all vertebrates surveyed, the GnRH-II system consists of only one molecular form of GnRH, the chicken-II GnRH (cGnRH-II) (Figure 2.1), and amphibians are no exception to this rule. The GnRH-II system emerges earlier in ontogeny than the GnRH-I system and was first detected in the mesencephalon of premetamorphic tadpoles between stages 25 and 28 (D'Aniello et al., 1995), before the appearance of the forelimbs. At the time when the GnRH-II system was detected, GnRH-I was not seen. It is now well accepted that GnRH-II neurons do not share the same origin as the GnRH-I system. Two lines of evidence support this notion. First, the ablation of olfactory placodes from whence GnRH-I neurons originate did not affect the presence of GnRH-II neurons (Northcutt & Muske, 1994). Second, the earliest detection of GnRH-II neurons is consistently within the midbrain (D'Aniello et al., 1995; Iela et al., 1996). These observations lead to the hypothesis that GnRH-II neurons in amphibians may arise from the proliferation zones surrounding the midbrain ventricle (D'Aniello et al., 1995). This notion is corroborated by later studies in teleosts showing that GnRH-II neurons originate in the ependymal cells lining

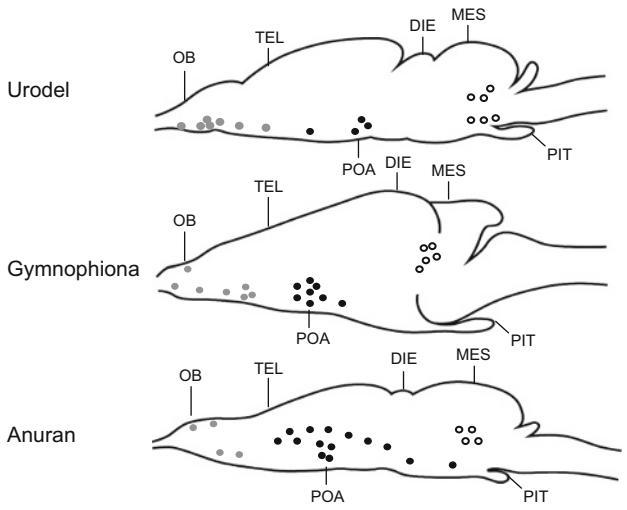

FIGURE 2.2 Representative diagrams illustrating neuroanatomical distribution of intracranial TN-GnRH-I neurons (gray circles), POA–hypothalamic GnRH-I neurons (black circles), and GnRH-II neurons (open circles) in the brains of three amphibian taxa. The brains are represented mid-sagittally with the rostral end facing the left. DIE, diencephalon; MES, mesencephalon; OB, olfactory bulb; PIT, pituitary; POA, preoptic area; TEL, telencephalon. *Compiled, modified, and redrawn from Rastogi, Meyer, Pinelli, Fiorentino, and D'Aniello, (1998).* Anatomical locations of intracranial TN-GnRH-I neurons are based on Muske and Moore (1988) and Pinelli et al. (1997).

the third ventricle (Parhar, Soga, Ishikawa, Nagahama, & Sakuma, 1998; White & Fernald, 1998).

By and large, the GnRH-II system in amphibian brains is more caudally distributed than GnRH-I. Considerable species differences in anatomical distribution exist in the GnRH-II system (Figure 2.2). One generalization that could be made for GnRH-II is its consistent presence in the midbrain tegmentum of all amphibian species surveyed (Muske & Moore, 1990; Conlon et al., 1993; Muske & Moore, 1994; Northcutt & Muske, 1994; D'Aniello et al., 1995; Iela et al., 1996; Pinelli et al., 1997; Rastogi et al., 1998). Compared to anurans and caecilians, urodeles appear to have a more rostrally distributed GnRH-II system that can also be found in the infundibular hypothalamus, paraventricular organ, and posterior tubercle (Rastogi et al., 1998) (Figure 2.2). In the bullfrog, GnRH-II fibers also are found in the hindbrain and the spinal cord. This is consistent with the role of GnRH-II as an excitatory preganglionic neurotransmitter in the bullfrog sympathetic ganglia (Jones, 1987). In the majority of cases, there is little neuroanatomical overlap between the distribution of GnRH-I and GnRH-II cell bodies, but, like GnRH-I fibers, some GnRH-II fibers project to the median eminence, suggesting a hypophysiotropic role for GnRH-II (Rastogi et al., 1998). The concentrations of GnRH-II in the brain also vary according to sex and reproductive stages (Rastogi, King, Di Fiore, D'Aniello, & Pinelli, 1997; Li & Lin, 2000). Thus, although GnRH-I is the dominant hypophysiotropic form in the amphibian brain, a role of GnRH-II in the neuroendocrine regulation of the pituitary cannot be excluded. Further, given the wide presence of GnRH-II projections in the brain, it also may participate in the regulation of amphibian reproductive behavior in a similar way to that shown in birds (Maney, Richardson, & Wingfield, 1997) and mammals (Temple, Millar, & Rissman, 2003). However, these are actions of GnRH-II as neurotransmitter/neuromodulator; they are beyond the scope of our current discussion on the actions of GnRH as a neurohormone.

2.3. The Pattern of GnRH Release from the Amphibian Brain

Successful reproduction in vertebrates is dependent not only upon the mere presence of GnRH-I but also the pattern of its release. In mammals, GnRH-I release from the median eminence occurs in discrete pulses (Karsch, Bowen, Caraty, Evans, & Moenter, 1997). Gonadotropin secretion in response to GnRH-I can only be maintained by this pulsatile mode of stimulation. If exposed continuously to GnRH-I, male and female mammalian pituitaries become desensitized to GnRH-I and consequently lose their ability to secrete GTHs (Smith & Vale, 1981), resulting in sterility (Belchetz, Plant, Nakai, Keogh, Knobil, 1978; Nett, Crowder, Moss, Duello, 1981). Interestingly, this phenomenon of pituitary desensitization is very widespread among vertebrates. Birds, some reptiles, and fishes are equally or even more susceptible to desensitization by continuous exposure to GnRH-I than are mammals (Licht & Porter, 1985; King, Davidson, & Millar, 1986; Habibi, 1991; Tsai & Licht, 1993). *In-vitro*, pituitary desensitization to GnRH-I can occur within the time frame of a few minutes (King et al., 1986; Tsai & Licht, 1993) to several hours (Smith & Vale, 1981; Badger, Loughlin, & Naddaff, 1983). Thus, the requirement for a pulsatile GnRH-I input appears to be a common feature shared by the majority of vertebrates examined so far.

In marked contrast to vertebrates, mentioned above, the episodic mode of GnRH-I stimulation is not required for the maintenance of pituitary functions in a few animals. The most striking example of deviation from such a requirement comes from studies on anuran amphibians. In bullfrogs, *R. catesbeiana*, plasma GTH levels can remain elevated for at least four days in response to constant GnRH-I infusion; in fact, the continuous GnRH-I treatment regimen that would lead to desensitization in mammals actually culminated in an ovulatory LH surge and triggered ovulation in the female bullfrog (McCreery & Licht, 1983). To demonstrate that this continuous *in-vivo* GTH elevation was not due to slow GTH clearance from the circulation, subsequent *in-vitro* perifusion studies were conducted. Again, even in an *in-vitro* system, pituitaries from two species of ranid frog (*R. catesbeiana* and *R. pipiens*) secreted GTH continuously for at least 48 hours under chronic GnRH-I stimulation (Porter & Licht, 1985; 1986a). Most unexpectedly, pituitaries from these frogs rapidly desensitized to five-minute GnRH-I pulses given every 30 minutes (Porter & Licht, 1986a). After 20 hours of GnRH treatments *in vitro*, GTH output remained maximal in pituitaries receiving continuous GnRH-I, but plummeted to baseline levels in glands receiving pulsatile GnRH-I stimulation. Thus, the dynamics of the hypothalamic–pituitary system in ranid frogs contrast sharply with those observed in mammals, birds, and some reptiles and teleosts. From these experiments, it seems likely that sustained elevation in GTH secretion in ranid frogs can be supported more effectively by continuous, rather than pulsatile, GnRH-I stimulation.

In addition to the pituitary data, studies further downstream of the pituitary also indirectly support the absence of a requirement for the pulsatile GnRH-I input in ranid frogs. In mammals, the temporal pattern of GTH secretion is tightly correlated with that of GnRH-I release, since GTH is directly stimulated by GnRH-I (Knobil, 1981). Likewise, continuous stimulation of mammalian gonads with GTH is normally less effective in inducing ovulation and in stimulating steroid secretion than episodic GTH stimulation (McNeilly, O'Connell, & Baird, 1982; Peluso, Gruenberg, &

Steger, 1984). Again in sharp contrast to mammals, ovarian fragments from both *R. catesbeiana* and *R. pipiens* release more steroid hormones with continuous, rather than episodic, GTH treatment (Hubbard & Licht, 1986). Most importantly, *in-vitro* oocyte maturation can only be induced under continuous GTH treatment; pulsatile GTH treatment was completely ineffective (Hubbard & Licht, 1986). These observations reflect a gonadal preference for a continuous stimulatory input that may be driven by the same mode of GnRH-I input two tiers upstream. Thus, two different systems of preference for the mode of GnRH-I challenge have emerged. The mammalian system, as well as that of birds, some reptiles, and fishes, is more effectively driven by pulsatile GnRH-I stimulation, and the system of ranid anurans more effectively driven by continuous GnRH-I stimulation.

In light of these observations, one might expect GnRH-I release in ranid frogs during the reproductive season to be tonically high and non-pulsatile. However, this has not been verified. Only one study has attempted to measure the pattern of GnRH-I release from amphibian hypothalamic explants (Tsai, Moenter, & Cavolina, 2003b) and the results do not support the continuous nature of GnRH-I release. In the study, acute hypothalamic explants were isolated from field-caught bullfrogs during the peak of the reproductive season and the *in-vitro* GnRH-I release over time was measured using a sensitive GnRH enzyme immunoassay. The level of GnRH-I release was very low and bordered the limit of assay detection. None of the explants exhibited continuous GnRH-I release at detectable levels, but 37.5% (three out of eight) field-caught frogs showed a single distinct pulse during the two-hour period of incubation (Figure 2.3) (Tsai et al., 2003b).

Overall, the low levels of GnRH-I released from the anuran hypothalamus pose a significant challenge to understanding the dynamic patterns of GnRH-I secretion. One may be able to use LH as a surrogate to infer the pattern of endogenous GnRH-I secretion, but such a study has not been done. The peculiar preference of the ranid frog pituitary to continuous GnRH-I stimulation is an unusual phenomenon that warrants further investigation at the cellular and molecular levels. To the author's knowledge, such a preference for continuous GnRH-I has not been studied in any other amphibians except ranid frogs.

2.4. Structure and Function of GnRH Receptors in Amphibians

Like all other vertebrate GnRH receptors (GnRHRs), amphibian GnRHRs are rhodopsin-like seven-transmembrane G-protein-coupled receptors (GPCRs). In order to understand amphibian GnRHRs, the nomenclature for GnRHRs must be defined first. The classification of GnRHRs in the literature has unfortunately been inconsistent and confusing due to the *ad hoc* assignment of names after cloning. In 2004, a three-clade nomenclature for vertebrate GnRHR types (types 1, 2, and 3) was proposed based on gene organization, the presence of three clades of vertebrate GnRHs, and the presence of a cytoplasmic tail at the carboxyl (C)-terminal of GnRHRs (Millar et al., 2004). However, this nomenclature has not been adopted consistently (Guilgur, Moncaut, Canario, & Somoza, 2006; Kah et al., 2007) because it lacks phylogenetic, structural, or functional support (Guilgur et al., 2006). The more recent reviews tend to classify GnRHRs into two types (types 1 and 2), based on the length of the C-terminal tail (Guilgur et al., 2006) or their affinities for GnRH-I and GnRH-II (Kah et al., 2007). For simplicity and consistency, this chapter will use the nomenclature proposed by Kah et al.

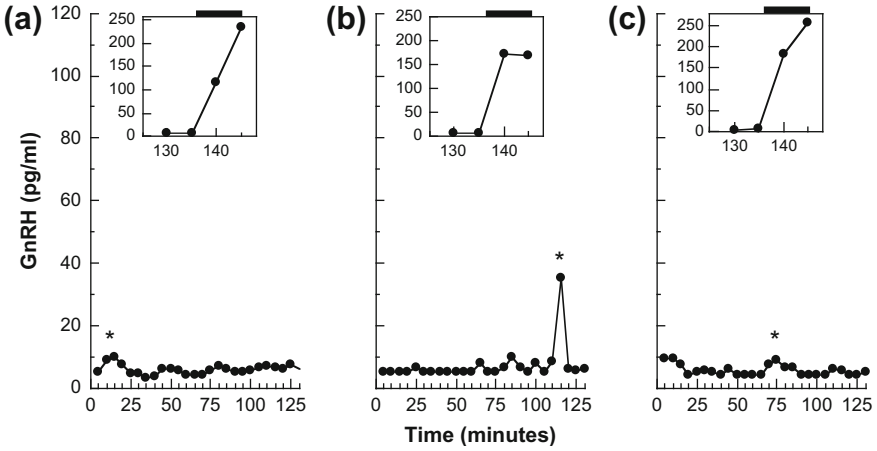

FIGURE 2.3 Representative profiles of *in-vitro* GnRH-I release from acute bullfrog hypothalamic explants. Each asterisk denotes a significant pulse. Inset: GnRH-I release in response to a veratridine challenge (thick black bar) to ensure the viability of explants at the end of the experiment. *Adapted from Tsai, Moenter, and Cavellina (2003).*

(2007), which classifies GnRHRs with higher affinity for GnRH-I as type 1 GnRHR, and those with higher affinity for GnRH-II as type 2 GnRHR. The nomenclature of type 3 GnRHR will not be used here because it received little support from the recent phylogenetic analysis (Guilgur et al., 2006).

To the author's knowledge, amphibian GnRHRs have been cloned and sequenced from three species of ranid frog*:* *R. catesbeiana* (bullfrog; Wang et al. (2001)), *R. ridibunda* (GenBank accession number AY260153-260155), and *R. esculenta* (GenBank accession number FM163433-163435). Gonadotropin-releasing hormone receptors were also cloned from the African clawed frog *X. laevis* (Troskie, Hapgood, Millar, and Illing (2000); GenBank accession number AF257320) and a caecilian *Typhlonectes natans* (GenBank accession number AF174481). Each of the ranid frogs possesses three forms of GnRHRs (GnRHR1, 2, and 3), but only two and one form of GnRHR have been cloned from *X. laevis* and *T. natans*, respectively. Available functional studies indicate that all anuran GnRHRs have higher affinities for GnRH-II than GnRH-I (Troskie et al., 2000; Wang et al., 2001) and should be categorized as type 2 GnRHRs. This classification is strictly functional and bears no relationship to the names originally given to three anuran receptors (GnRHR1, 2, and 3). The most complete functional studies were conducted on bullfrog GnRHRs (bfGnRHRs); therefore, our discussion will be focused largely on these receptors. However, structural similarities in GnRHRs among the ranid frog species suggest functional data from the bullfrog may be extrapolated to *R. ridibunda* and *R. esculenta*.

All three bfGnRHRs are classified as type 2 GnRHRs because of their preference for GnRH-II (Kah et al., 2007). The three bfGnRHRs share sequence similarities of 53% or less (Wang et al., 2001). bfGnRHR1 is expressed predominantly in the pituitary, and bfGnRHR2 and 3 are expressed primarily in the brain (Wang et al., 2001). Functional studies show that bfGnRHRs interact with specific G-proteins. bfGnRHR2 preferentially couples to G_s, whereas bfGnRHR1 and 3 couple to G_s and $G_{q/11}$ equally (Oh et al., 2003). This scenario differs from the mammalian GnRHR, which preferentially couples to $G_{q/11}$, at least in the gonadotropes (Millar et al., 2004). Both the calcium ionophore (A23187) and a diacylglycerol analog (phorbol myristate acetate) stimulated LH release from cultured pituitaries isolated from the leopard frog (Porter & Licht, 1986b). Thus, LH secretion from both ranid and mammalian pituitaries is responsive to the activation of the phosphatidylinositol pathway, despite their differential preference for G-protein coupling.

Structural analysis indicates that all amphibian GnRHRs, including bullfrog GnRHRs, possess a C-terminal tail. This is intriguing considering the C-terminal tail is the site that, upon phosphorylation, binds to β-arrestin to induce the uncoupling of GPCRs from the G protein, thereby facilitating homologous desensitization (Naor, 2009). As discussed earlier, the bullfrog pituitary does not become refractory to continuous GnRH stimulation and can secrete LH for days. This is in stark contrast with the observation that inositol phosphate accumulation in bfGnRHR-transfected HEK293 cells is susceptible to homologous desensitization (Archarjee et al., 2002). Further, all three bfGnRHRs undergo internalization *in vivo*, albeit at different rates (Archarjee et al., 2002). The internalization of all three receptors is dynamin-dependent, but only the internalization of bfGnRHR1 is also β-arrestin-dependent (Archarjee et al., 2002). In this regard, the primary GnRHR (bfGnRHR1) responsible for pituitary GnRH-I responsiveness appears to be highly susceptible to homologous desensitization. If so, how could one explain the persistent secretion of LH from the bullfrog pituitary in the face of continuous GnRH challenge (Porter & Licht, 1985; 1986a)?

At present, this question cannot be answered definitively. The most plausible explanation for this paradox lies downstream of receptor activation. Mammalian GnRHR lacks a C-terminal tail and is resistant to homologous desensitization, but LH secretion and inositol phosphate accumulation do become attenuated under constant exposure to GnRH-I (Naor, 2009). It is thought that this refractory state reflects the attenuation of downstream signaling events rather than the actual receptor desensitization (Naor, 2009). Along this line of reasoning, one could hypothesize that the post-receptor signaling in the bullfrog pituitary may be unusually resistant to the reduction in receptor efficacy, or GnRHR can be readily replenished and/or recycled in the presence of GnRH-I. In this regard, bullfrog gonadotropes may offer unique insights into receptor dynamics and signaling events that contribute to continuous GnRH-I responsiveness.

3. REGULATION OF THE GnRH-I SYSTEM

3.1. An Overview

Although several neurochemicals have been shown to alter reproductive function in amphibians (Rastogi et al., 2002), only the ones with the potential to influence the GnRH-I system are discussed below. As a result of the difficulty in measuring GnRH-I release in amphibians, relatively few functional studies have examined factors that might govern the activity of the GnRH-I system. In most cases, LH levels, GnRH-I neuronal presence and morphology, and GnRH mRNA have been used as surrogates for GnRH-I neuronal activity. The regulatory roles of neuropeptides upon the GnRH-I system are inferred from the types of afferents that project to GnRH-I neurons and the presence of neuropeptide receptors on GnRH-I neurons.

3.2. Modulation of the GnRH-I System by Gonadal Steroid Hormones

The GnRH-I system in vertebrates is highly sensitive to the actions of steroid hormones. In particular, gonadal steroids, such as 17β-estradiol (E_2) and 5α-dihydrotestosterone (DHT), alter the function of the GnRH system either directly or indirectly via other steroid-sensitive synaptic afferents (Terasawa & Fernandez, 2001). Earlier *in-vivo* and *in-vitro* studies on ranid frogs support a role of E_2 and DHT in the negative feedback of the HPG axis (McCreery & Licht, 1984; Pavgi & Licht, 1989; 1993). These studies established the pituitary as a direct target for the inhibitory actions of gonadal steroids; however, their effects upon the GnRH-I system were not investigated.

Previously, a study showed that the presence of GnRH-I neurons in the male and female ranid frog, *R. esculenta*, is highly dependent on gonadal steroids (Iela, D'Aniello, Di Meglio, & Rastogi, 1994). Ten weeks after gonadectomy, the presence of GnRH-I neurons was drastically reduced. Replacement with E_2 and/or DHT restored the presence of GnRH-I neurons (Iela et al., 1994). This study shows that accumulation of GnRH-I in neuronal cell bodies is enhanced by gonadal steroids. In this respect, both estrogens and androgens appear indispensable for the long-term maintenance of adequate levels of GnRH-I. Similarly, parallel studies in mammals show that long-term castrated male rodents had markedly diminished GnRH in the hypothalami, and estrogen or androgen replacement could restore GnRH-I contents back to pre-castration levels (Gross, 1980; Kalra, P., & Kalra, S., 1980; Roselli, Kelly, & Ronnekleiv, 1990).

Interestingly, gonadal steroids also have inhibitory effects on the GnRH-I system in mammals (Spratt & Herbison, 1997; Toranzo et al., 1989). There is evidence to suggest similar inhibitory effects in the amphibian GnRH-I system. Tsai and Jones (2005) showed that either E_2 or DHT implants for 20 days significantly increased the size of GnRH-I soma in gonadally intact male leopard frogs (Figure 2.4). To better interpret the biological significance of enlarged GnRH-I soma, circulating LH and testicular mass were measured in these implanted frogs to assess reproductive activity. Concomitant with neuronal enlargement was a significant reduction in the levels of circulating LH and testicular mass. These findings suggest that enlarged GnRH-I neurons likely release lower levels of GnRH-I since they are associated with reduced LH output and gonad size. The large soma size could therefore be a consequence of GnRH accumulation in the cell body due to the lack of release (Tsai & Jones, 2005). Consistent with

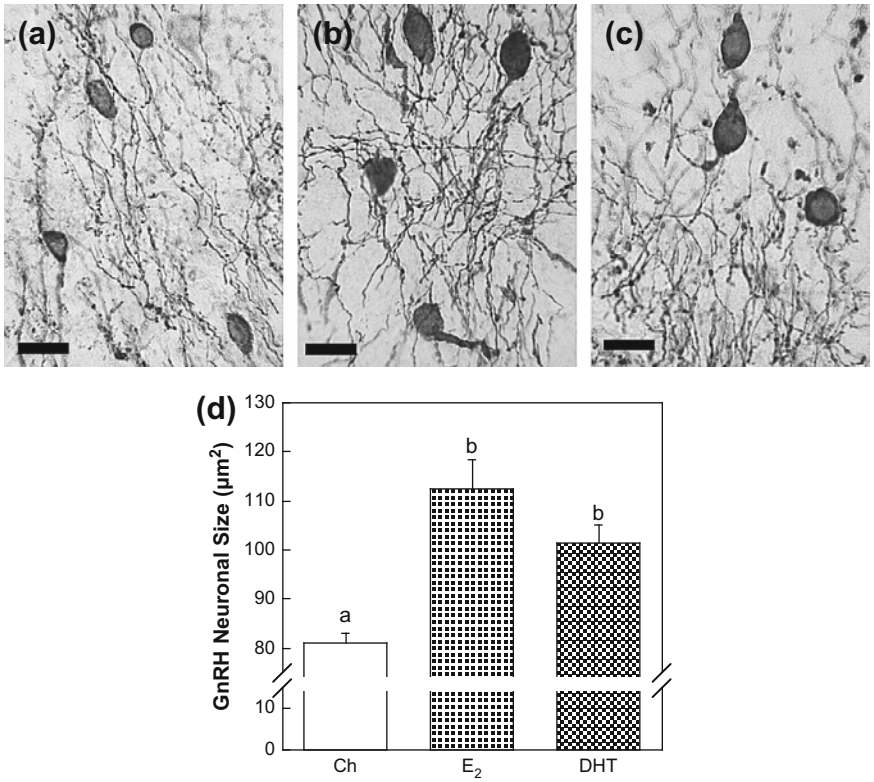

FIGURE 2.4 GnRH-I neurons in the POA of (a) control, (c) E_2-implanted, and (c) DHT-implanted male leopard frogs. (d) Morphometric analysis shows that the soma size of GnRH-I neurons is significantly enlarged in steroid-implanted animals. *Adapted from Tsai and Jones (2005).*

this interpretation, acute hypothalamic explants isolated from male leopard frogs that were implanted for three weeks with E_2 released significantly more GnRH-I when challenged with veratridine, an agent that opens voltage-gated Na^+ channels. These results suggest that E_2 promotes the accumulation of releasable GnRH-I, possibly through the inhibition of spontaneous GnRH-I release (Tsai, Lunden, & Jones, 2003a). In this respect, both E_2 and DHT are likely acting as negative feedback regulators on the GnRH-I system to suppress downstream reproductive activities. However, one should note that gonadal steroids are also indispensable for the long-term maintenance of GnRH-I synthetic activity. Future studies that examine the presence of estrogen or androgen receptors in amphibian GnRH-I neurons are needed to ascertain whether gonadal steroids can directly affect these neurons.

3.3. Modulation of the GnRH-I System by Dopamine (DA)

In mammals, dopamine (DA) innervation of GnRH-I neurons has been described (Ajika, 1980; Jennes, Stumpf, & Tappaz, 1983), and DA exerts significant effects on GnRH-I release (Weiner, Findell, & Kordon, 1988). The inhibitory effects of DA on GTH secretion also have been documented in diverse taxa ranging from fishes to birds (reviewed by Dufour et al., 2005). An ultrastructural study conducted on the newt *Triturus alpestris* demonstrated axo-axonal apposition between fibers positive for GnRH-I and tyrosine-hydroxylase (the rate-limiting enzyme in catecholamine synthesis) in the median eminence (Corio, Thibault, & Peute, 1990), suggesting regulation of GnRH terminals by dopaminergic input.

A few studies have investigated the neuroendocrine regulation of the amphibian GnRH-I system by DA, and these were conducted on the ranid frog, *Rana temporaria*. Sotowska-Brochocka (1988) showed that ovulation in hibernating *R. temporaria* can be advanced by lesioning the infundibular hypothalamus. It was shown later that these lesions resulted in the elevation of GnRH-I, leading to elevated LH and ovulation (Sotowska-Brochocka & Licht, 1992). Whereas a DA antagonist advanced ovulation during hibernation, a DA agonist significantly suppressed the lesion-induced rise in LH and blocked advanced ovulation (Sotowska-Brochocka, Martynska, & Licht, 1994). The authors suggested that DA could play a role in constraining GnRH-I release during reproductive quiescence (hibernation). However, a direct inhibitory effect of DA on pituitary GTH cannot be excluded. To this author's knowledge, no other functional studies exist to corroborate these observations in other amphibian species. The significant inhibitory role of DA in fish reproduction (Dufour et al., 2005) certainly encourages further exploration on the involvement of DA in the neuroendocrine control of amphibian reproduction.

3.4. Modulation of the GnRH-I System by Endocannabinoids

Endocannabinoids have recently gained attention as important regulators of vertebrate reproductive function (Wang, Dey, & Maccarrone, 2006). In addition to their well-established effects on sperm physiology (Rossato, Pagano, & Vettor, 2008), their involvement in the neuroendocrine regulation of reproduction has begun to emerge (Gammon, Freeman, Xie, Petersen, & Wetsel, 2005). Immortalized mouse GnRH neuronal cell lines were shown to synthesize two forms of endocannabinoids and directly respond to an exogenous cannabinoid agonist with attenuated GnRH release (Gammon et al., 2005). Interestingly, such a neuroendocrine role has recently been described in anurans.

Immunoreactivity for CB1, an endocannabinoid receptor was present in the telencephalon of both *X. laevis* and *R. esculenta* (Cottone, Guastalla, Mackie, & Franzoni, 2008; Meccariello et al., 2008). A fraction (~20%) of GnRH-I neurons are colocalized with CB1 in *R. esculenta* (Cottone et al., 2008; Meccariello et al., 2008). Levels of CB1 and GnRH-I mRNA exhibit a reciprocal relationship over the course of seasonal reproduction. *In-vitro* treatment of acute hypothalamic explants with a cannabinoid agonist significantly lowered the GnRH-I message, whereas treatment with a GnRH agonist significantly lowered the GnRH-I message and elevated the CB1 message (Meccariello et al., 2008). Thus, the GnRH-I and endocannabinoids form a feedback loop of reciprocal inhibition. The authors postulated that there may be a negative feedback circuit in which endogenous GnRH-I could regulate its own synthesis via the endocannabinoid system (Meccariello et al., 2008).

3.5. Modulation of the GnRH-I System by RFamides

RFamides are short peptides possessing the common C-terminal motif of Arg-Phe-NH_2. These peptides form a large family of bioactive molecules with diverse physiological actions (Chartrel et al., 2006). The recent discovery of two amphibian RFamides (R-RFa and 26RFa) with hypophysiotropic activities (Koda et al., 2002; Chartrel et al., 2003) sparked further interest in a role of these peptides in the neuroendocrine regulation of amphibian physiology.

Although neither R-RFa nor 26RFa has been shown to stimulate GTH release in amphibian bioassays (Koda et al., 2002; Chartrel et al., 2003), several lines of indirect

evidence suggest possible effects of RFamides on GnRH-I neurons. First, R-RFa is an ortholog of avian GTH-inhibiting hormone (GnIH) (Chartrel et al., 2006). Gonadotropin-inhibiting hormone not only inhibits GTH secretion but is also postulated to inhibit GnRH activity in birds (Tsutsui et al., 2007). Second, R-RFa-ir fibers project to the POA, where GnRH-I neurons reside, and to the external zone of the median eminence (Koda et al., 2002; Chartrel et al., 2003). Such neuroanatomical distribution suggests that this peptide is positioned to interact with GnRH-I soma and axon terminals. Third, an earlier study showed that anuran GnRH-I neurons are surrounded, and on occasion innervated, by fibers that are immunoreactive for FMRFamide (Rastogi et al., 2002). Although the identity of FMRFamide-ir antigen is unknown, it is most likely an RFamide. These data provide indirect support for a possible role of RFamides in regulating the GnRH-I system.

Recently, exciting new evidence has emerged to show that kisspeptins, products of the KiSS genes, are in fact present in a wide range of vertebrates including amphibians. Kisspeptins, also called metastins, belong to the RFamide family and were first discovered for their roles in suppressing tumor metastasis (Roa, Aguilar, Dieguez, Pinilla, & Tena-Sempere, 2008). Kisspeptins are now commonly hailed as the 'gatekeepers of puberty' because of their potent stimulatory actions on the mammalian GnRH-I system (Roa et al., 2008). Null or loss-of-function mutations in KiSS or the gene for its cognate receptor, GPR54, result in hypogonadotropic hypogonadism in mammals (de Roux et al., 2003; Funes et al., 2003; Seminara et al., 2003; d'Anglemont de Tassigny et al., 2007). The reproductive effects of kisspeptins in non-mammalian vertebrates are poorly explored, but several lines of evidence in amphibians encourage this way of thinking.

To date, several forms of KiSS and GPR54 have been cloned from anurans: two and three forms of KiSS from *X. laevis* and *Xenopus tropicalis*, respectively, and three and one form of GPR54 from *X. tropicalis* and *R. catesbeiana*, respectively (Biran, Ben-Dor, & Levavi-Sivan, 2008; Lee et al., 2009; Moon et al., 2009). In *Xenopus*, KiSS genes are categorized into two families, KiSS-1 and KiSS-2. KiSS-1 can further be categorized into two isoforms, KiSS-1a and 1*b*, in *X. tropicalis* (Lee et al., 2009). Of these isoforms, kisspeptin-2-ir cell bodies are found in the POA and ventral hypothalamus, and kisspeptin-2-ir fibers project to the median eminence. This neuroanatomical distribution is highly suggestive of a role of kisspeptin-2 in the neuroendocrine regulation of the pituitary. Indeed, two forms of GPR54 are expressed in the pituitary, suggesting that kisspeptin-2 is very likely hypophysiotropic. Further, kisspeptin-2-ir fibers project to the POA, where GnRH-I neurons reside, supporting a role of kisspeptin-2 in the regulation of the GnRH-I system. Overall, recent advances in comparative kisspeptin biology have shed light on the conservation and functional significance of these well-conserved peptides. An understanding of kisspeptin biology in amphibians, where progress is being made rapidly, will no doubt help us understand how these peptides evolved to become the indispensable reproductive gatekeepers seen in mammals.

3.6. Modulation of the GnRH-I System by Mesotocin/Opsin-positive Neurons

Amphibian reproduction is highly seasonal (Rastogi et al., 2002). The seasonal bouts of reproductive activity are cued by environmental signals such as temperature and photoperiod. In addition to photoreceptors in the retina, deep-brain opsin-based photoreceptors may play a role in transducing photoperiodic signals to the appropriate brain regions for reproductive activation (Bellingham & Foster, 2002).

In *X. laevis*, cone-opsin and melanopsin are found in the neuroendocrine neurons of the nucleus magnocellularis preopticus (Foster, Grace, Provencio, Degrip, & Garcia-Fernandez, 1994; Provencio, Jiang, De Grip, Hayes, & Rollag, 1998). Interestingly, many cone-opsin-positive neurons are also positive for mesotocin (MST) (Alvarez-Viejo, Cernuda-Cernuda, DeGrip, Alvarez-Lopez, & Garcia-Fernandez, 2003). Further, these cone-opsin and MST-ir fibers are co-mingled with GnRH-I fibers in the basal telencephalon, ventromedial hypothalamus, and median eminence (Alvarez-Viejo et al., 2003). These data raise the interesting possibility that the amphibian GnRH-I system receives inputs from photosensitive mesotocinergic afferents, and these inputs may contribute to the seasonal activation of the GnRH-I system.

3.7. Modulation of the GnRH-I System by Social and Environmental Cues

In many vertebrates, the GnRH-I system responds to environmental signals. The number and size of GnRH-I neurons can be altered dynamically by social, auditory, and courtship cues (Propper & Moore, 1991; Francis, Soma, & Fernald, 1993; Dellovade & Rissman, 1994; Cheng, Peng, & Johnson, 1998; Burmeister & Wilczynski, 2005). Several studies suggest that the amphibian GnRH-I system is also sensitive to such environmental stimuli. For example, male green treefrogs, *Hyla cinerea*, form lek-like aggregations during the mating season to create a chorus for attracting females. When reproductively regressing male frogs were exposed to a mating chorus for 10 days, the number of their GnRH-I neurons increased significantly compared to males exposed to control tones. Androgen levels also increased significantly in chorus-exposed males. The neurocircuitry underlying this change has been postulated (Burmeister & Wilczynski, 2005). In anurans, the

auditory thalamus and a midbrain auditory nucleus send strong projections to the ventral hypothalamus and weaker projections to the POA (Neary, 1988; Wilczynski, 1988; Allison & Wilczynski, 1991; Wilczynski, Allison, & Marler, 1993). In this regard, GnRH-I neurons in the POA may receive inputs either directly from the auditory afferents or indirectly from the ventral hypothalamus. The dynamic alteration of the GnRH-I system by sensory cues speaks to the extraordinary plasticity underlying the activation of this system. Further, it suggests that sensory and neuroendocrine circuitries can be integrated to achieve reproductive activation.

In another study conducted on the female newt, *T. granulosa*, exposure to courtship behavior resulted in an increase in the TN-GnRH-I concentration and circulating E_2 (Propper & Moore, 1991). The time-course for this increase was surprisingly rapid, with changes seen within 20 minutes of exposure. However, GnRH-I concentrations in the POA and hypothalamus were not altered. The amphibian TN-GnRH-I neurons (Figure 2.2) represent a subpopulation of olfactory placode-derived GnRH-I neurons that presumably terminated migration before reaching the POA—hypothalamic area. Like the more caudal GnRH-I neurons, TN-GnRH-I neurons are also regulated by gonadal steroids in amphibians (Wirsig-Wiechmann & Lee, 1999). A hypophysiotropic role of TN-GnRH-I neurons in amphibians has not been established, since it is unclear whether TN-GnRH-I fibers actually target the median eminence. However, a role for TN-GnRH-I in amphibian olfaction has been shown (discussed below). Thus, one might hypothesize that courtship rapidly alters the TN-GnRH-I system, which could further alter important sensory feedback such as olfaction during courtship and subsequent mating.

4. THE ROLES OF PERIPHERAL GnRH IN REPRODUCTION

4.1. Overview

Although the most well-defined reproductive role of central GnRH-I is the stimulation of GTH secretion, GnRH (both the GnRH-I and II forms) is also found in the periphery. The peripheral GnRH appears to participate in reproductive functions not directly related to pituitary regulation. It is unlikely that GnRH acts as a neurohormone while assuming these less conventional peripheral roles. However, because of the novel nature of these roles, they will be discussed briefly below.

4.2. GnRH-I and Chemosensory Function

With the exception of birds, chemosensory signals are universally cues for vertebrate reproduction. The TN is an anterior cranial nerve that appears to participate in the regulation of reproductive behavior and chemosensory perception in many vertebrates (Wirsig & Leonard, 1987; Propper & Moore, 1991; Yamamoto, Oka, & Kawashima, 1997). A common marker for the TN in developing and postnatal vertebrates is the presence of GnRH neurons (Wirsig-Wiechmann et al., 2002). In amphibians, a population of TN-GnRH-I neurons is located outside the brain and embedded within the olfactory and vomeronasal nerves in the nasal cavity; these peripheral TN-GnRH-I neurons and their fibers, along with a few neurochemical markers, constitute the extracranial TN (Wirsig-Wiechmann, Wiechmann, & Eisthen, 2002). Because of the proximity of extracranial TN-GnRH-I neurons to chemosensory structures and afferents, they are suitably positioned for the regulation of chemosensory functions.

In salamanders, TN-GnRH-I fibers were found to terminate in the vicinity of the olfactory epithelium (Eisthen, Delay, Wirsig-Wiechmann, & Dionne, 2000). Although no direct contact between GnRH fibers and olfactory epithelium was observed (Eisthen et al., 2000; Koza & Wirsig-Wiechmann, 2001), their close proximity suggests that GnRH-I could be released in a paracrine fashion to modulate the function of olfactory sensory neurons (OSNs). The electrophysiological effects of GnRH-I on OSNs have been studied in a number of salamander species. The effects are diverse, ranging from excitatory to inhibitory (Eisthen et al., 2000; Park & Eisthen, 2003; Zhang & Delay, 2007). For interest, GnRH-I may modulate the responsiveness of OSNs in a manner that is both concentration- and odorant-dependent (Park & Eisthen, 2003). Overall, these studies provide further impetus for exploring a novel and relatively unstudied role of GnRH-I as a chemosensory modulator. The effects of GnRH-I on OSNs appear to be context-dependent and could play a role in the sensory feedback during courtship and mating.

4.3. Peripheral GnRH in Gonadal Regulation

The peripheral expression of GnRH has been shown in many tissues (Walters, Wegorzewska, Chin, Parikh, & Wu, 2008). Of special interest is the expression of GnRH in gonadal tissues, which appears to locally regulate steroidogenesis and gametogenesis. The origin of these peripheral GnRH cells is not known; thus, neither the terminology of GnRH-I nor GnRH-II will be used here. GnRH-ir materials have been demonstrated in amphibian gonads (Cariello et al., 1989; Battisti et al., 1994; Di Matteo, Vallarino, & Pierantoni, 1996), and this gonadal GnRH presumably regulates several aspects of gonadal physiology.

Studies on *R. esculenta* demonstrated that the injection of GnRH or a GnRH agonist induced spermiation

(Minucci, Di Matteo, Chieffi Baccari, & Pierantoni, 1989; Minucci, Fasano, D'Antonio, & Pierantoni, 1993), androgen production (Di Matteo et al., 1988; 1990), and spermatogonial proliferation (Minucci et al., 1986; Di Matteo et al., 1988; Minucci, Di Matteo, Fasano, Baccari, & Pierantoni, 1992) in hypophysectomized male frogs. These results indicate that GnRH could bypass the pituitary to achieve these outcomes. The effects of a GnRH agonist could be direct upon the testes, since GnRH binding sites were identified in the testes of these frogs (Fasano, de Leeuw, Pierantoni, Chieffi, & van Oordt, 1990). The direct actions of GnRH upon the gonads were in fact verified by *in-vitro* studies showing that exogenously administered GnRH or a GnRH agonist could stimulate steroid production and spermatogonial proliferation in testicular and ovarian fragments (Pierantoni et al., 1984; Minucci et al., 1986; Di Matteo et al., 1988; Minucci et al., 1989; Di Matteo et al., 1990; Zerani, Gobbetti, & Polzonetti-Magni, 1991). Although the precise mechanisms underlying the direct gonadal effects of GnRH are not clear, it has been suggested that prostaglandin $F_{2\alpha}$, produced under GnRH stimulation, could mediate GnRH's effects on steroidogenesis (Gobbetti & Zerani, 1992).

It should be noted that the stimulatory effects of GnRH directly upon the gonads are reported primarily for one species of ranid frog, *R. esculenta*. There may be considerable species variations in these effects, since GnRH failed to alter steroidogenesis in *R. catesbeiana* and *R. pipiens* (Hubbard & Licht, 1985) but had inhibitory effects on steroid production in the toad, *Bufo arenarum* (Canosa, Pozzi, Somoza, & Ceballos, 2002). Overall, the direct effect of GnRH upon the gonads is a phenomenon that needs to be verified in other amphibian taxa.

5. NONAPEPTIDES AND NEUROSTEROIDS

Nonapeptides and steroids have been shown to regulate diverse amphibian reproductive behaviors ranging from calling, courtship, and amplexus, to female sexual receptivity (Moore & Miller, 1983; Wetzel & Kelley, 1983; Boyd, 1994; 1997; Klomberg & Marler, 2000; Thompson & Moore, 2000; 2003). The precise actions of nonapeptides and steroids on amphibian behavior are discussed elsewhere (see Chapter 8, this volume). However, recent studies have revealed interesting interactions between the nonapeptides and neurosteroid production, shedding light on the neuroendocrine circuitry involved in the regulation of some reproductive behaviors.

It has been known for some time that amphibian brains have the capability to synthesize neurosteroids (Gobbetti, Zerani, & Cardellini, 1992; Mensah-Nyagan et al., 1994; 1999; Takase, Ukena, Yamazaki, Kominami, & Tsutsui, 1999). In fact, key enzymes involved in both Δ4 and Δ5 steroid synthesis pathways are present in amphibian brains and the *de novo* synthesis of neurosteroids has been demonstrated. These neurosteroids presumably could affect many steroid-sensitive brain regions to elicit neuroendocrine or behavioral effects. Several neurochemicals, including GABA and neuropeptide Y, have been shown to modulate neurosteroid production (do-Rego et al., 2000; Beaujean et al., 2002).

Recently, do-Rego et al. (2006) reported the ability of the nonapeptides arginine vasotocin (AVT) and MST to stimulate the formation of several neurosteroids, including progesterone and dehydroepiandrosterone, in the brain of *R. esculenta*. Abundant AVT and MST receptors are found in the anterior POA, lateral and medial amygdala, posterior tuberculum, nucleus of the periventricular organ, and dorsal and ventral hypothalamic nuclei, all of which express steroidogenic enzymes (do-Rego et al., 2006). Further, neurons containing steroidogenic enzymes (3β-hydroxysteroid dehydrogenase and $P450_{c17}$) in the anterior POA, posterior tuberculum, and medial amygdala are contacted by vasotocinergic fibers (do-Rego et al., 2006). From these data, the authors hypothesized that the behavioral effects of nonapeptides could be mediated, in part, through the synthesis of neurosteroids. These results are exciting in that they provide evidence for a direct involvement of nonapeptides in the *de novo* synthesis of neurosteroids. Downstream activation of steroid-sensitive brain regions can then occur to elicit diverse effects on reproductive behavior, GnRH-I system regulation, or even sexual differentiation of the reproductive brain.

6. FUTURE DIRECTIONS

Although great progress has been made regarding our understanding of reproductive physiology in amphibians, much of the effort has focused on peripheral reproductive tissues. The way in which the brain controls reproductive function via neurohormones remains a largely uncharted territory and is a field that has just begun to gain attention. The known and potential neuroendocrine regulators of amphibian reproduction are summarized in Figure 2.5.

GnRH-I in amphibians, as in other vertebrates, assumes a central and a critical role in the activation of the HPG axis. However, there is virtually nothing known about how the GnRH-I system is controlled by afferent inputs from other brain regions. Important questions remain unanswered regarding whether the amphibian GnRH-I system is directly steroid-responsive, what neurotransmitter receptors are expressed in GnRH-I neurons, and what are the upstream regulators of the GnRH-I system. The recent discovery of kisspeptins in *Xenopus* introduces a promising candidate that could act as a higher-tier activator of the GnRH-I system, but this line of research has just begun. Further, the roles of TN-GnRH-I and GnRH-II as

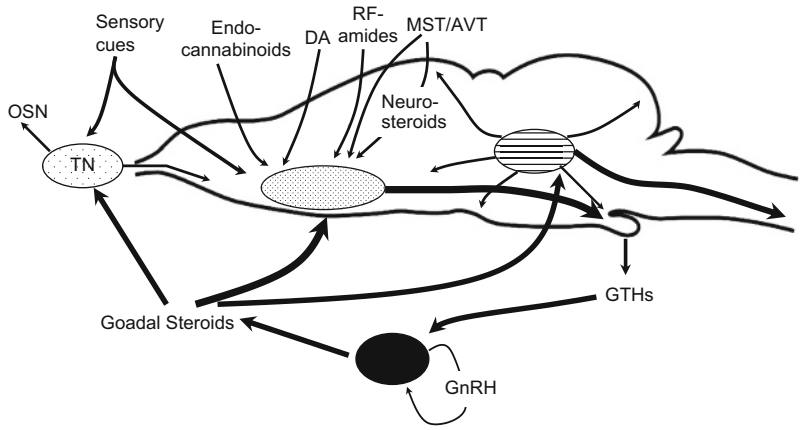

FIGURE 2.5 Schematic diagram illustrating the regulation and functions of central and peripheral GnRH systems of amphibians. The TN-GnRH-I system is represented by the sparsely dotted zone; the forebrain GnRH-I system is represented by the densely dotted zone; the midbrain GnRH-II system is represented by the horizontally hatched zone; the gonadal GnRH system is represented by the solid black zone. The projections of the TN- and central GnRH fibers are indicated by arrows emanating from the respective zones. The thickness of the arrows represents the strength of these efferent projections. The paracrine effects of gonadal GnRH are indicated by a closed-loop arrow in this zone. Lastly, regulatory inputs upon the TN- and central GnRH systems are indicated by arrows that point to these zones. The thickness of these arrows represents the strength of regulation.

reproductive regulators remain poorly explored. Lastly, with the exception of GnRH-I, the neuroendocrine factors that alter pituitary GTH secretion remain virtually unknown, since homologous GTH RIAs are unavailable in all amphibians except the bullfrog.

In light of the declining amphibian population, the focus on amphibian reproduction needs to be shifted from peripheral tissues, such as the gonad and the pituitary, to the brain. The reproductive brain is extraordinarily plastic and could be highly sensitive to hormone mimetics and other disrupting chemicals from the environment (see Chapter 11, this volume). In fact, with environmental perturbation, neuroendocrine functions could be disrupted even before deleterious changes in the gonad are seen. Impaired neuroendocrine function could then lead to the dysregulation in the sensory perception of reproductive cues, courtship and mating, and pituitary GTH secretion. In this regard, impaired neuroendocrine function will lead to suboptimal or absent reproduction even in animals with normal gonads. Understanding neuroendocrine control of amphibian reproduction is therefore not a goal that pertains merely to the satisfaction of intellectual curiosity. It promises to shed light on how we can gauge the reproductive performance of declining amphibian populations in the face of multiple environmental challenges.

ABBREVIATIONS

AVT	Arginine vasotocin
cGnRH-II	Chicken-II gonadotropin-releasing hormone
DA	Dopamine
DHT	5α-dihydrotestosterone
E_2	17β-estradiol
FMRFamide	Phenylalanine−methionine−arginine−phenylalanine amide
FSH	Follicle-stimulating hormone
GnIH	GTH-inhibiting hormone
GnRH	Gonadotropin-releasing hormone
GnRHR	Gonadotropin-releasing hormone receptor
GPCR	G-protein-coupled receptor
GPR54	Kisspeptin receptor
GTH	Gonadotropin
HPG	Hypothalamus−pituitary−gonadal
HPLC	High-performance liquid chromatography
ir	Immunoreactive
LH	Luteinizing hormone
LHRH	see GnRH
mGnRH	Mammalian gonadotropin-releasing hormone
MST	Mesotocin
OSN	Olfactory sensory neuron
POA	Preoptic area
RFa	RFamides
rfGnRH	Ranid frog gonadotropin-releasing hormone
RIA	Radioimmunoassay
sGnRH	Salmon gonadotropin-releasing hormone
TN	Terminal nerve

REFERENCES

Acharjee, S., Maiti, K., Soh, J. M., Im, W. B., Seong, J. Y., & Kwon, H. B. (2002). Differential desensitization and internalization of three different bullfrog gonadotropin-releasing hormone receptors. *Mol. Cells, 14*, 101−107.

Ajika, K. (1980). Relationship between catecholaminergic neurons and hypothalamic hormone-containing neurons in the hypothalamus. In L. Martini, & W. F. Ganong (Eds.), *"Frontiers in Neuroendocrinology", Vol. 6* (pp. 1−32). New York: Raven Press.

Allison, J. D., & Wilczynski, W. (1991). Thalamic and midbrain auditory projections to the preoptic area and ventral hypothalamus in the green treefrog (*Hyla cinerea*). *Brain Behav. Evol., 38*, 322−331.

Alvarez-Viejo, M., Cernuda-Cernuda, R., DeGrip, W. J., Alvarez-Lopez, C., & Garcia-Fernandez, J. M. (2003). Co-localization of mesotocin and opsin immunoreactivity in the hypothalamic preoptic nucleus of *Xenopus laevis*. *Brain Res., 969*, 36−43.

Badger, T. M., Loughlin, J. S., & Naddaff, P. G. (1983). The luteinizing hormone-releasing hormone (LHRH)-desensitized rat pituitary: luteinizing hormone responsiveness to LHRH *in vitro*. *Endocrinology, 112*, 793−799.

Battisti, A., Vallarino, M., Carnevali, O., Fasano, S., Polzonetti-Magni, A., & Pierantoni, R. (1994). Detection and localization of gonadotrophin-releasing hormone (GnRH)-like material in the frog, *Rana esculenta*, ovary. *Comp. Biochem. Physiol. A Physiol., 109*, 1097−1103.

Beaujean, D., Do-Rego, J. L., Galas, L., Mensah-Nyagan, A. G., Fredriksson, R., Larhammar, D., Fournier, A., Luu-The, V., Pelletier, G., & Vaudry, H. (2002). Neuropeptide Y inhibits the biosynthesis of sulfated neurosteroids in the hypothalamus through activation of Y(1) receptors. *Endocrinology, 143*, 1950−1963.

Belchetz, P. E., Plant, T. M., Nakai, Y., Keogh, E. J., & Knobil, E. (1978). Hypophysial responses to continuous and intermittent delivery of hypopthalamic gonadotropin-releasing hormone. *Science, 202*, 631−633.

Bellingham, J., & Foster, R. G. (2002). Opsins and mammalian photoentrainment. *Cell Tissue Res., 309*, 57−71.

Biran, J., Ben-Dor, S., & Levavi-Sivan, B. (2008). Molecular identification and functional characterization of the kisspeptin/kisspeptin receptor system in lower vertebrates. *Biol Reprod., 79*, 776−786.

Boyd, S. K. (1994). Arginine vasotocin facilitation of advertisement calling and call phonotaxis in bullfrogs. *Horm. Behav., 28*, 232−240.

Boyd, S. K. (1997). Brain vasotocin pathways and the control of sexual behaviors in the bullfrog. *Brain Res. Bull., 44*, 345−350.

Burmeister, S. S., & Wilczynski, W. (2005). Social signals regulate gonadotropin-releasing hormone neurons in the green treefrog. *Brain Behav. Evol., 65*, 26−32.

Canosa, L. F., Pozzi, A. G., Somoza, G. M., & Ceballos, N. R. (2002). Effects of mGnRH on testicular steroidogenesis in the toad *Bufo arenarum*. *Gen. Comp. Endocrinol., 127*, 174−180.

Cariello, L., Romano, G., Spagnuolo, A., Zanetti, L., Fasano, S., Minucci, S., Di Matteo, L., Pierantoni, R., & Chieffi, G. (1989). Molecular forms of immunoreactive gonadotropin-releasing hormone in hypothalamus and testis of the frog, *Rana esculenta*. *Gen. Comp. Endocrinol., 75*, 343−348.

Chartrel, N., Bruzzone, F., Leprince, J., Tollemer, H., Anouar, Y., Do-Rego, J. C., et al. (2006). Structure and functions of the novel hypothalamic RFamide neuropeptides R-RFa and 26RFa in vertebrates. *Peptides, 27*, 1110−1120.

Chartrel, N., Dujardin, C., Anouar, Y., Leprince, J., Decker, A., Clerens, S., et al. (2003). Identification of 26RFa, a hypothalamic neuropeptide of the RFamide peptide family with orexigenic activity. *Proc. Natl. Acad. Sci. USA, 100*, 15247−15252.

Cheng, M. F., Peng, J. P., & Johnson, P. (1998). Hypothalamic neurons preferentially respond to female nest coo stimulation: demonstration of direct acoustic stimulation of luteinizing hormone release. *J. Neurosci., 18*, 5477−5489.

Collin, F., Chartrel, N., Fasolo, A., Conlon, J. M., Vandesande, F., & Vaudry, H. (1995). Distribution of two molecular forms of gonadotropin-releasing hormone (GnRH) in the central nervous system of the frog *Rana ridibunda*. *Brain Res., 703*, 111−128.

Conlon, J. M., Tonon, M. C., & Vaudry, H. (1993). Isolation and structural characterization of calcitonin gene-related peptide from the brain and intestine of the frog, *Rana ridibunda*. *Peptides, 14*, 581−586.

Corio, M., Thibault, J., & Peute, J. (1990). Topographical relationships between catecholamine- and neuropeptide-containing fibers in the median eminence of the newt, *Triturus alpestris*. An ultrastructural immunocytochemical study. *Cell Tissue Res., 259*, 561−566.

Cottone, E., Guastalla, A., Mackie, K., & Franzoni, M. F. (2008). Endocannabinoids affect the reproductive functions in teleosts and amphibians. *Mol. Cell Endocrinol., 286*, S41−45.

d'Anglemont de Tassigny, X., Fagg, L. A., Dixon, J. P., Day, K., Leitch, H. G., Hendrick, A. G., et al. (2007). Hypogonadotropic hypogonadism in mice lacking a functional Kiss1 gene. *Proc. Natl. Acad. Sci. USA, 104*, 10714−10719.

D'Aniello, B., Pinelli, C., Di Fiore, M. M., Tela, L., King, J. A., & Rastogi, R. K. (1995). Development and distribution of gonadotropin-releasing hormone neuronal systems in the frog (*Rana esculenta*) brain: immunohistochemical analysis. *Brain Res. Dev. Brain Res., 89*, 281−288.

Daniels, E., & Licht, P. (1980). Effects of gonadotropin-releasing hormone on the levels of plasma gonadotropins (FSH and LH) in the bullfrog, *Rana catesbeiana*. *Gen. Comp. Endocrinol., 42*, 455−463.

Daniels, E. L., Licht, P., Farmer, S. W., & Papkoff, H. (1977). Immunochemical studies on the pituitary gonadotropins (FSH and LH) from the bullfrog, *Rana catesbeiana*. *Gen. Comp. Endocrinol., 32*, 146−157.

Dellovade, T. L., & Rissman, E. F. (1994). Gonadotropin-releasing hormone-immunoreactive cell numbers change in response to social interactions. *Endocrinology, 134*, 2189−2197.

de Roux, N., Genin, E., Carel, J. C., Matsuda, F., Chaussain, J. L., & Milgrom, E. (2003). Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. *Proc. Natl. Acad. Sci. USA, 100*, 10972−11097.

Di Matteo, L., Vallarino, M., & Pierantoni, R. (1996). Localization of GnRH molecular forms in the brain, pituitary, and testis of the frog, *Rana esculenta*. *J. Exp. Zool., 274*, 33−40.

Di Matteo, L., Minucci, S., Fasano, S., D'Antonio, M., Pierantoni, R., & Chieffi, G. (1990). Indirect evidence for a physiological role exerted by a "testicular gonadotropin-releasing hormone" in the frog, *Rana esculenta*. *Gen. Comp. Endocrinol., 79*, 147−153.

Di Matteo, L., Minucci, S., Fasano, S., Pierantoni, R., Varriale, B., & Chieffi, G. (1988). A gonadotropin-releasing hormone (GnRH) antagonist decreases androgen production and spermatogonial multiplication in frog (*Rana esculenta*): indirect evidence for the existence of GnRH or GnRH-like material receptors in the hypophysis and testis. *Endocrinology, 122*, 62−67.

do-Rego, J. L., Acharjee, S., Seong, J. Y., Galas, L., Alexandre, D., Bizet, P., et al. (2006). Vasotocin and mesotocin stimulate the biosynthesis of neurosteroids in the frog brain. *J. Neurosci., 26*, 6749−6760.

do-Rego, J. L., Mensah-Nyagan, G. A., Beaujean, D., Vaudry, D., Sieghart, W., Luu-The, V., Pelletier, G., et al. (2000). gamma-Aminobutyric acid, acting through gamma-aminobutyric acid type A receptors, inhibits the biosynthesis of neurosteroids in the frog hypothalamus. *Proc. Natl. Acad. Sci. USA, 97*, 13925−13930.

Dufour, S., Weltzien, F.-A., Sebert, M.-E., Le Belle, N., Vidal, B., Vernier, P., et al. (2005). Dopaminergic inhibition of reproduction in teleost fishes. *Ann. NY Acad. Sci., 1040*, 9–21.

Eisthen, H. L., Delay, R. J., Wirsig-Wiechmann, C. R., & Dionne, V. E. (2000). Neuromodulatory effects of gonadotropin releasing hormone on olfactory receptor neurons. *J. Neurosci., 20*, 3947–3955.

Fasano, S., Goos, H. J., Janssen, C., & Pierantoni, R. (1993). Two GnRHs fluctuate in correlation with androgen levels in the male frog *Rana esculenta*. *J. Exp. Zool., 266*, 277–283.

Fasano, S., de Leeuw, R., Pierantoni, R., Chieffi, G., & van Oordt, P. G. (1990). Characterization of gonadotropin-releasing hormone (GnRH) binding sites in the pituitary and testis of the frog, *Rana esculenta*. *Biochem. Biophys. Res. Commun., 168*, 923–932.

Fernald, R. D., & White, R. B. (1999). Gonadotropin-releasing hormone genes: phylogeny, structure, and functions. *Front Neuroendocrinol., 20*, 224–240.

Foster, R. G., Grace, M. S., Provencio, I., Degrip, W. J., & Garcia-Fernandez, J. M. (1994). Identification of vertebrate deep brain photoreceptors. *Neurosci. Biobehav. Rev., 18*, 541–546.

Francis, R. C., Soma, K., & Fernald, R. D. (1993). Social regulation of the brain–pituitary–gonadal axis. *Proc. Natl. Acad. Sci. USA, 90*, 7794–7798.

Frost, D. (12 February, 2009). Amphibian Species of the World: an Online Reference. *Version, 5.3*. Electronic Database accessible at. http://research.amnh.org/herpetology/amphibia/2009.

Funes, S., Hedrick, J. A., Vassileva, G., Markowitz, L., Abbondanzo, S., Golovko, A., et al. (2003). The KiSS-1 receptor GPR54 is essential for the development of the murine reproductive system. *Biochem. Biophys. Res. Commun., 312*, 1357–1363.

Gammon, C. M., Freeman, G. M., Jr., Xie, W., Petersen, S. L., & Wetsel, W. C. (2005). Regulation of gonadotropin-releasing hormone secretion by cannabinoids. *Endocrinology, 146*, 4491–4499.

Gobbetti, A., & Zerani, M. (1992). Mammalian GnRH involvement in prostaglandin F2 alpha and sex steroid hormones testicular release in two amphibian species: the anuran water frog, *Rana esculenta*, and the urodele crested newt, *Triturus carnifex*. *Gen. Comp. Endocrinol., 87*, 240–248.

Gobbetti, A., Zerani, M., & Cardellini, L. B. (1992). Relationships among mammalian gonadotropin-releasing hormone, prostaglandins, and sex steroids in the brain of the crested newt, *Triturus carnifex*. *Prostaglandins, 44*, 209–218.

Gross, D. S. (1980). Effect of castration and steroid replacement on immunoreactive gonadotropin-releasing hormone in hypothalamus and preoptic area. *Endocrinology, 106*, 1442–1450.

Guilgur, L. G., Moncaut, N. P., Canario, A. V., & Somoza, G. M. (2006). Evolution of GnRH ligands and receptors in gnathostomata. *Comp. Biochem. Physiol. A Mol. Integr. Physiol., 144*, 272–283.

Habibi, H. R. (1991). Homologous desensitization of gonadotropin-releasing hormone (GnRH) receptors in the goldfish pituitary: effects of native GnRH peptides and a synthetic GnRH antagonist. *Biol. Reprod., 44*, 275–283.

Hayes, W. P., Wray, S., & Battey, J. F. (1994). The frog gonadotropin-releasing hormone-I (GnRH-I) gene has a mammalian-like expression pattern and conserved domains in GnRH-associated peptide, but brain onset is delayed until metamorphosis. *Endocrinology, 134*, 1835–1845.

Hubbard, G. M., & Licht, P. (1985). *In vitro* study of the direct ovarian effects of gonadotropin-releasing hormone (GnRH) in the frogs, *Rana pipiens* and *Rana catesbeiana*. *Gen. Comp. Endocrinol., 60*, 154–161.

Hubbard, G. M., & Licht, P. (1986). *In vitro* ovarian responses to pulsatile and continuous gonadotrophin administration on steroid secretion and oocyte maturation in the frogs, *Rana pipiens* and *Rana catesbeiana*. *Gen. Comp. Endocrinol., 61*, 417–423.

Iela, L., D'Aniello, B., Di Meglio, M., & Rastogi, R. K. (1994). Influence of gonadectomy and steroid hormone replacement therapy on the gonadotropin-releasing hormone neuronal system in the anterior preoptic area of the frog (*Rana esculenta*) brain. *Gen. Comp. Endocrinol., 95*, 422–431.

Iela, L., Powell, J. F., Sherwood, N. M., D'Aniello, B., Rastogi, R. K., & Bagnara, J. T. (1996). Reproduction in the Mexican leaf frog, *Pachymedusa dacnicolor*. VI. Presence and distribution of multiple GnRH forms in the brain. *Gen. Comp. Endocrinol., 103*, 235–243.

Jennes, L., Stumpf, W. E., & Tappaz, M. L. (1983). Anatomical relationships of dopaminergic and GABAergic systems with the GnRH-systems in the septo-hypothalamic area. Immunohistochemical studies. *Exp. Brain. Res., 50*, 91–99.

Jones, S. W. (1987). Chicken II luteinizing hormone-releasing hormone inhibits the M-current of bullfrog sympathetic neurons. *Neurosci. Lett., 80*, 180–184.

Kah, O., Lethimonier, C., Somoza, G., Guilgur, L. G., Vaillant, C., & Lareyre, J. J. (2007). GnRH and GnRH receptors in metazoa: a historical, comparative, and evolutive perspective. *Gen. Comp. Endocrinol., 153*, 346–364.

Kalra, P. S., & Kalra, S. P. (1980). Modulation of hypothalamic luteinizing hormone-releasing hormone levels by intracranial and subcutaneous implants of gonadal steroids in castrated rats: effects of androgen and estrogen antagonists. *Endocrinology, 106*, 390–397.

Karsch, F. J., Bowen, J. M., Caraty, A., Evans, N. P., & Moenter, S. M. (1997). Gonadotropin-releasing hormone requirements for ovulation. *Biol. Reprod., 56*, 303–309.

King, J. A., & Millar, R. P. (1986). Identification of His5, Trp7, Tyr8-GnRH (chicken GnRH II) in amphibian brain. *Peptides, 7*, 827–834.

King, J. A., Davidson, J. S., & Millar, R. P. (1986). Desensitization to gonadotropin-releasing hormone in perifused chicken anterior pituitary cells. *Endocrinology, 119*, 1510–1518.

King, J. A., Steneveld, A. A., & Millar, R. P. (1994). Differential regional distribution of gonadotropin-releasing hormones in amphibian (clawed toad, *Xenopus laevis*) brain. *Regul. Pept., 50*, 277–289.

Klomberg, K. F., & Marler, C. A. (2000). The neuropeptide arginine vasotocin alters male call characteristics involved in social interactions in the grey treefrog, *Hyla versicolor*. *Anim. Behav., 59*, 807–812.

Knobil, E. (1981). Patterns of hypophysiotropic signals and gonadotropin secretion in the rhesus monkey. *Biol. Reprod., 24*, 44–49.

Koda, A., Ukena, K., Teranishi, H., Ohta, S., Yamamoto, K., Kikuyama, S., & Tsutsui, K. (2002). A novel amphibian hypothalamic neuropeptide: isolation, localization, and biological activity. *Endocrinology, 143*, 411–419.

Koza, J. M., & Wirsig-Wiechmann, C. R. (2001). A subpopulation of nervus terminalis neurons projects to the olfactory mucosa in *Xenopus laevis*. *J. Neurosci. Rev., 66*, 8–15.

Lee, Y. R., Tsunekawa, K., Moon, M. J., Um, H. N., Hwang, J. I., Osugi, T., Otaki, N., Sunakawa, Y., Kim, K., Vaudry, H., Kwon, H. B., Seong, J. Y., & Tsutsui, K. (2009). Molecular evolution of multiple forms of kisspeptins and GPR54 receptors in vertebrates. *Endocrinology, 150*, 2837–2846.

Li, Y., & Lin, H. (2000). Differences in mGnRH and cGnRH-II contents in pituitaries and discrete brain areas of *Rana rugulosa* W. according to age and stage of maturity. *Comp. Biochem. Physiol. C Toxicol. Pharmacol., 125*, 179−188.

Licht, P., & McCreery, B. R. (1985). Regulation of secretion and physiological actions of gonadotropins in amphibians. In B. Lofts, & W. N. Holmes (Eds.), *"Current Trends in Comparative Endocrinology"* (pp. 191−194). Hong Kong: Hong Kong University Press.

Licht, P., & Porter, D. A. (1987). Role of gonadotropin-releasing hormone in regulation of gonadotropin secretion from amphibian and reptilian pituitaries. In D. O. Norris, & R. E. Jones (Eds.), *"Hormones and Reproduction in Fishes, Amphibians, and Reptiles"* (pp. 61−85). New York: Plenum Publishing Co.

Licht, P., & Porter, D. A. (1985). LH secretion in response to gonadotropin releasing hormone (GnRH) by superfused pituitaries from two species of turtles. *Gen. Comp. Endocrinol., 59*, 442−448.

Licht, P., Tsai, P. S., & Sotowska-Brochocka, J. (1994). The nature and distribution of gonadotropin-releasing hormones in brains and plasma of ranid frogs. *Gen. Comp. Endocrinol., 94*, 186−198.

Licht, P., Millar, R., King, J. A., McCreery, B. R., Mendonca, M. T., Bona-Gallo, A., et al. (1984). Effects of chicken and mammalian gonadotropin-releasing hormones (GnRH) on *in vivo* pituitary gonadotropin release in amphibians and reptiles. *Gen. Comp. Endocrinol., 54*, 89−96.

Maney, D. L., Richardson, R. D., & Wingfield, J. C. (1997). Central administration of chicken gonadotropin-releasing hormone-II enhances courtship behavior in a female sparrow. *Horm. Behav., 32*, 11−18.

McCreery, B. R. (1984). Pituitary gonadotropin release by graded electrical stimulation of the preoptic area in the male bullfrog, *Rana catesbeiana*. *Gen. Comp. Endocrinol., 55*, 367−372.

McCreery, B. R., & Licht, P. (1983). Induced ovulation and changes in pituitary responsiveness to continuous infusion of gonadotropin-releasing hormone during the ovarian cycle in the bullfrog, *Rana catesbeiana*. *Biol. Reprod., 29*, 863−871.

McCreery, B. R., & Licht, P. (1984). Effects of gonadectomy and sex steroids on pituitary gonadotrophin release and response to gonadotrophin-releasing hormone (GnRH) agonist in the bullfrog, *Rana catesbeiana*. *Gen. Comp. Endocrinol., 54*, 283−296.

McCreery, B. R., Licht, P., Barnes, R., Rivier, J. E., & Vale, W. W. (1982). Actions of agonistic and antagonistic analogs of gonadotropin releasing hormone (Gn-RH) in the bullfrog *Rana catesbeiana*. *Gen. Comp. Endocrinol., 46*, 511−520.

McNeilly, A. S., O'Connell, M., & Baird, D. T. (1982). Induction of ovulation and normal luteal function by pulsed injections of luteinizing hormone in anestrous ewes. *Endocrinology, 110*, 1292−1299.

Meccariello, R., Franzoni, M. F., Chianese, R., Cottone, E., Scarpa, D., Donna, D., et al. (2008). Interplay between the endocannabinoid system and GnRH-I in the forebrain of the anuran amphibian *Rana esculenta*. *Endocrinology, 149*, 2149−2158.

Mensah-Nyagan, A. G., Do-Rego, J. L., Beaujean, D., Luu-The, V., Pelletier, G., & Vaudry, H. (1999). Neurosteroids: expression of steroidogenic enzymes and regulation of steroid biosynthesis in the central nervous system. *Pharmacol. Rev., 51*, 63−81.

Mensah-Nyagan, A. G., Feuilloley, M., Dupont, E., Do-Rego, J. L., Leboulenger, F., Pelletier, G., et al. (1994). Immunocytochemical localization and biological activity of 3 beta-hydroxysteroid dehydrogenase in the central nervous system of the frog. *J. Neurosci., 14*, 7306−7318.

Millar, R. P., Lu, Z. L., Pawson, A. J., Flanagan, C. A., Morgan, K., & Maudsley, S. R. (2004). Gonadotropin-releasing hormone receptors. *Endocr. Rev., 25*, 235−275.

Minucci, S., Di Matteo, L., Chieffi Baccari, G., & Pierantoni, R. (1989). A gonadotropin releasing hormone analog induces spermiation in intact and hypophysectomized frogs, *Rana esculenta*. *Experientia, 45*, 1118−1121.

Minucci, S., Di Matteo, L., Fasano, S., Baccari, G. C., & Pierantoni, R. (1992). Intratesticular control of spermatogenesis in the frog, *Rana esculenta*. *J. Exp. Zool., 264*, 113−118.

Minucci, S., Di Matteo, L., Pierantoni, R., Varriale, B., Rastogi, R. K., & Chieffi, G. (1986). *In vivo* and *in vitro* stimulatory effect of a gonadotropin-releasing hormone analog (HOE 766) on spermatogonial multiplication in the frog, *Rana esculenta*. *Endocrinol., 119*, 731−736.

Minucci, S., Fasano, S., D'Antonio, M., & Pierantoni, R. (1993). Dopamine regulation of testicular activity in intact and hypophysectomized frogs, *Rana esculenta*. *Experientia, 49*, 65−67.

Moon, J. S., Lee, Y. R., Oh da, Y., Hwang, J. I., Lee, J. Y., Kim, J. I., Vaudry, H., Kwon, H. B., & Seong, J. Y. (2009). Molecular cloning of the bullfrog kisspeptin receptor GPR54 with high sensitivity to *Xenopus* kisspeptin. *Peptides, 30*, 171−179.

Moore, F. L., & Miller, L. J. (1983). Arginine vasotocin induces sexual behavior of newts by acting on cells in the brain. *Peptides, 4*, 97−102.

Muller, C. H., & Licht, P. (1980). Gonadotropin specificity of androgen secretion by amphibian testes. *Gen. Comp. Endocrinol., 42*, 365−377.

Murakami, S., Kikuyama, S., & Arai, Y. (1992). The origin of the luteinizing hormone-releasing hormone (LHRH) neurons in newts (*Cynops pyrrhogaster*): the effect of olfactory placode ablation. *Cell Tissue Res., 269*, 21−27.

Muske, L. E., & Moore, F. L. (1988). The nervus terminalis in amphibians: anatomy, chemistry and relationship with the hypothalamic gonadotropin-releasing hormone system. *Brain Behav. Evol., 32*, 141−150.

Muske, L. E., & Moore, F. L. (1990). Ontogeny of immunoreactive gonadotropin-releasing hormone neuronal systems in amphibians. *Brain Res., 534*, 177−187.

Muske, L. E., & Moore, F. L. (1994). Antibodies against different forms of GnRH distinguish different populations of cells and axonal pathways in a urodele amphibian, *Taricha granulosa*. *J. Comp. Neurol., 345*, 139−147.

Naor, Z. (2009). Signaling by G-protein-coupled receptor (GPCR): studies on the GnRH receptor. *Front Neuroendocrinol., 30*, 10−29.

Narayanan, C. H., & Narayanan, Y. (1978). Determination of the embryonic origin of the mesencephalic nucleus of the trigeminal nerve in birds. *J. Embryol. Exp. Morphol., 43*, 85−105.

Neary, T. J. (1988). Forebrain auditory pathways in ranid frogs. In B. Fritzsch, M. J. Ryan, W. Wilczynski, T. E. Hetherington, & W. Walkowiak (Eds.), *"The Evolution of the Amphibian Auditory System"* (pp. 233−252). New York, NY: John Wiley & Sons.

Nett, T. M., Crowder, M. E., Moss, G. E., & Duello, T. M. (1981). GnRH-receptor interaction. V. Down-regulation of pituitary receptors for GnRH in ovariectomized ewes by infusion of homologous hormone. *Biol. Reprod., 24*, 1145−1155.

Northcutt, R. G., & Muske, L. E. (1994). Multiple embryonic origins of gonadotropin-releasing hormone (GnRH) immunoreactive neurons. *Brain Res. Dev. Brain Res., 78*, 279−290.

Oguchi, A., Tanaka, S., Aida, T., Yamamoto, K., & Kikuyama, S. (1997). Enhancement by prolactin of the GnRH-induced release of LH from dispersed anterior pituitary cells of the bullfrog (*Rana catesbeiana*). *Gen. Comp. Endocrinol., 107*, 128−135.

Oh, D. Y., Wang, L., Ahn, R. S., Park, J. Y., Seong, J. Y., & Kwon, H. B. (2003). Differential G protein coupling preference of mammalian and nonmammalian gonadotropin-releasing hormone receptors. *Mol. Cell. Endocrinol., 205*, 89−98.

Parhar, I. S., Soga, T., Ishikawa, Y., Nagahama, Y., & Sakuma, Y. (1998). Neurons synthesizing gonadotropin-releasing hormone mRNA subtypes have multiple developmental origins in the medaka. *J. Comp. Neurol., 401*, 217−226.

Park, D., & Eisthen, H. L. (2003). Gonadotropin releasing hormone (GnRH) modulates odorant responses in the peripheral olfactory system of axolotls. *J. Neurophysiol., 90*, 731−738.

Pavgi, S., & Licht, P. (1989). Effects of gonadectomy and steroids on pituitary gonadotropin secretion in a frog, *Rana pipiens*. *Biol. Reprod., 41*, 40−48.

Pavgi, S., & Licht, P. (1993). Inhibition of *in vitro* pituitary gonadotropin secretion by 17 beta-estradiol in the frog *Rana pipiens*. *Gen. Comp. Endocrinol., 89*, 132−137.

Peluso, J. J., Gruenberg, M. L., & Steger, R. W. (1984). Regulation of ovarian follicular growth and steroidogenesis by low-amplitude LH pulses. *Am. J. Physiol., 246*, R184−189.

Pierantoni, R., Fasano, S., Di Matteo, L., Minucci, S., Varriale, B., & Chieffi, G. (1984). Stimulatory effect of a GnRH agonist (buserelin) in *in vitro* and *in vivo* testosterone production by the frog (*Rana esculenta*) testis. *Mol. Cell Endocrinol., 38*, 215−219.

Pinelli, C., D'Aniello, B., Fiorentino, M., Bhat, G., Saidapur, S. K., & Rastogi, R. K. (1997). Distribution of gonadotropin-releasing hormone immunoreactivity in the brain of *Ichthyophis beddomei* (Amphibia: Gymnophiona). *J. Comp. Neurol., 384*, 283−292.

Porter, D. A., & Licht, P. (1985). Pituitary responsiveness to superfused GnRH in two species of ranid frogs. *Gen. Comp. Endocrinol., 59*, 308−315.

Porter, D. A., & Licht, P. (1986a). Effects of temperature and mode of delivery on responses to gonadotropin-releasing hormone by superfused frog pituitaries. *Gen. Comp. Endocrinol., 63*, 236−244.

Porter, D. A., & Licht, P. (1986b). The cellular basis of the calcium dependence of GnRH-stimulated gonadotropin release from frog, *Rana pipiens*, pituitaries. *J. Exp. Zool., 240*, 353−361.

Propper, C. R., & Moore, F. L. (1991). Effects of courtship on brain gonadotropin hormone-releasing hormone and plasma steroid concentrations in a female amphibian (*Taricha granulosa*). *Gen. Comp. Endocrinol., 81*, 304−312.

Provencio, I., Jiang, G., De Grip, W. J., Hayes, W. P., & Rollag, M. D. (1998). Melanopsin: An opsin in melanophores, brain, and eye. *Proc. Natl. Acad. Sci. USA, 95*, 340−345.

Rastogi, R. K., Iela, L., di Meglio, M., Di Fiore, M. M., D'Aniello, B., Pinelli, C., et al. (2002). In H. Heatwole (Ed.), *"Amphibian Biology. Hormonal regulation of reproductive cycles in amphibians", Vol. 6* (pp. 2045−2177). Chipping Norton, NSW Australia: Surrey Beatty & Sons.

Rastogi, R. K., King, J. A., Di Fiore, M. M., D'Aniello, B., & Pinelli, C. (1997). Sex and reproductive status related brain content of mammalian and chicken-II GnRHs in *Rana esculenta*. *J. Neuroendocrinol., 9*, 519−522.

Rastogi, R. K., Meyer, D. L., Pinelli, C., Fiorentino, M., & D'Aniello, B. (1998). Comparative analysis of GnRH neuronal systems in the amphibian brain. *Gen. Comp. Endocrinol., 112*, 330−345.

Richter, T. A., & Terasawa, E. (2001). Neural mechanisms underlying the pubertal increase in LHRH release in the rhesus monkey. *Trends Endocrinol. Metab., 12*, 353−359.

Roa, J., Aguilar, E., Dieguez, C., Pinilla, L., & Tena-Sempere, M. (2008). New frontiers in kisspeptin/GPR54 physiology as fundamental gatekeepers of reproductive function. *Front Neuroendocrinol., 29*, 48−69.

Roselli, C. E., Kelly, M. J., & Ronnekleiv, O. K. (1990). Testosterone regulates progonadotropin-releasing hormone levels in the preoptic area and basal hypothalamus of the male rat. *Endocrinology, 126*, 1080−1086.

Rossato, M., Pagano, C., & Vettor, R. (2008). The cannabinoid system and male reproductive functions. *J. Neuroendocrinol., 20*(Suppl 1), 90−93.

Schwanzel-Fukuda, M., & Pfaff, D. W. (1989). Origin of luteinizing hormone-releasing hormone neurons. *Nature, 338*, 161−164.

Seminara, S. B., Messager, S., Chatzidaki, E. E., Thresher, R. R., Acierno, J. S., Jr., Shagoury, J. K., et al. (2003). The GPR54 gene as a regulator of puberty. *N. Engl. J. Med., 349*, 1614−1627.

Sherwood, N. M., Zoeller, R. T., & Moore, F. L. (1986). Multiple forms of gonadotropin-releasing hormone in amphibian brains. *Gen. Comp. Endocrinol., 61*, 313−322.

Smith, M. A., & Vale, W. W. (1981). Desensitization to gonadotropin-releasing hormone observed in superfused pituitary cells on Cytodex beads. *Endocrinology, 108*, 752−759.

Somoza, G. M., Paz, D. A., Stefano, A. V., & Affanni, J. M. (1996). Identification of immunoreactive mammalian gonadotropin-releasing hormone in the brain of metamorphic larvae of *Bufo arenarum* Hensel (Amphibia: Anura). *Int. J. Dev. Neurosci., 14*, 663−672.

Sotowska-Brochocka, J. (1988). The stimulatory and inhibitory role of the hypothalamus in the regulation of ovulation in grass frog, *Rana temporaria* L. *Gen. Comp. Endocrinol., 70*, 83−90.

Sotowska-Brochocka, J., & Licht, P. (1992). Effect of infundibular lesions on GnRH and LH release in the frog, *Rana temporaria*, during hibernation. *Gen. Comp. Endocrinol., 85*, 43−54.

Sotowska-Brochocka, J., Martynska, L., & Licht, P. (1994). Dopaminergic inhibition of gonadotropic release in hibernating frogs, *Rana temporaria*. *Gen. Comp. Endocrinol., 93*, 192−196.

Spratt, D. P., & Herbison, A. E. (1997). Regulation of preoptic area gonadotrophin-releasing hormone (GnRH) mRNA expression by gonadal steroids in the long-term gonadectomized male rat. *Brain Res. Mol. Brain Res., 47*, 125−133.

Stamper, D. L., & Licht, P. (1993a). Further studies on the influence of GnRH on the biosynthesis of gonadotropins in female frogs (*Rana pipiens*). *Gen. Comp. Endocrinol., 92*, 104−112.

Stamper, D. L., & Licht, P. (1993b). *In vitro* exposure of frog (*Rana pipiens*) pituitaries to gonadotropin-releasing hormone results in a persistent stimulation of gonadotropin biosynthesis. *J. Exp. Zool., 265*, 646−652.

Stamper, D. L., & Licht, P. (1994). Influence of androgen on the GnRH-stimulated secretion and biosynthesis of gonadotropins in the pituitary of juvenile female bullfrogs, *Rana catesbeiana*. *Gen. Comp. Endocrinol., 93*, 93−102.

Takase, M., Ukena, K., Yamazaki, T., Kominami, S., & Tsutsui, K. (1999). Pregnenolone, pregnenolone sulfate, and cytochrome P450 side-chain cleavage enzyme in the amphibian brain and their seasonal changes. *Endocrinology, 140*, 1936−1944.

Temple, J. L., Millar, R. P., & Rissman, E. F. (2003). An evolutionarily conserved form of gonadotropin-releasing hormone coordinates energy and reproductive behavior. *Endocrinology, 144*, 13−19.

Terasawa, E., & Fernandez, D. L. (2001). Neurobiological mechanisms of the onset of puberty in primates. *Endocr. Rev., 22*, 111−151.

Thompson, R. R., & Moore, F. L. (2000). Vasotocin stimulates appetitive responses to the visual and pheromonal stimuli used by male roughskin newts during courtship. *Horm. Behav., 38*, 75−85.

Thompson, R. R., & Moore, F. L. (2003). The effects of sex steroids and vasotocin on behavioral responses to visual and olfactory sexual stimuli in ovariectomized female roughskin newts. *Horm. Behav., 44*, 311−318.

Toranzo, D., Dupont, E., Simard, J., Labrie, C., Couet, J., Labrie, F., & Pelletier, G. (1989). Regulation of pro-gonadotropin-releasing hormone gene expression by sex steroids in the brain of male and female rats. *Mol. Endocrinol., 3*, 1748−1756.

Troskie, B., King, J. A., Millar, R. P., Peng, Y. Y., Kim, J., Figueras, H., & Illing, N. (1997). Chicken GnRH II-like peptides and a GnRH receptor selective for chicken GnRH II in amphibian sympathetic ganglia. *Neuroendocrinology, 65*, 396−402.

Troskie, B. E., Hapgood, J. P., Millar, R. P., & Illing, N. (2000). Complementary deoxyribonucleic acid cloning, gene expression, and ligand selectivity of a novel gonadotropin-releasing hormone receptor expressed in the pituitary and midbrain of *Xenopus laevis*. *Endocrinology, 141*, 1764−1771.

Tsai, P. S., & Jones, J. T. (2005). Steroid-induced changes in the morphology of GnRH neurons in the male leopard frog, *Rana pipiens*: correlation with plasma gonadotropin and gonadal size. *Gen. Comp. Endocrinol., 141*, 152−160.

Tsai, P. S., & Licht, P. (1993). GnRH-induced desensitization of *in vitro* luteinizing hormone secretion in the turtle, *Trachemys scripta*. *Gen. Comp. Endocrinol., 89*, 238−247.

Tsai, P. S., Lunden, J. B., & Jones, J. T. (2003a). Effects of steroid hormones on spermatogenesis and GnRH release in male Leopard frogs, *Rana pipiens*. *Gen. Comp. Endocrinol., 134*, 330−338.

Tsai, P. S., Moenter, S. M., & Cavolina, J. M. (2003b). Development of a novel enzyme immunoassay for the measurement of *in vitro* GnRH release from rat and bullfrog hypothalamic explants. *Comp. Biochem. Physiol. A Mol. Integr. Physiol., 136*, 693−700.

Tsutsui, K., Bentley, G. E., Ubuka, T., Saigoh, E., Yin, H., Osugi, T., et al. (2007). The general and comparative biology of gonadotropin-inhibitory hormone (GnIH). *Gen. Comp. Endocrinol., 153*, 365−370.

Walters, K., Wegorzewska, I. N., Chin, Y. P., Parikh, M. G., & Wu, T. J. (2008). Luteinizing hormone-releasing hormone I (LHRH-I) and its metabolite in peripheral tissues. *Exp. Biol. Med. (Maywood), 233*, 123−130.

Wang, H., Dey, S. K., & Maccarrone, M. (2006). Jekyll and hyde: two faces of cannabinoid signaling in male and female fertility. *Endocr. Rev., 27*, 427−448.

Wang, L., Yoo, M. S., Kang, H. M., Im, W. B., Choi, H. S., Bogerd, J., & Kwon, H. B. (2001). Cloning and characterization of cDNAs encoding the GnRH1 and GnRH2 precursors from bullfrog (*Rana catesbeiana*). *J. Exp. Zool., 289*, 190−201.

Weiner, R. I., Findell, P. R., & Kordon, C. (1988). Role of classi and peptide neuromediators in the neuroendocrine regulation of LH and prolacint. In E. Knobil, & J. D. Neill (Eds.), *"The Physiology of Reproduction"* (pp. 1235−1281). New York: Raven Press.

Wetzel, D. M., & Kelley, D. B. (1983). Androgen and gonadotropin effects on male mate calls in South African clawed frogs, *Xenopus laevis*. *Horm. Behav., 17*, 388−404.

White, R. B., & Fernald, R. D. (1998). Ontogeny of gonadotropin-releasing hormone (GnRH) gene expression reveals a distinct origin for GnRH-containing neurons in the midbrain. *Gen. Comp. Endocrinol., 112*, 322−329.

Wilczynski, W. (1988). Brainstem auditory pathways in anuran amphibians. In B. Fritzsch, M. J. Ryan, W. Wilczynski, T. E. Hetherington, & W. Walkowiak (Eds.), *"The Evolution of the Amphibian Auditory System"* (pp. 209−231). New York: John Wiley & Sons.

Wilczynski, W., Allison, J. D., & Marler, C. A. (1993). Sensory pathways linking social and environmental cues to endocrine control regions of amphibian forebrains. *Brain Behav. Evol., 42*, 252−264.

Wirsig, C. R., & Leonard, C. M. (1987). Terminal nerve damage impairs the mating behavior of the male hamster. *Brain Rev., 417*, 293−303.

Wirsig-Wiechmann, C. R., & Lee, C. E. (1999). Estrogen regulates gonadotropin-releasing hormone in the nervus terminalis of *Xenopus laevis*. *Gen. Comp. Endocrinol., 115*, 301−308.

Wirsig-Wiechmann, C. R., Wiechmann, A. F., & Eisthen, H. L. (2002). What defines the nervus terminalis? Neurochemical, developmental, and anatomical criteria. *Prog. Brain Rev., 141*, 45−58.

Wray, S. (2002). Development of gonadotropin-releasing hormone-1 neurons. *Front Neuroendocrinol., 23*, 292−316.

Wray, S., Grant, P., & Gainer, H. (1989). Evidence that cells expressing luteinizing hormone-releasing hormone mRNA in the mouse are derived from progenitor cells in the olfactory placode. *Proc. Natl. Acad. Sci. USA, 86*, 8132−8136.

Yamamoto, N., Oka, Y., & Kawashima, S. (1997). Lesions of gonadotropin-releasing hormone-immunoreactive terminal nerve cells: effects on the reproductive behavior of male dwarf gouramis. *Neuroendocrinology, 65*, 403−412.

Yoo, M. S., Kang, H. M., Choi, H. S., Kim, J. W., Troskie, B. E., Millar, R. P., & Kwon, H. B. (2000). Molecular cloning, distribution and pharmacological characterization of a novel gonadotropin-releasing hormone ([Trp8] GnRH) in frog brain. *Mol. Cell Endocrinol., 164*, 197−204.

Zerani, M., Gobbetti, A., & Polzonetti-Magni, A. (1991). *In vitro* steroid production by follicles of frog *Rana esculenta*: mammalian gonadotropin-releasing hormone effects. *Acta. Physiol. Scand., 142*, 495−501.

Zhang, W., & Delay, R. J. (2007). Gonadotropin-releasing hormone modulates voltage-activated sodium current and odor responses in *Necturus maculosus* olfactory sensory neurons. *J. Neurosci. Res., 85*, 1656−1667.

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Chapter 3

Testicular Structure and Control of Sperm Development in Amphibians

Catherine R. Propper
Northern Arizona University, Flagstaff, AZ, USA

SUMMARY
In the three clades of amphibians, Gymnophiona, Urodela, and Anura, the testes produce sperm through a progression of events similar to development pathways seen in the rest of vertebrates. However, there are differences within the three clades in the general structural foundation of the testes, and there are differences and similarities in how the sperm undergo maturation and release. This chapter reviews the structure of the testes in all three clades and discusses how the interstitial and seminiferous tubule compartments function to lead to sperm development. The pituitary signaling events known for each group are reviewed, along with the intratesticular paracrine interactions between steroids and non-steroidal molecular signals that facilitate communication between the different testicular compartments. Research in each clade has led to clade-dependent discoveries and cross-clade identification of similarities. These similarities and differences are summarized at the end of the chapter.

1. INTRODUCTION

Descriptions of the structure of amphibian testes have been updated over the last century, including in a series of recent books (Jamieson, 2003; Sever, 2003; Exbrayat, 2006). In this chapter, the structure of the testes is briefly described based on these reviews and the newest literature in the field, and across-clade comparisons are accented. Within each section, the chapter covers new and functionally important literature on amphibian testicular activity and regulation.

In all three clades of amphibians, urodeles (salamanders), anurans (frogs and toads), and gymnophionans (caecilians or apodans), sperm development has three distinct stages, defined by van Oordt (1956). The first stage is spermatogenesis, in which the primary germ cells, known as spermatogonia, undergo mitotic cell division leading to clonal secondary spermatogonia. The spermatogonia undergo a final mitotic division to become primary spermatocytes that begin the meiotic process. After the first meiotic division, the cells are called secondary spermatocytes, and, following meiosis II, they are smaller round haploid spermatids. Next, during spermiogenesis, spermatids mature via cell remodeling and elongation events to form spermatozoa. Spermiation occurs when spermatozoa are released from their close Sertoli cell connections into the lumen matrix of the seminiferous lobules. The differences and similarities in this sperm maturation process among the amphibian clades in both morphology and functional physiology will be described in this chapter.

2. URODELES

2.1. Testicular Structure

2.1.1. General gross anatomical structure

In urodele amphibians, the testes are elongated bilateral structures. In some clades the testes are single long structures and in others they are divided into lobes attached by connective tissue. The number of lobes increases with age (Humphery, 1922; Valdivieso & Tamsitt, 1965). Within each lobe are lobules containing cysts where groups of cells at the same stage of spermatogenesis are found.

The reproductive duct system of urodeles has been reviewed recently (Uribe Aranzabal, 2003). Sperm are released into the lumen of lobules and are carried to a system of intratesticular ducts located between each lobule. The ducts empty into transverse efferent collecting ducts, also known as vasa efferentia, which are located between the testes and the anterior portion of the kidney where they open into nephrons. The nephronic collecting ducts empty into the vas deferens, also called the primary urinary duct, which carries sperm to the cloaca. This system varies slightly in the Hynobiidae and the Plethodontidae, in

which the transverse efferent ducts empty into the vas deferens directly rather than emptying first into the nephrons.

2.1.2. Histomorphic structure and sperm development

The histomorphic structure of the testes in urodeles is interesting and unique to this clade (reviewed in Uribe Aranzabal, 2003). Within each testis lobule in salamanders, there are germinal cysts, each made up of synchronously developing cells in the same stage of spermatogenesis. Such cystic development during spermatogenesis is similar in all amphibians and is a primitive characteristic shared with fish. In salamanders, sperm development occurs in a 'wave,' beginning in the cranial region of the testes with cysts in early stages of spermatogenesis and ending in the caudal testicular region with cysts containing sperm in the final stage of spermiogenesis. The apparent progression or wave of cysts from the cranial to caudal orientation in the testes is probably due to the formation of new early stage cysts in the cranial testicular region (Pudney, 1995) and disintegration of cysts as spermiation occurs in the caudal region, rather than a result of the movement of cysts through the testes.

In salamanders, there are basic structural changes that occur during spermatogenesis (reviewed in Uribe Aranzabal, 2003). Generally, in the cranial region of the testes, the primary germ cells are found in connective tissue. These cells form an association with Sertoli cells and develop into individual cysts where spermatogenesis then proceeds synchronously within each cyst as these large primary spermatogonia undergo proliferation via mitosis to form secondary spermatogonia. At this stage of spermatogenesis, within each cyst, a lumen appears as the secondary spermatogonia come to the end of their proliferation phase. At completion of the mitotic divisions, secondary spermatogonia enter the initial stage of meiosis I with chromosome duplication. These germ cells are now considered primary spermatocytes, which undergo the different stages of meiosis I prophase (leptotene, zygotene, pachytene, and diplotene). At the end of meiosis I, after the first cell division, the two resulting sister cells are considered secondary spermatocytes. These cells are significantly smaller than either spermatogonia or primary spermatocytes and they quickly undergo meiosis II to become four haploid spermatids per original secondary spermatogonium. Spermatids next enter spermiogenesis, and elongate and mature into sperm appearing as a set of long cells oriented in the same direction or as whorls within the cyst. During spermiation, these sperm move into the lumen of the cysts and empty into the intralobular ductules and ultimately into efferent ducts.

During spermiation in the caudal lobes of the testes, the direct connection between the maturing spermatids and spermatozoa and the Sertoli cells is lost (Pudney, 1995). The Sertoli cells either are sloughed into the lumen of the lobules or simply degenerate (Pudney, 1995). In these lobules, following spermiation, only degenerating or defective sperm are seen. Eventually the depleted lobules also degenerate. Figure 3.1 shows some of these details.

The interstitial or Leydig cells of salamanders are also under a maturation process in the testes. Early in the last century, Humphrey (1921) provided a detailed review and description of these cells that was expanded upon by Callard, Canick, and Pudney (1980), Ucci (1982), and two papers by Pudney and colleagues (Pudney, Canick, Mak, & Callard, 1983; Pudney & Callard, 1984), mostly using *Necturus maculosus* as a model salamander species. The development of the glandular Leydig tissue follows the wave of spermiation. Immature interstitial cells that surround the lobules containing cysts undergoing spermatogenesis have a fibroid appearance. However, in the distal and caudal region of the testes where spermiation has already occurred, these cells and their nuclei become

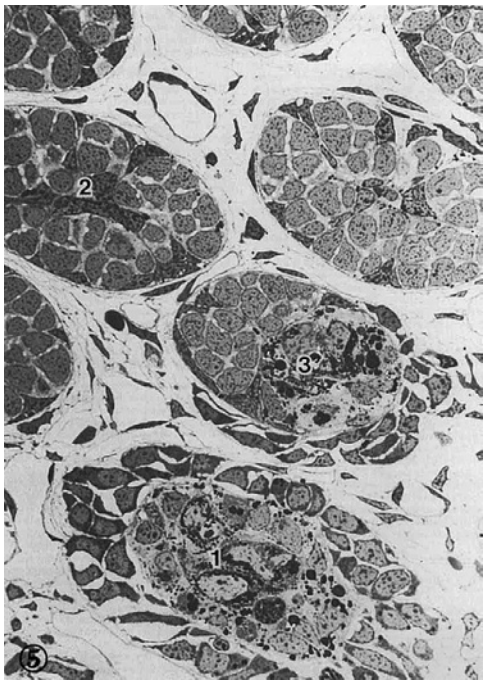

FIGURE 3.1 Seminiferous lobules from *Necturus* testes demonstrating the rostral to caudal nature of sperm development. The lobule labeled '2' shows all cysts at the same stage of sperm development. The lobule labeled '1' shows cysts undergoing spermiation. The lobule labeled '3' demonstrates an intermediate lobule in the process of undergoing final spermatid maturation in preparation for spermiation. *Pudney and Callard (1984) Figure 5, p. 92. Copyright, 1984 Wiley and Sons. Reprinted with permission of John Wiley & Sons, Inc.*

hypertrophied and rounded. Further, myoid cells form a layer between the glandular interstitium and the degenerating lobules and even surround groups of Leydig cells (Pudney & Callard, 1984). This interstitial tissue is not only closely associated with blood vessels but is also innervated with unmyelinated neurons containing substance P and neurotensin (Pudney & Callard, 1984). The close physical relationship between lobules and interstitium, along with the correlated physical changes occurring in the lobules during spermiation and maturation of the glandular tissues, strongly suggests that there are complex and localized signaling mechanisms occurring within the testes between these different tissue types. Perhaps the neuronal associations with the testes lead to regional glandular tissue control within the testes that would possibly allow for a stronger local regulation.

Seasonal changes in interstitial cell morphology correlated with spermatogenesis are found in the marbled newt, *Triturus marmoratus* (Fraile, Saez, & Paniagua, 1990). The Leydig cells are small during the period of sperm maturation, but become large and lipid-rich following spermiation. In *Taricha granulosa* (Specker & Moore, 1980), interstitial hypertrophy is closely associated with seasonal rises in testosterone (T) levels. This work further supports the link between the interstitial compartment and the lobules containing developing germ cells.

There appears to be a blood–testis barrier in salamanders most likely formed from Sertoli cells (Franchi, Camatini, & Decurtis, 1982; Bergmann, Greven, & Schindelmeiser, 1983; Jin, Uchida, Eto, Kitano, & Abe, 2008). In both *Triturus cristatus* (Franchi et al., 1982; Bergmann et al., 1983) and *Salamandra salamandra*, immature cysts appear to have little or no barrier, as the tracer, lanthanum, can penetrate into the cyst lumen (Franchi et al., 1982). However, in mature cysts containing spermatids and sperm, the tracer cannot penetrate the lumen. In *Cynops pyrrogaster*, the barrier forms from tight junctions between Sertoli cells lining the cysts: Jin et al. (2008) found that, although larger markers were excluded only from mature cysts, smaller markers were able to penetrate all cysts, demonstrating that the barrier does not exclude smaller molecules. Jin et al. (2008) also found that two forms of the protein occludin were involved in adhesion of the Sertoli cells to each other but were probably not responsible for the size-specific exclusion properties of Sertoli–Sertoli cell barriers.

Smooth muscle cells also are found in the testes of salamanders. In *S. salamandra*, these smooth muscle cells exist under the peritoneal epithelium within the connective tissue (Bergmann, Greven, & Schindelmeiser, 1982). The muscle cells appear to be more mature and contractile in the caudal portion of the testes that contains the more mature cysts, suggesting that they may be involved in spermiation.

2.2. Regulation of Testicular Activity

2.2.1. Endocrine and molecular events associated with spermatogenesis and spermiogenesis

Several studies in the salamander *C. pyrrhogaster* have led to a greater understanding of urodele spermatogenesis. Spermatogonia in this species undergo seven cycles of mitosis prior to entering into meiosis (Abe, 2004), and several hormones and chemical mediators play a role in both cell proliferation and progression to the primary spermatocyte stage.

Spermatogenesis in *C. pyrrhogaster* is under regulation by anterior pituitary release of follicle-stimulating hormone (FSH). Follicle-stimulating hormone impacts mitotic division of the spermatogonia and is necessary for the formation of secondary spermatocytes and then for the transformation through meiosis II from secondary spermatocytes to spermatids (Ji, Kubokawa, & Abe, 1995). The effects of FSH are shown through its actions on Sertoli cells (Maekawa, Zai-Si Ji, & Shin-Ichi Ab, 1995; Abe, 2004), which express FSH receptors from early spermatogenesis through the spermatid stage (Nakayama, Yamamoto, Oba, Nagahama, & Abe, 2000). In *Ambystoma tigrinum*, treatment of larvae with FSH induces formation of secondary spermatogonial cysts (Moore, 1975), demonstrating again that FSH is involved in the induction of mitosis of early stage germ cells and maintenance of early germ cell development to the spermatid stage.

The anterior pituitary hormone luteinizing hormone (LH) also plays a role in testicular function in salamanders. In *C. pyrrhogaster*, treatment with bullfrog LH (*Rana* LH) induces spermiation and T secretion but does not impact early spermatogenesis (Abe & Ji, 1994; Maekawa et al., 1995; Tanaka et al., 2004). In salamanders, therefore, FSH is important in early spermatogenesis whereas LH plays a role in later spermiation and T secretion.

Steroid signaling plays an essential role in the initiation of spermatogenesis. In *T. marmoratus*, estrogen receptors (ERα and ERβ) and the androgen receptor (AR) show dramatic and different patterns of immunocytochemical staining throughout the maturing cysts, demonstrating the direct role of steroid hormone action on both germ and Sertoli cells (Arenas et al., 2001). Early in spermatogenesis, ARs are expressed in the developing germ cells but not in the Sertoli cells, but, as sperm maturation progresses, ARs are expressed weakly in the Sertoli cells but not in the mature sperm. Early in spermatogenesis, ERα immunoreactivity is visible in the early maturing sperm cells but, like AR, ER expression fades as spermiogenesis progresses past the spermatid stage. However, in the Sertoli cells, ERα staining increases from the spermatid stage of spermatogenesis through spermiation. Estrogen receptor-β shows

a different pattern of staining with all spermatogenetic cells staining except for mature sperm. However, only Sertoli cells are immunoreactive for ERβ once spermiation occurs. This differential timing of sensitivity to androgens and estrogens in the spermatogenetic cells and Sertoli cells suggests the importance of steroid signaling in coordinating events linked to spermatogenesis and spermiation. Besides receptor-related regulation, a specific androgen binding protein (ABP) exists in the testes of *N. maculosus* (Singh & Callard, 1989; 1992), demonstrating that there are complex steroid regulatory mechanisms at work in the salamander testes. Future investigations will be needed to determine the exact role of both steroid and gonadotropin hormones within the different testicular compartments, and coordinate these roles with Leydig cell production of both androgens and estradiol (E_2).

Other endocrine and/or paracrine factors are involved in germ cell proliferation. For example, insulin-like growth factor 1 (IGF-1) signaling may be involved in testicular function. In *C. pyrrhogaster*, testes cultures exposed to FSH show increased expression of IGF-1 mRNA in the Sertoli cells of cysts containing secondary spermatogonia. Also, treatment of testes fragments *in vitro* with IGF-1 and insulin-like growth factor 2 (IGF-2) induces differentiation of secondary spermatogonia into primary spermatocytes (Nakayama, Yamamoto, & Abe, 1999; Yamamoto, Nakayama, & Abe, 2001). Spermatogonial proliferation is under regulation of stem cell factor (SCF). *In-vitro* treatment with a human recombinant form of this factor leads to mitotic division of spermatogonia, but not the initiation of meiosis (Abe K., Jin, Yamamoto, & Abe S., 2002). An organ culture system in *C. pyrrhogaster* also has identified epidermal growth factor (EGF) as an endocrine modulator of spermatogonia mitosis (Abe K., Eto, & Abe S., 2008). Further, there are EGF receptors (ErbB1, ErbB2, and ErbB4) found on both Sertoli cells and on spermatogonia (Abe et al., 2008). Results from this recent work suggest that EGF impacts spermatogonial proliferation via a pathway that involves endocrine induction of gene expression of SCF, Erb4, and an immunoglobulin-like domain containing neuregulin1 (Ig-NRG1). These studies demonstrate that there are complex signaling systems involved in the induction of the first phase of sperm production.

Other gene products involved in cell cycle regulation may be implicated in arresting or activating mitosis at appropriate times during the breeding season. In the marbled newt, expression of p53 and p21 in primary spermatogonia and Sertoli cells during the quiescent phase of testicular activity suggests that these proteins may play a role in limiting mitosis at this time of year. However, during active cell proliferation, greater spermatogonial expression of retinoblastoma (Rb) and phospho-Rb, a protein involved in cell proliferation, is correlated with lower p53 and p21 levels. Studies in marbled newts of the expression of retinoid acid receptors (RXR-α, -β, -γ) and farnesoid X-activated receptor (FXR) find that these genomic receptors are expressed with greatest intensity in proliferating primary spermatogonia, but not in later stages of spermatogenesis (Alfaro et al., 2002). Wilms' tumor gene (WT1) expression in Sertoli cells is greater in those lobules containing cysts at the early stages of spermatogenesis and mitosis than in cysts in the later stages of spermatogonial proliferation (Del Rio-Tsonis, Covarrubias, Kent, Hastie, & Panagiotis, 1996), suggesting that WT1 may play a role in the early initiation of mitosis and cell division. Changes in gene and protein expression associated with the onset of active spermatogenesis demonstrate coordination of molecular signaling involved in sperm development (Ricote et al., 2002) at the appropriate time of year.

2.2.2. Control of spermiation

Endocrine control of spermiation is less studied in salamanders than it is in anurans (see Section 3.2.). In the newt *C. pyrrhogaster*, exposure to LH but not FSH appears to induce spermiation and androgen secretion (Tanaka et al., 2004). These results suggest that, while FSH may induce spermatogenesis by promoting mitosis of spermatogonia, LH acts towards the end of the sperm development process to cause spermiation.

2.2.3. Control of the interstitial compartment

There are distinct changes in the activity and morphological structure of the interstitial component of the testes associated directly with sperm development, and gonadotropin-releasing hormone (GnRH) may have direct actions on these processes. Gonadotropin-releasing hormone treatment in *Pleurodeles waltl* during the breeding season leads not only to a decrease in the amount of lipid in interstitial cells but also to an increase in cell and nuclear size and the amount of smooth endoplasmic reticulum, suggesting that GnRH induces steroidogenesis in Leydig cells during the breeding season (Moya, Guerrero, Navas, & Garcia-Herdugo, 1987). Gonadotropin-releasing hormone may act directly to impact steroidogenesis (Gobbetti & Zerani, 1992). In *Triturus carnifex*, testes tested *in vitro* secrete the highest levels of androgens and have the highest response to GnRH exposure during the reproductive season. Estradiol levels were highest during the post-reproductive phase, and testes treated *in vitro* with GnRH exhibited increased E_2 secretion compared to controls when treated during this stage of reproduction. Gonadotropin-releasing hormone also induced changes in testicular tissue prostaglandin F2α secretion (Gobbetti & Zerani, 1992), and prostaglandin E_2 affects androgen synthesis through complicated signaling pathways that impact androgen production at the beginning

of the breeding season through actions via phospholipase C and estradiol production at the end of the breeding season through adenylate cyclase signaling (Gobbetti & Zerani, 1995).

As in other vertebrates, LH and FSH are important in regulating interstitial testicular function. In *T. granulosa*, the rough-skinned newt, *in-vivo* treatment of the testis with ovine LH induced increased T release and *in-vitro* treatment induced greater T release from the caudal, more mature region of the testes than it did from the less mature region (Moore, Muller, & Specker, 1979), indicating that the Leydig compartment's sensitivity to GTHs associated with the stage of sperm development in the lobules. In a study by Muller and Licht (1980), induction of androgen release in *A. tigrinum* testes was more sensitive to homologous LH exposure than to FSH. However, the authors also tested several other species with non-homologous forms of LH and FSH. The general trend was greater sensitivity to LH than FSH, but there were exceptions. A recent study in the newt *C. pyrrhogaster* demonstrated that LH derived from *Rana catesbeiana* was a much more potent inducer of androgen release than was *Rana* FSH (Tanaka et al., 2004). Overall, when GTHs from closely related amphibian species are evaluated, LH has the greatest impact on interstitial steroid secretion.

Changes in steroid secretion from the interstitium correlate well with changes in Leydig cell morphology. In *N. maculosus*, the mature Leydig cells at the caudal and distal portion of the testes contain greater concentrations of enzymes involved in androgen and estrogen production (Pudney et al., 1983). Aromatase activity is greatest in those regions of the testis undergoing the greatest degree of lobular disintegration, suggesting that estrogen production may be directly responsible for terminating androgen production at the end of a reproductive cycle and for increasing estrogen signaling important to spermiation (see below). However, in the less mature anterior regions of the testes, Leydig cells have some capacity for producing androgens even while the caudal regions are undergoing spermiation, suggesting that regionalized androgen secretion may be important not only to early spermatogenesis but also to maintenance of primary and secondary sex characteristics during the breeding season (Pudney et al., 1983). This hypothesis is supported in *T. granulosa*, in which seasonal changes in androgens correspond to seasonal changes in testis morphology (Specker & Moore, 1980). In this species, androgen levels are low during spermatogenesis but rise during spermiogenesis and again after spermiation and during the period leading to the breeding season when secondary sex characteristics are developing.

The distribution of ARs and ERs in the interstitium during the testicular cycle has been studied in *T. marmoratus* (Arenas et al., 2001) Androgen receptor staining in the Leydig cells is only found following spermiation and not earlier in the spermatogenetic cycle, suggesting that there may be some autoregulation of Leydig cells by androgens at this reproductive stage. Estrogen receptor-α immunostaining is positive throughout the cycle, whereas ERβ is only highly expressed in the mature hypertrophied glandular tissue. Again, this study supports the earlier work of Callard and colleagues (Callard et al., 1980; Mak, Callard, I.P., & Callard, G.V., 1983; Pudney et al., 1983) that demonstrates the presence of an ER. Clearly, there are complex regulatory mechanisms between androgenic and estrogenic mechanisms functioning in localized compartments within the testes of salamanders.

Several other molecular regulators appear to be important in controlling interstitium development during the annual reproductive cycle. In *T. marmoratus*, during the testicular quiescent period, the Leydig cells are large and actively steroidogenic, but not proliferating. During this time, these cells stain positive for RXRs (Alfaro et al., 2002). Also in this species, two intracellular regulators of cell proliferation, p53 and p21, are present in the active interstitial tissues (Ricote et al., 2002), suggesting that these evolutionarily conserved markers of cell proliferation might also be involved in the annual development of glandular tissue in salamanders. Proteoglycans also appear to play an important role in cell differentiation and Saez and colleagues (Saez, Madrid, Aparicio, Hernandez, & Alonso, 2001; Saez, Madrid, Cardoso, Gomez, & Hernandez, 2004) have found that, as the Leydig cells differentiate in the glandular tissue of *P. waltl*, there are changes in the glycan composition of the cells, suggesting that proteoglycans may play a role in the development of the steroidogenic tissues.

In summary, urodele testes undergo three phases during the annual cycle. There is the proliferative phase, during which new lobules form and spermatogenesis occurs via recruitment and proliferation of spermatogonia. This period of testicular function appears to be under the regulation of FSH with local regulation of spermatogenesis also under the control of EFG, IGF-1, IGF-2, SCF, and possibly local low-level secretion of androgens. Less is known about the stage of sperm maturation that involves continuation of meiosis; however, RAD51 may be involved (Yamamoto, Hikino, Nakayama, & Abe, 1999). Spermiation appears to be under the regulation of LH and, following spermiation, there is a dramatic differentiation of the cells surrounding the lobules into glandular tissue forming a steroid secreting interstitium. Both androgens and estrogens are secreted; the androgens induce development of secondary sex characteristics and E_2 possibly acts as an intratesticular signaling mediator to initiate the end of the breeding season and beginning of new lobular formation. The whole cycle occurs in waves where the more mature areas of sperm development are found in the caudal and distal region of the

testes and the early formation of lobules occurs in the cranial and proximal regions. There are complex interactions that occur between the interstitial cells and the other testicular compartments that demonstrate local regulatory control of testicular function.

3. ANURANS

3.1. Testicular Structure

3.1.1. General gross anatomical structure

In anuran amphibians, testes are single bilateral compact ovular organs, as opposed to the multilobed structures found in salamanders. As in salamanders, the lobes consist of cyst-filled lobules. However, there is no regional localization of maturation as is seen in salamanders. In most anuran species, sperm are released from the testicular lobules into several efferent ductules that carry the sperm to outer lateral kidney canals at the cranial portion of the kidney. Sperm pass through the mesonephros via connecting ducts that pass the sperm to the primary urinary duct, the vas deferens, which is derived from the Wolffian duct, and then to the cloaca. Both sperm and urinary products are carried in this primary urinary duct. However, in *Alytes*, the efferent duct empties directly into the urinary duct without passing through any portion of the mesonephros (Blüm, 1937).

3.1.2. Histomorphic structure and sperm development

The histological structure of the testis has been described in more than 18 anuran species (see citations within Scheltinga & Jamieson, 2003). In anurans, there are seminiferous lobules similar to the lobules described above for urodeles. These lobules are contained by a basement membrane and spermatogenesis is initiated within cysts. As the spermatids develop, the cysts rupture and the lobules resemble seminiferous tubules of amniotes with the maturing spermatozoa embedded in Sertoli cells lining the basement membrane of the lobules. Anuran testes also differ from those of urodeles in that, within a lobule, there can be cysts containing germ cells at different stages of development, especially in continuously breeding species.

The details of spermatogenesis have been described in several species and have been extensively reviewed in Pudney (1995), Ko, Kang, Im, and Kwon (1998), and Scheltinga and Jamieson (2003). In summary, in most species, spermatogenesis is initiated immediately following the breeding season and is completed prior to the inactive period of the frogs (i.e., completed by fall for spring breeding anurans) (Figure 3.2). However, in continuously breeding animals in tropical regions, and in laboratory-kept *Xenopus laevis*, all stages of spermatogenesis can be found even within a single lobule (van Oordt, 1960; Sasso-Cerri, de Faria, Freymuller, & Miraglia, 2004) (Figure 3.3). The sperm are stored in the seminiferous lobules until the initiation of breeding. In discontinuous breeders, during and immediately following the breeding season when early spermatogenesis occurs, primary spermatogonia that line the seminiferous tubules become closely associated with one or more Sertoli cells in a cyst. Within each cyst, there may be two or more Sertoli cells. As spermatogenesis continues within the cyst, each primary spermatogonium undergoes mitosis to form clones of secondary spermatogonia within the cyst and ultimately sets of primary spermatocytes. Following breeding, primary spermatocytes complete meiosis I and form secondary spermatocytes. The secondary spermatocytes continue through meiosis II to form spermatids, which remain embedded in clusters in the Sertoli cells. During spermiogenesis, spermatids begin elongation with their heads remaining in the Sertoli cells

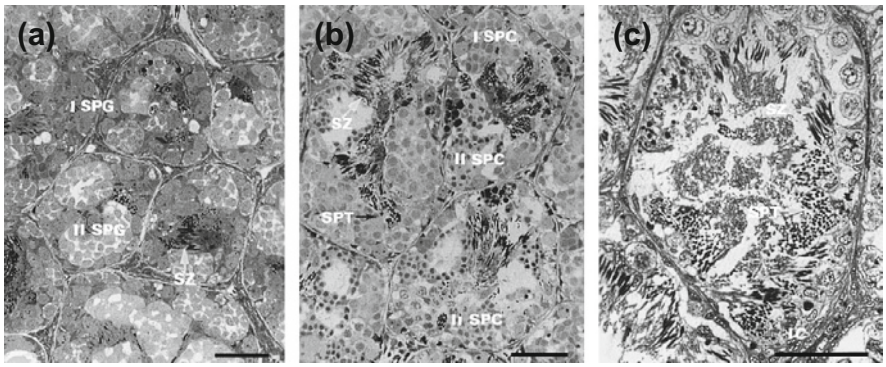

FIGURE 3.2 Seminiferous lobules from the seasonally breeding *Rana nigromaculata*. (a) From the month of June. Representing lobules containing cysts with mostly primary spermatogonia (I SPG) and secondary spermatogonia (II SPG). Interstial Leydig cells (IC) can be seen between two lobules. (b) Lobules from a frog collected in October, showing cysts in the secondary spermatocytes stage (II SPC), some spermatids (SPT), and spermatozoa (SZ). (c) A lobule from an animal collected in April demonstrating that the cysts have begun to break down and the spermatozoa have been released into the lobule lumen. Some IC cells are found between lobules. *Figure adapted with permission from Elsevier from Ko, Kang, Im and Kwon (1998), Figure 4, p. 354.*

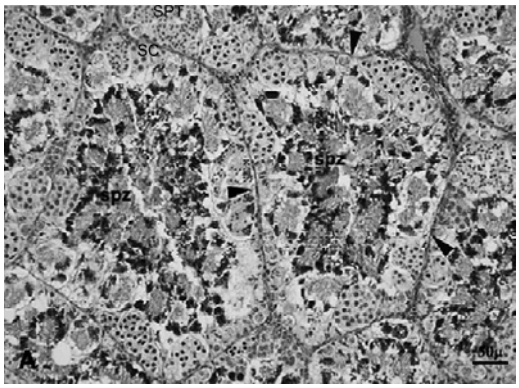

FIGURE 3.3 Section through the testis of *Xenopus laevis* demonstrating that all stages of spermatogenesis are found within a single lobule. Spz, spermatozoa; SC, spermatocytes; SPT, spermatids. The arrowheads demonstrate where connective tissue separates two lobules. *Figure adapted with permission from Elsevier from Cevasco et al. (2008), Figure 1a, p. 245.*

and their tails elongating into the lumen of the cysts. Cysts are attached to the basement membrane of seminiferous lobules and Sertoli cells within cysts are attached to the basement membrane. In the explosively breeding spadefoot toad *Scaphiopus couchii*, changes in spermatogenesis can occur over a matter of days (Harvey, Propper, Woodley, & Moore, 1997). Although within each lobule there are cysts containing all stages of spermatogenesis, the proportion of cysts with each type of germ cell shifts. Shortly after emergence following powerful rainstorms, this desert species exhibits greater numbers of cysts containing spermatids and sperm. Following breeding there is a clear reduction in spermatids and sperm. Early stage germ cells show the opposite changes with spermatogonia proliferating immediately following breeding. Therefore, in anurans, the basics of spermatogenesis are the same across species. However, there are clear differences in timing of each phase of sperm development dependent on evolutionary adaptations to the environment.

Sertoli cells form close connections with primary spermatogonia and retain these connections until just prior to spermiation. As spermatids form flagella, cysts break down and open into the lumen of the seminiferous lobules. As the breeding season nears, spermiation occurs and spermatozoa are released from their Sertoli cell connections into the lobule lumen (Pudney, 1995). However, initially lobules do not open into the efferent ductules until spermiation is underway. Gonadotropins (GTHs) binding to Sertoli cells that are around the interface between the seminiferous lobules and efferent ductules may play a role in the release of sperm into the efferent ductules (Kobayashi & Iwasawa, 1989). The apical part of each Sertoli cell associated with sperm breaks down and is released into the seminiferous lobule lumen at spermiation, but the nucleus remains and the Sertoli cells form a permanent epithelial layer that supports the next wave of spermatogenesis. This pattern of retention of Sertoli cells is not seen in urodele amphibians, which recruit new follicular cells that become Sertoli cells with the formation of new anterior lobules of the testes.

Changes in Leydig cell morphology have been described for several species and are reviewed in Ko et al. (1998) and Sasso-Cerri et al. (2004). Similarly to the urodeles, in the anuran testes, maturation of the interstitium occurs during spermiogenesis and is complete during and just after spermiation, corresponding with the beginning of the breeding season. Therefore, in spring breeders, winter testicular lobules contain spermatids and sperm embedded in Sertoli cells, and interstitial Leydig cells occur in groups or even whorls of cells between lobules and, although small, show some steroidogenic activity. Following spermiation, the Leydig cells rapidly complete maturation and secrete androgens important for the development of secondary sex characteristics. It has been suggested that, as with the urodeles, there are complex paracrine interactions between the lobules and the interstitium that regulate development of the Leydig cells, which in turn release androgens that further regulate the final stages of spermiogenesis and spermiation and possibly lead to the next early initiation of spermatogenesis (Ucci, 1982; Sasso-Cerri et al., 2004). In *X. laevis*, where all stages of spermatogenesis through spermiation can occur within an individual lobule, there appear to be active Leydig cells throughout the testes at all times of the year (Unsicker, 1975; Kalt, 1976). Therefore, the Leydig compartment also varies in function according to the evolutionary history of the species investigated.

As with salamanders, toads (*Bufo arenarum)* appear to have a blood–testis barrier that is limited to seminiferous lobules containing maturing germ cells (Cavicchia & Moviglia, 1983). Intracellular markers for tight junctions identified by freeze-fracture techniques identify occluding junctions between Sertoli cells in tubules that contain spermatids, but not in those containing earlier spermatogenetic stages. The barrier is confirmed, as with salamanders (see Section 2.1.2) by lanthanum injection. Again, the dye is excluded from the more mature tubules. These results demonstrate in amphibians that the blood–testis barrier forms and possibly protects maturing germ cells from plasma-borne pathogens and/or toxins.

3.2. Regulation of Testicular Activity

3.2.1. Endocrine and molecular events associated with spermatogenesis and spermiogenesis

In anurans, a few studies have evaluated the impact of the different GTHs on spermatogenesis. In the green frog *Rana*

esculenta, an FSH-like hormone is secreted under conditions of low T through lack of negative feedback (Rastogi, Iela, Saxena, & Chieffi, 1976). Treatment of hypophysectomized frogs with this homologous GTH induces the onset of spermatogenesis via spermatogonial proliferation and differentiation into secondary spermatocytes. The role of FSH after this stage is not well characterized; however, in the toad *B. arenarum*, human recombinant forms of FSH, LH, and chorionic gonadotropin (hCG) all induce spermiation, with FSH being the most effective. Rasotogi et al. (1976) hypothesize that LH plays a role in the spermiogenesis process of spermatid formation through induction of androgen production by the Leydig cells. This hypothesis is supported by the finding that in *B. arenarum* (Pozzi, Rosemblit, & Ceballos, 2006) and *X. laevis* (Lecouteux, 1988), LH is the most potent gonadotropic stimulator of androgen secretion. However, it will be important to test homologous GTHs in more species to better understand how FSH and LH regulate spermatogenesis in this clade.

The impact of androgens and E_2 on spermatogenesis and spermiogenesis has been studied in some anuran species. *In-vivo* treatment with dihydrotestosterone (DHT) in *Rana pipiens* induces an apparent increase in the number of spermatogonia differentiating into spermatocytes and entering meiosis I, while inhibiting these cells' maturation through meiosis II (Tsai, Lunden, & Jones, 2003). Also, blocking androgen synthesis via cyproterone acetate partly blocks spermatogenesis, which is consistent with the above results, suggesting some role of androgens in early spermatogenesis (Rastogi et al., 1976). The role of androgens in spermiogenesis or passage of the secondary spermatocytes to spermatids is supported by the finding that blocking androgen synthesis completely inhibits spermatid formation in *R. esculenta* (Rastogi et al., 1976), while long-term DHT treatment facilitates the formation of post-meiotic germ cells (Tsai, Kessler, Jones, & Wahr, 2005), suggesting that androgens play an important role in spermiogenesis. Lastly, seasonal increases in androgens are positively correlated both with increased spermatogenesis and spermiogenesis (Raucci & Di Fiore, 2007). Taken together, these studies demonstrate that androgens are important both in the mitotic and meiotic phases of sperm production in anurans.

Estradiol has profound and stage-specific effects on spermatogenesis. During early spermatogenesis, E_2 induces an increase in the differentiation of primary spermatogonia (Chieffi, Colucci-D'Amato, Staibano, Franco, & Tramontano, 2000a) possibly through an ERK 1/2 signaling pathway. Estradiol also induces proliferation of primary spermatogonia through phosphorylation of Fos proteins (Cobellis et al., 1999; Cobellis, Meccariello, Fienga, Pierantoni, & Fasano, 2002). However, long-term E_2 appears to inhibit differentiation of pre-meiotic germ cells into later developmental stages (Tsai et al., 2003; 2005). Therefore, E_2 plays an important role in early spermatogenesis by inducing cell proliferation, but may inhibit the transformation of primary spermatogonia into secondary spermatogonia. Interestingly, as with salamanders, the production and release of both androgens and estrogens, as well as prostaglandins, may be under the direct regulation of GnRH (Gobbetti & Zerani, 1992), and there is evidence for a GnRH receptor in frog testicular membrane preparations (Fasano, de Leeuw, Pierantoni, Chieffi, & van Oordt, 1990), although little is understood about whether the source of GnRH that acts on these receptors is gonadal or hypothalamic. These studies demonstrate that steroids play complex signaling roles in testicular activity in anurans.

Several other chemical mediators of testicular function have been studied in *R. esculenta*. For example, the renin−angiotensin system modulates testicular steroidogenesis and prostaglandin production (Miano et al., 1999). Generally, angiotensin I is converted to angiotensin II (AII) through the enzymatic action of angiotensin-converting enzyme (ACE). In this study, AII acted independently from ACE conversion activity to affect testicular E_2 and androgen production. The activity of endogenous ACE decreased the amount of E_2 produced by the testicular tissue while increasing the amount of androgens produced; however, exogenously administered AII had the opposite action on both steroids. These results suggest that ACE can modulate aromatase activity independently of its effects on AII production. Further evidence that ACE may play a role in testicular function is suggested by the finding that there is a seasonal cycle in testicular ACE activity (Bramucci, Quassinti, Maccari, Murri, & Amici, 2004). These results suggest that more research is necessary to understand both the role of the angiotensin system in reproduction and the non-angiotensin II-producing role of ACE in biological systems.

In *R. esculenta*, an amino acid, D-aspartate (D-Asp), appears to also play an important role in testicular function (Raucci et al., 2004). During the annual cycle of this frog, D-Asp levels in the testes are directly and positively correlated with T levels. Treatment with D-Asp induces a rise in plasma T *in vitro* and ultimately leads to increases in proliferating cell nuclear antigen (PCNA) staining in spermatogonia and spermatocytes. Proliferating cell nuclear antigen has been found only in proliferating pre-meiotic cells in frog testes, and it therefore may be a useful marker in evaluating the impact of different hormones on stages of germ cell (or spermatid) differentiation (Chieffi, Franco, Fulgione, & Staibano, 2000b). The interplay between D-Asp and T in the induction of germ cell proliferation deserves further investigation.

A few intracellular molecular markers of spermatogenesis have been identified in frogs. For example, some nuclear proto-oncogenes are associated with specific stages of sperm development in *R. esculenta* (Chieffi,

Minucci, Cobellis, Fasano, & Pierantoni, 1995), and their expression also depends on the period of the breeding season. For example, c-myc oncoprotein (Myc) is found in the nucleus of spermatogonia, primary and secondary spermatocytes, and Sertoli cells that are associated with both primary spermatogonia and mature spermatozoa. However, there is no evidence of Myc expression in the Leydig cells, suggesting that this oncoprotein is not important for interstitial development and function. C-fos oncoprotein (Fos) is also detected only in spermatogonia (though not in all spermatogonia). Sertoli cells adjacent to non-staining spermatogonia stain positive for Fos. Interstitial tissue also stained positive for nuclear Fos, especially in the winter. C-Jun oncoprotein (Jun) was found in the nucleus of some spermatogonia and secondary spermatocytes, but not in primary spermatocytes. As with Myc, Sertoli cells adjacent to primary spermatocytes that did not stain for Jun were immunopositive for this oncoprotein, as were Sertoli cells involved in spermiation. Leydig cells had consistent staining for Jun. Staining for one other protein, c-mos oncoprotein, was found consistently in the cytoplasm of spermatogonia and in both primary and secondary spermatocytes. The timing of the expression of these oncogenes in the nucleus as opposed to the cytoplasm suggests that expression of these proteins is critical to the seasonal onset of the spermatogenetic wave seen in this species. C-Jun oncoprotein in particular may be important to the onset of the second meiotic division as it is found in the nucleus of secondary spermatocytes. Chieffi et al. (1995) suggest that the immunostaining for Jun and Myc in Sertoli cells associated with cysts undergoing spermiation provides evidence that these two oncogene proteins may be involved in Sertoli−germ cell communication.

Other oncogenes have been studied in some anuran species. In *X. laevis*, A-myb appears to be mostly associated with spermatogenesis and cell proliferation (Sleeman, 1993). In the frog, *R. esculenta*, E_2 induces proliferation of primary spermatogonia, possibly through induction of extracellular signal-regulated kinase 1 and 2 (ERK1/2) activity, which increases during the time of year when active spermatogenesis occurs (Chieffi et al., 2000a). Another oncogene protein that may be linked to sperm development is c-kit receptor protein (Risley, 1983; Raucci & Di Fiore, 2007), which binds to stem cell factor (SCF). This transmembrane tyrosine kinase receptor is expressed most intensely in all germ cell types during the peak of the reproductive season when T levels are high in *R. esculenta*. The seasonal pattern of expression suggests that this protein may be key in the pathway of signaling between androgen secretion from Leydig cells and germ cell proliferation and maturation. Clearly, early spermatogenesis is associated with increases in intracellular machinery involved in mitotic cell proliferation events.

3.2.2. Control of spermiation

The impact of GTHs on spermiation is only slightly better understood in anurans than in urodeles. Early studies with purified ovine LH and FSH demonstrated that the two GTHs were equally potent in inducing spermiation in several frog species (Licht, 1973). In the toad *B. arenarum*, spermiation was induced by human recombinant LH, FSH, and hCG, with FSH being the most potent (Pozzi et al., 2006).

Information on the impact of other hormones on spermiation is limited. Treatment with E_2 leads to increased activity of myoid cells surrounding the lobules, which in turn induces release of sperm from the Sertoli cells in *R. esculenta* (Cobellis et al., 2008). This finding demonstrates that steroids have functions on testicular tissue other than the gametes, Sertoli cells, and Leydig compartment.

3.2.3. Control of the interstitial compartment

Studies of GnRH and GTHs on androgen secretion in frogs demonstrate that all may play an important role. In *B. arenarum*, a GnRH receptor has been identified in the testes, and *in-vitro* treatment with GnRH causes a decrease in both basal and human chorionic gonadotropin-induced secretion of androgens (Canosa, Pozzi, Somoza, & Ceballos, 2002). When the homologous hormones are used in bullfrogs, LH is a more potent stimulator of androgen secretion (Muller & Licht, 1980). However, in three other frog species (*Bufo marinus*, *Pseudoeurycea smithi*, and *X. laevis*), *Rana* LH was only slightly more potent than FSH in stimulating androgen release (Muller & Licht, 1980). Dose response curves generated in *B. arenarum* to human recombinant LH and FSH demonstrate that the toad testes secrete androgens in response to both gonadotropins *in vitro*, but are more sensitive to LH (Pozzi et al., 2006) .

In general, both LH and FSH in anurans may play a role in spermiation and androgen secretion, and both hormones are secreted during the breeding season in bullfrogs (Licht, McCreery, Barnes, & Pang, 1983). However, as with the actions of these pituitary hormones on germ cell development, separating the functions of LH and FSH on amphibian Leydig tissue will require investigations that use homologous hormones.

Melatonin (MEL) plays a direct role in regulating early spermatogenesis (d'Istria, Palmiero, Serino, Izzo, & Minucci, 2003; d'Istria et al., 2004), possibly by impacting Leydig cell communication with Sertoli cells. In *R. esculenta*, treatment with MEL *in vivo* or *in vitro* causes dramatic morphological changes in Leydig cell and Sertoli cell appearance, inhibits GnRH-induced T secretion, and reduces frog testicular relaxin gene expression. It also inhibits spermatogonial cell proliferation. These results suggest that MEL induces changes in Leydig cell function

that lead to paracrine interactions with Sertoli cell control of germ cell proliferation. By this hypothesized mechanism, MEL secretion may fine tune testicular development to appropriate environmental conditions.

4. GYMNOPHIONA

4.1. Testicular Structure

4.1.1. General gross anatomical structure

The literature regarding the anatomical structure of the testes in the clade Gymnophiona has been extensively reviewed by Exbrayat and Estabel (2006) and Smita, Jancy, Akbarsha, Oommen, and Exbrayat (2006). Early detailed studies were conducted by Seshachar (e.g., Seshachar, 1937). In caecilians, the paired testes are made up of one or a few lobes to a string of up to 22 lobes; those species with the fewest lobes come from the most derived group, suggesting an evolutionary trend in those clades towards fusion of the lobes (Smita et al., 2006). The number and size of the lobes is species-specific although there is within-species variation (Wake, 1968; Ebersole, 1998; Smita, Oommen, George, & Akbarsha, 2003). The lobes are joined by connective tissue strands that contain efferent sperm ductules, which transfer sperm to kidney ductules and subsequently to the urinary ducts that exit to the cloaca (Wake, 1968; Smita, Oommen, Jancy, & Akbarsha, 2004).

4.1.2. Histomorphic structure and sperm development

Within each lobe of the testis are several basement membrane-contained seminiferous lobules (Wake, 1968; Smita et al., 2003; 2004b; Exbrayat & Estabel, 2006). Between the lobules are connective tissue, blood vessels, nerves, and interstitial tissue. Within each lobule there are cysts with germ cells undergoing spermatogenesis, spermiogenesis, and ultimately spermiation, as reviewed in Smita et al. (2006).

Germ cells develop in cysts within the lobules of the testes, as with the other amphibian clades. Within each cyst, the germ cells are at the same stage of maturation (as reviewed by Smita et al., 2006). However, within each lobule, the cysts may be at different stages of development, although lobules with cysts all at one stage have been documented (Wake, 1968).

The early stages of spermatogenesis were extensively documented in the works of Seshachar in the 1930s and 40s (reviewed by Wake, 1968; Exbrayat & Estabel, 2006; Smita et al., 2006). The primary spermatogonia appear to migrate into the lobules from the efferent ductules and form cysts along the lobule walls in association with Sertoli cells. From this stage, the specific maturation stages of spermatogenesis in two species of caecilian, *Ichthyophis tricolor* and *Uraeotyphlus* cf. *narayani*, have been described in great detail (Smita et al., 2004b). Early in the spermatogenetic process, Sertoli cells associated with primary spermatogonia arise around the periphery of the lobules next to the basement membrane. These Sertoli cells appear to have migrated from the ductules along with the spermatogonia. The proliferation of these primary spermatogonia leads to the formation of cysts or cell nests containing usually smaller secondary spermatogonia also found in groups of cells along the inside periphery of the lobule. The secondary spermatogonia undergo further cell division to form two rows of compact smaller primary spermatocytes around the edges of the cysts. As the differentiation into spermatocytes occurs, the cysts move away from the periphery of the lobule and toward the central stroma, although in some species the lobular lumen does not appear to develop until the spermatids begin to mature (Wake, 1968). As these primary spermatocytes undergo the first meiotic division to form secondary spermatocytes, the cysts begin to move towards the center of the lobule and then the cells undergo the second meiotic division to form spermatids, which remain in close association with the Sertoli cells. The spermatids form a layer of cells more towards the center of the lobule and, once the lumen forms, these germ cells line the lumen of the cyst. Then spermatids undergo elongation and transformation to mature sperm, as described in detail by Smita, George, Girija, Akbarsha, and Oommen (2004a) and summarized in Smita et al. (2006). Figure 3.4 shows one active lobule.

Very little research on the interstitial compartment has been conducted outside of basic morphological and enzymatic descriptions (see Smita, Beyo, George, Akbarsha, & Oommen, 2005). As with other vertebrates, the interstitial tissue is located between the lobules (Smita et al., 2004b). It contains myoid cells, fibrous elements, capillaries, fibroblasts, and Leydig cells. In *I. tricolor*, the interstitial tissue containing the Leydig cells appears to be larger in areas when the testes are quiescent or showing only early stages of spermatogenesis than it is during the most active phase of sperm development. In comparison, the opposite is found in the New World *Dermophis mexicanus*, in which the interstitial compartment increases in volume during active phases of spermatogenesis and is regressed when the testes are completely quiescent (Wake, 1995; Smita et al., 2005).

Evaluation of Sertoli cell location, structure, and function has undergone differing interpretations of morphological findings (e.g., Seshachar, 1942; Smita et al., 2003; Exbrayat & Estabel, 2006). All of these investigators agree that the Sertoli cells appear to originate from precursor follicular cells in the tissue surrounding the lobular efferent ductules. These cells move within the cysts toward the inside wall of the lobules and have close contact with primary spermatogonia. Exbrayat and colleagues (2006) found from testicular evaluation of *Typhlonectes* spp. that

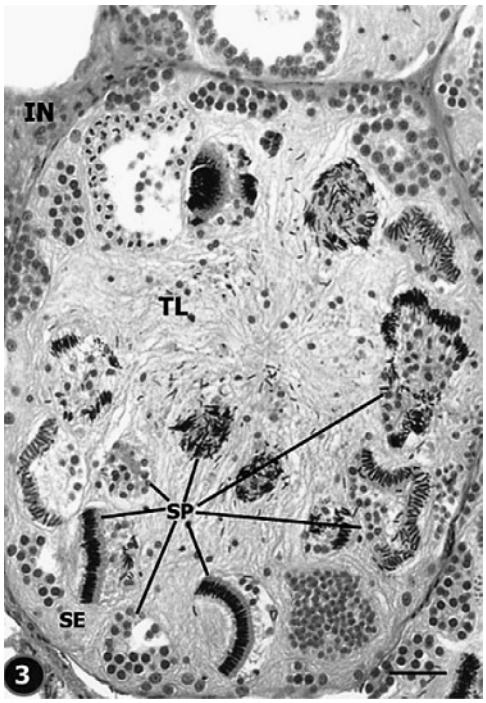

FIGURE 3.4 Histological image of a section through the testis of *Uraeotyphlus narayani*. TL, testis lobule; SP, spermatogenic cysts; IN, interstitial cells; SE, Sertoli cell. Note that within the lobule there are cysts containing all stages of spermatogenesis. Scale bar = 112 µm. *From George, Smita, Oommen, and Akbarsha (2004), Figure 3, p. 35.*

the Sertoli cells move away from the lobular wall and follow the developing cysts towards the center of the lobule as spermatogenesis progresses. The degenerate Sertoli cells are released into the lobule lumen along with sperm. If this interpretation of Sertoli cell development is correct then, during sperm development, the degenerate Sertoli cells are sloughed into the general matrix of the lobular ducts similarly to what is seen in the urodeles. However, other investigators have a different explanation for how the Sertoli cells support sperm development in this clade (Smita et al., 2003). Through electron and light microscopy studies in *I. tricolor*, evaluation of lobule morphology suggest that the Sertoli cells remain attached to the basal membrane throughout sperm development until sperm release, with the developing germ cells intimately associated and often embedded in the Sertoli cell membranes. The Sertoli cells then are available to support early stage spermatogonia during the next round of spermatogenesis. This interpretation of Sertoli cell changes in the testes of caecilians is more similar to that seen in anurans. Detailed studies of more species may be necessary to determine whether the differences in interpretation of these data are due to species specificity in testicular function or are based on interpretive differences in the collected images.

4.2. Regulation of Testicular Activity

4.2.1. Endocrine and molecular events associated with spermatogenesis and spermiogenesis

Because of the secretive nature of caecilians, little is known about endocrine regulation of testicular function. There are no published experimental studies on the role of pituitary GTHs on spermatogenesis, spermiogenesis, or spermiation, or on interstitial cell compartment regulation. One study has demonstrated steroidogenic enzyme activity in the testes of *Typhlonectes compressicauda* (Anjubault & Exbrayat, 1999) and another study has examined the role that prolactin (PRL) may play in testicular activity in the same species, where PRL receptor mRNA is found to be expressed in germ cells, Sertoli cells, and Leydig cells (Exbrayat & Morel, 2003). However, aside from the presence of the PRL receptor throughout gonadal compartments, little is known about the role of endocrine signaling within caecilian testes.

5. SUMMARY

There are similarities and differences across the three clades of Lissamphibia in terms of morphology, the sperm maturation process, and hormonal regulation of the testes. Salamanders and caecilians have multilobed testes, whereas anurans tend to have single compact testes. Sperm maturational processes differ between the three groups. In salamanders, spermatogenesis, spermiogenesis, and spermiation occur in a wave from the cranial to the caudal end of each testis, with the developing germ cells being in a similar stage of development across cysts within the same region of the testis, whereas in anurans, cysts within one lobule can be at differing stages of development. A similar finding to that for anurans is seen in caecilians, although, in seasonal breeders, the cysts in the testes may be at one or only a few stages of sperm maturation at any one time of year. There are dramatic differences in the fate of the Sertoli cells across the three groups. In salamanders, the Sertoli cells degenerate along with the lobular tissue following spermiation. In frogs and toads, the Sertoli cells reform following spermiation and remain to support the next round of germ cell production. In caecilians, evidence from some studies suggests that these animals follow an anuran pattern while others suggest that the Sertoli cells are lost in spermiation.

Although there is clear overlap in GTH potency on all testicular functions, generally, FSH appears to impact Sertoli cells to regulate the onset and continuation of germ cell recruitment, mitosis, and meiosis, while LH acts on the Leydig cells of the interstitium to influence androgen production in both anurans and urodeles. Little is

understood about the endocrine regulation of testicular activity in caecilians and more work with this clade is necessary to understand the regulatory processes involved in male reproduction.

Steroids are clearly important to spermatogenesis in all groups of lissamphibians, with androgens being important for spermatogenesis, spermiogenesis, and especially spermiation. Estradiol may also play a role following spermiation in the recruitment and proliferation of new germ cells at the end of the breeding cycle. In salamanders and frogs, it is clear that endocrine, paracrine, and intracellular modulators of cell proliferation regulate the onset of spermatogenesis, but more efforts are necessary to understand how the GTHs and steroids interact with these factors in all clades in order to better understand sperm maturation in this group and whether these intratesticular processes can inform research in other vertebrate clades.

ABBREVIATIONS

ABP	Androgen-binding protein
ACE	Angiotensin-converting enzyme
AII	Angiotensin II
AR	Androgen receptor
D-Asp	D-aspartate
DHT	Dihydrotestosterone
E_2	Estradiol
EGF	Epidermal growth factor
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
Fos	C-fos oncoprotein
FSH	Follicle-stimulating hormone
FXR	Farnesoid X-activated receptor
GnRH	Gonadotropin-releasing hormone
GTH	Gonadotropin
hCG	Chorionic gonadotropin
IGF-1	Insulin-like growth factor 1
IGF-2	Insulin-like growth factor 2
Ig-NRG1	Neuregulin1
Jun	C-Jun oncoprotein
LH	Luteinizing hormone
MEL	Melatonin
Myc	C-myc oncoprotein
PCNA	Proliferating cell nuclear antigen
PRL	Prolactin
Rb	Retinoblastoma
RXR-α, -β, -γ	Retinoid acid receptors
SCF	Stem cell factor
T	Testosterone
WT1	Wilms' tumor

REFERENCES

Abe, S. (2004). Hormonal control of meiosis initiation in the testis from Japanese newt, *Cynops pyrrhogaster. Zool. Sci., 21*, 691−704.

Abe, S. I., & Ji, Z. S. (1994). Initiation and stimulation of spermatogenesis *in vitro* by mammalian follicle-stimulating hormone in the Japanese newt, *Cynops pyrrhogaster. Int. J. Dev. Biol., 38*, 201−208.

Abe, K., Eto, K., & Abe, S. (2008). Epidermal growth factor mediates spermatogonial proliferation in newt testis. *Reprod. Biol. Endocrinol., 6*, 7−20.

Abe, K., Jin, Y., Yamamoto, T., & Abe, S. (2002). Human recombinant stem cell factor promotes spermatogonial proliferation, but not meiosis initiation in organ culture of newt testis fragments. *Biochem. Biophys. Res. Commun., 294*, 695−699.

Alfaro, J. M., Ricote, M., Lobo, M. V., Royuela, M., Fraile, B., Paniagua, R., & Arenas, M. I. (2002). Immunohistochemical detection of the retinoid acid receptors (RXR-Alpha, -Beta, -Gamma) and Farnesoid X-Activated receptor (FXR) in the marbled newt along the annual cycle. *Mol. Reprod. Dev., 62*, 216−222.

Anjubault, E., & Exbrayat, J. M. (1999). Yearly cycle of leydig-like cells in testes of *Typhlonectes compressicaudus* (Amphibia:Gymnophiona). In C. Miaud, & R. Guyetant (Eds.), *"Current Studies in Herptetology"* (pp. 53−58). France: Le Bourget Du Lac.

Arenas, M. I., Royuela, M., Lobo, M. V., Alfaro, J. M., Fraile, B., & Paniagua, R. (2001). Androgen Receptor (AR), Estrogen Receptor-Alpha (ER-Alpha) and Estrogen Receptor-Beta (ER-Beta) expression in the testis of the newt, *Triturus marmoratus marmoratus* during the annual cycle. *J. Anat., 199*, 465−472.

Bergmann, M., Greven, H., & Schindelmeiser, J. (1983). Observations on the blood-testis barrier in a frog and a salamander. *Cell Tissue Res., 232*, 189−200.

Bergmann, M., Greven, H., & Schindelmeiser, J. (1982). The fine structure of the testis wall in *Salamandra salamandra* (L.) (Amphibia, Urodela). *Arch. Histol. Jpn., 45*, 317−324.

Blüm, V., 1937. Vergleichende reproduktionsbiologie der wirbeltiere. English %T vertebrate reproduction: a textbook / volker blum; translated from the German edition by A.C. Whittle.

Bramucci, M., Quassinti, L., Maccari, E., Murri, O., & Amici, D. (2004). Seasonal changes in angiotensin converting enzyme activity in male and female frogs (*Rana esculenta*). *Comp. Biochem. Physiol., Part A. Mol. Integr. Physiol., 137*, 605−610.

Callard, G. V., Canick, J. A., & Pudney, J. (1980). Estrogen synthesis in leydig cells: structural−functional correlations in *Necturus* testis. *Biol. Reprod., 23*, 461−479.

Canosa, L. F., Pozzi, A. G., Somoza, G. M., & Ceballos, N. R. (2002). Effects of mGnRH on testicular steroidogenesis in the toad *B. arenarum. Gen. Comp. Endocrinol., 127*, 174−180.

Cavicchia, J. C., & Moviglia, G. A. (1983). The blood−testis barrier in the toad (*Bufo arenarum* Hensel): a freeze-fracture and lanthanum tracer study. *Anat. Rec., 205*, 387−396.

Cevasco, A., Urbatzka, R., Bottero, S., Massari, A., Pedemonte, F., Kloas, W., & Mandich, A. (2008). Endocrine disrupting chemicals (EDC) with (anti)estrogenic and (anti)androgenic modes of action affecting reproductive biology of *Xenopus laevis*: II. Effects on gonad histomorphy. *Comp. Biochem. Physiol. C, 147*, 241−251.

Chieffi, P., Colucci-D'Amato, G. L., Staibano, S., Franco, R., & Tramontano, D. (2000a). Estradiol-induced mitogen-activated protein kinase (extracellular signal-regulated kinase 1 and 2) activity in the frog (*Rana esculenta*) testis. *J. Endocrinol., 167*, 77−84.

Chieffi, P., Franco, R., Fulgione, D., & Staibano, S. (2000b). PCNA in the testis of the frog, *Rana esculenta*: a molecular marker of the mitotic testicular epithelium proliferation. *Gen. Comp. Endocrinol., 119*, 11−16.

Chieffi, P., Minucci, S., Cobellis, G., Fasano, S., & Pierantoni, R. (1995). Changes in proto-oncogene activity in the testis of the frog, *Rana esculenta*, during the annual reproductive cycle. *Gen. Comp. Endocrinol., 99*, 127−136.

Cobellis, G., Cacciola, G., Chioccarelli, T., Izzo, G., Meccariello, R., Pierantoni, R., et al. (2008). Estrogen regulation of the male reproductive tract in the frog, *Rana esculenta*: a role in fra-1 activation in peritubular myoid cells and in sperm release. *Gen. Comp. Endocrinol., 155*, 838−846.

Cobellis, G., Meccariello, R., Fienga, G., Pierantoni, R., & Fasano, S. (2002). Cytoplasmic and nuclear fos protein forms regulate resumption of spermatogenesis in the frog, *Rana esculenta*. *Endocrinology, 143*, 163−170.

Cobellis, G., Pierantoni, R., Minucci, S., Pernas-Alonso, R., Meccariello, R., & Fasano, S. (1999). C-Fos activity in *Rana esculenta* testis: seasonal and estradiol-induced changes. *Endocrinology, 140*, 3238−3244.

Del Rio-Tsonis, K., Covarrubias, L., Kent, J., Hastie, N. D., & Panagiotis, A. T. (1996). Regulation of the wilms' tumor gene during spermatogenesis. *Develop. Dyn., 207*, 372−381.

d'Istria, M., Serino, I., Izzo, G., Ferrara, D., De Rienzo, G., & Minucci, S. (2004). Effects of melatonin treatment on leydig cell activity in the testis of the frog *Rana esculenta*. *Zygote, 12*, 293−299.

d'Istria, M., Palmiero, C., Serino, I., Izzo, G., & Minucci, S. (2003). Inhibition of the basal and oestradiol-stimulated mitotic activity of primary spermatogonia by melatonin in the testis of the frog, *Rana esculenta*, *in Vivo* and *in Vitro*. *Reproduction, 126*, 83−90.

Ebersole, T. J. (1998). Gonadal steroid production in the viviparous caecilian amphibian, *Typhlonectes natans*. *Am. Zool., 38*, 21A.

Exbrayat, J. M. 2006. Reproductive Biology and Phylogeny of Gymnophiona (Caecilians). Series Editor B.G.M. Jamieson. *Vol 5*. Enfield (NH), USA, Jersey, Plymouth, U.K.

Exbrayat, J. M., & Estabel, J. (2006). Anatomy with particular reference to the reproductive system. In J. M. Exbrayat (Ed.), *"Reproductive Biology and Phylogeny of Gymnophiona (Caecilians)", Vol. 5* (pp. 79−156). Enfield, NH: Science Publishers, Inc.

Exbrayat, J. M., & Morel, G. (2003). Visualization of gene expression of prolactin-receptor (PRL-R) by *in situ* hybridization in reproductive organs of *Typhlonectes compressicauda*, a gymnophionan amphibian. *Cell Tissue Res., 312*, 361−367.

Fasano, S., de Leeuw, R., Pierantoni, R., Chieffi, G., & van Oordt, P. G. (1990). Characterization of gonadotropin-releasing hormone (GnRH) binding sites in the pituitary and testis of the frog, *Rana esculenta*. *Biochem. Biophys. Res. Commun., 168*, 923−932.

Fraile, B., Saez, F. J., & Paniagua, R. (1990). The cycle of follicular and interstitial cells (Leydig Cells) in the testis of the marbled newt, *Triturus marmoratus* (Caudata, Salamandridae). *J. Morphol., 204*, 89−101.

Franchi, E., Camatini, M., & Decurtis, I. (1982). Morphological evidence of a permeability barrier in urodele testis. *J. Ultrastruct. Res., 80*, 253−263.

George, J. M., Smita, M., Oommen, V. O., & Akbarsha, M. A. (2004). Histology and ultrastructure of male mullerian gland of *Uraeotyphlus narayani* (Amphibia: Gymnophiona). *J. Morph., 260*, 33−56.

Gobbetti, A., & Zerani, M. (1992). Mammalian GnRH involvement in prostaglandin F2alpha and sex steroid hormones testicular release in two amphibian species; the anuran water frog, *Rana esculenta*, and the urodele crested newt, *Triturus carnifex*. *Gen. Comp. Endocrinol., 87*, 240−248.

Gobbetti, A., & Zerani, M. (1995). Androgen synthesis modulation by protaglandin E2 is differentially mediated via adenylate cyclase and phospholipase C in the testis of the crested newt, *Trituris carnifex*: *in vitro* studies. *Biochem. Biophys. Res. Comm., 211*, 1047−1052.

Harvey, L. A., Propper, C. R., Woodley, S. K., & Moore, M. C. (1997). Reproductive endocrinology of the explosively breeding desert spadefoot toad, *Scaphiopus couchii*. *Gen. Comp. Endocrinol., 105*, 102−113.

Humphery, R. R. (1921). The interstitial cells of the urodele testes. *Am. J. Anat., 29*, 213−279.

Humphery, R. R. (1922). The multiple testis in urodels. *Biol. Bull., 43*, 45−67.

Jamieson, B.G.M. (2003). Reproductive biology and phylogeny of the Anura. In B.G.M. Jamieson (Series Ed.), *Enfield (NH): Vol. 5* (pp. 452). USA, Jersey: Plymouth, U.K.

Ji, Z. S., Kubokawa, K., & Abe, S. (1995). Promotion of differentiation of newt primary spermatocytes into spermatids by mammalian FSH via sertoli cells. *J. Exp. Zool., 272*, 374−383.

Jin, Y., Uchida, I., Eto, K., Kitano, T., & Abe, S. (2008). Size-selective junctional barrier and Ca(2+)-independent cell adhesion in the testis of *cynops pyrrhogaster*: expression and function of occludin. *Mol. Reprod. Dev., 75*, 202−216.

Kalt, M. R. (1976). Morphology and kinetics of spermatogenesis in *Xenopus laevis*. *J. Exp. Zool., 195*, 393−407.

Ko, S. K., Kang, H. M., Im, W., & Kwon, H. B. (1998). Testicular cycles in three species of korean frogs: *Rana nigromaculata, Rana rugosa*, and *Rana dybowskii*. *Gen. Comp. Endocrinol., 111*, 347−358.

Kobayashi, T., & Iwasawa, H. (1989). Gonadotropin-induced luminal formation in the seminiferous tubules and efferent ductules in young frogs: light and electron microscopic study. *J. Exp. Zool., 252*, 301−307.

Lecouteux, A. (1988). Gonadotropin action on androgen synthesis in short-term incubation of explants and dispersed cells of the testis of *Xenopus laevis*. *J. Exp. Zool., 245*, 187−193.

Licht, P. (1973). Induction of spermiation in anurans by mammalian pituitary gonadotropins and their subunits. *Gen. Comp. Endocrinol., 20*, 522−529.

Licht, P., McCreery, B., Barnes, R., & Pang, R. (1983). Seasonal and stress related changes in plasma gonadotropins, sex steroids, and corticosterone in the bullfrog, *Rana catesbeiana*. *Gen. Comp. Endocrinol., 50*, 124−125.

Maekawa, K., Zai-Si Ji, & Shin-Ichi, Ab (1995). Proliferation of newt spermatogonia by mammalian FSH via sertoli cells *in vitro*. *J. Exp. Zool., 272*, 363−373.

Mak, P., Callard, I. P., & Callard, G. V. (1983). Characterization of an estrogen receoptor in the testis of the urodele Amphibian *Necturus maculosus*. *Biol. Reprod., 28*, 261−270.

Miano, A., Gobbetti, A., Zerani, M., Quassinti, L., Maccari, E., Murri, O., Amici, D., & Bramucci, M. (1999). Different modulation of aromatase activity in frog testis *in vitro* by ACE and ANG II. *Am. J. Physiol., 277*, R1261−R1267.

Moore, F. L. (1975). Spermatogenesis in larval ambystoma tigrinum: positive and negative interactions of FSH and testosterone. *Gen. Comp. Endocrinol., 26*, 525−533.

Moore, F. L., Muller, C. H., & Specker, J. L. (1979). Origin and regulation of plasma dihydrotestosterone and testosterone in the rough-skinned newt, *Taricha granulosa*. *Gen. Comp. Endocrinol., 38*, 451−456.

Moya, L., Guerrero, F., Navas, P., & Garcia-Herdugo, G. (1987). GNRH induces activation of leydig-like cells in pleurodeles waltlii. a morphometric study. *Histol. Histopathol., 2*, 193−198.

Muller, C. H., & Licht, P. (1980). Gonadotropin specificity of androgen secretion by amphibian testes. *Gen. Comp. Endocrinol., 42*, 365−377.

Nakayama, Y., Yamamoto, T., & Abe, S. I. (1999). IGF-I, IGF-II and insulin promote differentiation of spermatogonia to primary spermatocytes in organ culture of newt testes. *Int. J. Dev. Biol., 43*, 343−347.

Nakayama, Y., Yamamoto, T., Oba, Y., Nagahama, Y., & Abe, S. (2000). Molecular cloning, functional characterization, and gene expression of a follicle-stimulating hormone receptor in the testis of newt *Cynops pyrrhogaster*. *Biochem. Biophys. Res. Commun., 275*, 121−128.

Pozzi, A. G., Rosemblit, C., & Ceballos, N. R. (2006). Effect of human gonadotropins on spermiation and androgen biosynthesis in the testis of the toad *Bufo arenarum* (Amphibia, Anura). *J. Exp. Zoolog.A. Comp. Exp. Biol., 305*, 96−102.

Pudney, J. (1995). Spermatogenesis in nonmammalian vertebrates. *Microsc. Res. Tech., 32*, 459−497.

Pudney, J., & Callard, G. V. (1984). Organization of interstitial tissue in the testis of the salamander *Necturus maculosus* (Caudata: Proteidae). *J. Morphol., 181*, 87−95.

Pudney, J., Canick, J. A., Mak, P., & Callard, G. V. (1983). The differentiation of leydig cells, steroidogenesis, and the spermatogenetic wave in the testis of *Necturus maculosus*. *Gen. Comp. Endocrinol., 50*, 43−66.

Rastogi, R., Iela, L., Saxena, P., & Chieffi, G. (1976). The control of spermatogenesis in the green frog, *Rana esculenta*. *J. Exp. Zool., 196*, 151−166.

Raucci, F., & Di Fiore, M. M. (2007). The c-Kit receptor protein in the testis of green frog Rana esculenta: seasonal changes in relationship to testosterone titres and spermatogonial proliferation. *Reproduction, 133*, 51−60.

Raucci, F., Assisi, L., D'Aniello, S., Spinelli, P., Botte, V., & Di Fiore, M. M. (2004). Testicular endocrine activity is upregulated by D-aspartic acid in the green frog, *Rana esculenta*. *J. Endocrinol., 182*, 365−376.

Ricote, M., Alfaro, J. M., Garcia-Tunon, I., Arenas, M. I., Fraile, B., Paniagua, R., & Royuela, M. (2002). Control of the annual testicular cycle of the marbled-newt by p53, p21, and Rb gene products. *Mol. Reprod. Dev., 63*, 202−209.

Risley, M. S. (1983). Spermatogenic cell differentiation *in vitro*. *Gamete. Res., 7*, 331−346.

Saez, F. J., Madrid, J. F., Aparicio, R., Hernandez, F., & Alonso, E. (2001). Carbohydrate moieties of the interstitial and glandular tissues of the Amphibian *Pleurodeles waltl* testis shown by lectin histochemistry. *J. Anat., 198*, 47−56.

Saez, F. J., Madrid, J. F., Cardoso, S., Gomez, L., & Hernandez, F. (2004). Glycoconjugates of the urodele amphibian testis shown by lectin cytochemical methods. *Microsc. Res. Tech., 64*, 63−76.

Sasso-Cerri, E., de Faria, F. P., Freymuller, E., & Miraglia, S. M. (2004). Testicular morphological changes during the seasonal reproductive cycle in the bullfrog *Rana catesbeiana*. *J. Exp. Zoolog. A. Comp. Exp. Biol., 301*, 249−260.

Scheltinga, D. M., & Jamieson, B. G. M. (2003). Spermatogenesis and the mature spermatozoon: form, function and phylogenetic implications. In B. G. M. Jamieson (Ed.), *"Reproductive Biology and Phylogeny of Anura"* (pp. 119−251). Enfield, NH: Science Publishers, Inc.

Seshachar, B. R. (1937). Germ-cell origin in the adult caecilian *Ichthyophis glutinosus* (Linn). *Cell Tissue Res., 26*, 293−304.

Seshachar, B. R. (1942). The sertoli cells in apoda. *J. Mys. Univ. N. S., 3B*, 65−71.

Sever, D.M. (2003). Reproductive biology and phylogeny of urodela. In B.G.M. Jamieson (Series Ed.), Vol. 1. Science Publishers, Inc., Enfield (pp. 452) New Hampshire, U.S.A.

Singh, S., & Callard, G. (1992). Identification of an androgen receptor in the zonal testis of the salamander (*Necturus maculosus*). *Gen. Comp. Endocrinol., 86*, 220−230.

Singh, S., & Callard, G. V. (1989). A specific androgen-binding protein (ABP) in *Necturus* testis and its zonal distribution. *J. Exp. Zool., 250*, 73−81.

Sleeman, J. P. (1993). *Xenopus* A-Myb is expressed during early spermatogenesis. *Oncogene, 8*, 1931−1941.

Smita, M., George, J. M., Girija, R., Akbarsha, M. A., & Oommen, O. V. (2004a). Spermiogenesis in caecilians *Ichthyophis tricolor* and *Uraeotyphlus cf. narayani* (Amphibia: Gymnophiona): analysis by light and transmission electron microscopy. *J. Morphol., 262*, 484−499.

Smita, M., Jancy, G. M., Akbarsha, M. A., Oommen, O. V., & Exbrayat, J. M. (2006). Caecilian spermatogenesis. In J. M. Exbrayat (Ed.), *"Reproductive biology and phylogeny of gymnophiona (Caecilians)", Vol. 5* (pp. 231). New Hampshire: Science Publishers.

Smita, M., Oommen, O. V., Jancy, M. G., & Akbarsha, M. A. (2004b). Stages in spermatogenesis of two species of caecilians, *Ichthyophis tricolor* and *Uraeotyphlus cf. narayani* (Amphibia: Gymnophiona): light and electron microscopic study. *J. Morphol., 261*, 92−104.

Smita, M., Beyo, R. S., George, J. M., Akbarsha, M. A., & Oommen, O. V. (2005). Seasonal variation in spermatogenetic and androgenic activities in a caecilian testis (*Ichthyophis tricolor*). *J. of Zool., 267*, 45.

Smita, M., Oommen, O. V., George, J. M., & Akbarsha, M. A. (2003). Sertoli cells in the testis of caecilians, *Ichthyophis tricolor* and *Uraeotyphlus cf. narayani* (Amphibia: Gymnophiona): light and electron microscopic perspective. *J. Morphol., 258*, 317−326.

Specker, J. L., & Moore, F. L. (1980). Annual cycle of plasma androgens and testicular composition in the rough-skinned newt, *Taricha granulosa*. *Gen. Comp. Endocrinol., 42*, 297−303.

Tanaka, S., Sakai, M., Hattori, M. A., Kikuyama, S., Wakabayashi, K., & Hanaoka, Y. (2004). Effect of bullfrog LH and FSH on newt testes under different temperatures. *Gen. Comp. Endocrinol., 138*, 1−7.

Tsai, P. S., Kessler, A. E., Jones, J. T., & Wahr, K. B. (2005). Alteration of the hypothalamic−pituitary−gonadal axis in estrogen- and androgen-treated adult male leopard frog, *Rana pipiens*. *Reprod. Biol. Endocrinol., 3*, 2.

Tsai, P. S., Lunden, J. B., & Jones, J. T. (2003). Effects of steroid hormones on spermatogenesis and GnRH release in male leopard frogs, *Rana pipiens*. *Gen. Comp. Endocrinol., 134*, 330−338.

Ucci, A. A. (1982). A fine-structural study of interstitial cell changes in the testes of *Necturus maculosus* during a portion of the annual cycle,

and possible evidence for local feedback control by seminiferous epithelium. *Am. J. Anat., 165*, 27−38.

Unsicker, K. (1975). Fine structure of the male genital tract and kidney in the anura *Xenopus laevis* daudin, *Rana temporaria* L. and *Bufo bufo* L. under normal and experimental conditions. *Cell Tissue Res., 158*, 215−240.

Uribe Aranzabal, M. C. (2003). The testes, spermatogenesis and male reproductive ducts. In D. M. Sever (Ed.), *"Reproductive biology and phylogeny of urodela", Vol. 1* (pp. 183−202). Enfield, NH: Science Publishers, Inc.

Valdivieso, D., & Tamsitt, J. R. (1965). Reproduction in a neotropical salamander, *Bolitoglossa adspersa* (Peters). *Herpetological, 21*, 228−236.

van Oordt, P. G. W. J. (1960). The influence of the internal and external factors in the regulation of the spermatogenic cycle in amphibia. *Zool. Soc. London Symp., 2*, 29−52.

Van Oordt, P. G. W. J. (1956). *Regulation of the Spermatogenetic Cycle in the Common Frog (Rana temporaria)*. Arnhem: G. W. Van der Wiel and Co.

Wake, M. H. (1968). Evolutionary morphology of the caecilian urogenital system. I. the gonads and the fat bodies. *J. Morphol., 126*, 291−331.

Wake, M. H. (1995). The spermatogenic cycle of *Dermophis mexicanus* (Amphibia: Gymnophiona). *J. Herpetol., 29*, 119−122.

Yamamoto, T., Hikino, T., Nakayama, Y., & Abe, S. (1999). Newt RAD51: cloning of cDNA and analysis of gene expression during spermatogenesis. *Dev. Growth Differ., 41*, 401−406.

Yamamoto, T., Nakayama, Y., & Abe, S. I. (2001). Mammalian follicle-stimulating hormone and insulin-like growth factor I (IGF-I) up-regulate IGF-I gene expression in organ culture of newt testis. *Mol. Reprod. Dev., 60*, 56−64.

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Chapter 4

Hormones and the Female Reproductive System of Amphibians

Mari Carmen Uribe Aranzábal
Universidad Nacional Autónoma de México, Ciudad de México, Mexico

SUMMARY
Paired ovaries and oviducts form the female reproductive system of amphibians. This system changes seasonally in accordance with the reproductive cycle. The neuroendocrine systems influence the functions of the ovaries. The ovaries produce steroids, which control the oviductal changes. The ovaries are elongated and sacular structures containing oogonia and oocytes in different stages of oogenesis. Oogenesis is a complex process of cellular and molecular changes that occur during the formation, growth, and maturation of the female germinal cells. Follicle cells form a squamous or cuboidal layer around the oocyte during oogenesis. At this time, the oocyte is at diplotene, in meiotic arrest, which ends during oocyte maturation. Oogenesis includes primary growth or previtellogenesis, when organelles and diverse molecules increase in the ooplasm; secondary growth or vitellogenesis, when deposition of yolk platelets occurs; and oocyte maturation, when meiotic arrest is broken and the oocyte is released into the coelom during ovulation. The oviducts are paired, elongated ducts, extending on each side of the midline of the body cavity. The oviducts include an infundibulum—an ample opening that receives the eggs after ovulation; a pars recta, formed by a short and straight region; the pars convoluta, formed by the largest and glandular region; and the ovisac, an aglandular short and straight region with a thick muscle layer that opens to the cloaca.

1. INTRODUCTION

The female reproductive system of amphibians, as in most vertebrates, consists of paired ovaries and oviducts. Temperate zone amphibians and numerous tropical species exhibit cyclic ovarian and oviductal changes in response to changes in environmental conditions such as light, temperature, rainfall, and humidity. These environmental changes regulate the physiology of the neuroendocrine systems that control the reproductive process. The neuroendocrine systems influence the functions of the ovaries via hypothalamic releasing factors and hypophysial gonadotropins (GTHs), which stimulate the follicles during oogenesis (Redshaw, 1972; Norris, 2007). Females may produce one or several clutches of eggs during the reproductive season. Temperate species usually produce one clutch per year; however, tropical species may produce several clutches (Lofts, 1984; Iela, Rastogi, Delrio, & Bagnara, 1986; Norris & Lopez, 2005). In some species, oogenesis requires more than one year, as described in temperate and tropical amphibians that reproduce biennially such as urodeles of the genera *Desmognathus*, *Plethodon*, and *Pseudotriton* (Bruce, 1975; Licht & Porter, 1987; Whittier & Crews, 1987; Jørgensen, 1992; Norris, 2007). An adequate supply of nutrients is an essential factor in the reproductive process, particularly in females, which require considerable energy reserves to develop yolked eggs (Adams, 1940; Bruce, 1975; Whittier & Crews, 1987; Lofts, 1984; Jørgensen, 1992; Wake & Dickie, 1998).

The majority of amphibians are oviparous, but there are also viviparous species in all three orders: Gymnophiona, Urodela, and Anura (Wake, 1993). Numerous viviparous species occur among the caecilians (*Gymnopis multiplicta*, *Typhlonectes compressicaudata* (Wake, 1970; 1977; 1998), *Dermophis mexicanus* (Wake, 1998), *Scolecomorphus kirkii* (Wake, 1977; 1998), and *Scolecomorphus vittatus* (Loader, Wilkinson, Gower, & Msuya, 2003)); the urodeles (*Mertensiella luschani*, *Salamandra atra*, *Salamandra salamandra*, and *S. s. infraimmaculata* (Wake, 1985; Greven & Guex, 1994; Greven, 1998; Wake, 1998; Greven, 2003)), and a few species of anuran (*Nectophrynoides occidentalis* (Wake, 1985)). Even though oviparity is the most frequent type of reproduction in amphibians, internal fertilization occurs in a high percentage of species of urodeles (90%) and caecilians (100%) (Lofts, 1984; Duellman & Trueb, 1986; Wake, 1993; Wake & Dickie, 1998). The urodele species that have external fertilization belong to the families Hynobiidae, Cryptobranchidae, and Sirenidae (Lofts, 1984; Duellman & Trueb, 1986; Sever, Rania, & Krenz, 1996),

in which oviposition and the extrusion of sperm into spermatophores occur simultaneously and fertilization is external (Brandon, 1970). Internal fertilization is supported by the presence of spermathecae in female oviducts. The spermathecae are clusters of cloacal glands that store sperm (Trauth, 1983; 1984; Sever, 1994; Brizzi, Delfino, Selmi, & Sever, 1995; Brizzi, Delfino, & Jantra 2003; Sever, 2003). Internal fertilization occurs in the oviduct, usually taking place in its caudal part or in the cloacal cavity immediately before oviposition (Brandon, 1970; Duellman & Trueb, 1986; Joly, Chesnel, & Boujard, 1994; Wake & Dickie, 1998).

The structural and cyclical changes in the amphibian ovary are characterized by major morphological and physiological processes that are similar to those described in most vertebrates. These processes include (1) oogenesis, when oogonia initiate meiosis becoming oocytes and their differentiation into mature oocytes; (2) ovulation, when mature oocytes are released into the coelom and then move into the oviducts; and (3) endocrine events leading to the secretion of female sex hormones and the regulation of ovarian changes essential for reproduction (Lofts, 1984; Duellman & Trueb, 1986; Wake & Dickie, 1998; Fernández & Ramos, 2003; Sánchez and Villecco, 2003; Uribe, 2009).

The ovaries and oviducts of amphibians develop during early embryogenesis, when the coelomic epithelium of the mesonephros is colonized by primordial germ cells that form the genital ridges. As in other vertebrates, the origin of primordial germ cells is extragonadal. In amphibians, the origin of these cells is associated with endodermal cells of the embryonic gut. From there, the primordial germ cells migrate through the dorsal mesentery until they reach the coelomic epithelium of the mesonephros, giving rise to the germinal epithelium in the outermost layer of the developing ovary that is surrounded by coelom. During ovarian development, the oogonia are situated in the cortex. A cavitation occurs in the medulla and develops into the sacular structure of the ovary. In early morphogenesis of the ovary, the germinal epithelium consists of oogonia and somatic cells (Figure 4.1(a−c)). A kidney, one Wolffian duct (Figure 4.1(d)−(e)), and one Müllerian duct are adjacent to each ovary (Dodd, 1977; Lofts, 1984; Subtenly & Penkala, 1984; Tanimura & Iwasawa, 1988; 1989; Sharon, Degani, & Warburg, 1997; Uribe, 2003; 2009).

The ovaries of adult amphibians are located in the abdominal cavity and are suspended dorsally to the body wall by the mesovarium. The ovaries lie symmetrically on either side of the midline of the body, in parallel position to the kidneys, oviducts, Wolffian ducts, and fat bodies (Figure 4.2(a)) (Hope, Humphries, & Bourne, 1963; Lofts, 1984; Wake, 1985; Iela et al., 1986; Norris, 2007; Beyo, Sreejith, Divya, Oommen, & Akbarsha, 2007; Uribe, 2009).

2. OVARIAN STRUCTURE

The ovaries of adult amphibians are elongated and irregular in shape, lie in the abdominal cavity, and are composed of one or several lobes. Normally, both ovaries have the same number of lobes (Tyler, 2003). The size of the ovaries changes seasonally according to the sequential stages of oogenesis. During primary growth (previtellogenesis) and secondary growth (vitellogenesis), the ovaries increase in size progressively, attaining their maximal volume during the breeding season. At this time, the ovaries contain numerous previtellogenic and vitellogenic oocytes (Dumont, 1972; Dodd, 1977; Lofts, 1984; Dodd, 1986). At the onset of breeding, the large ovaries can occupy the entire length of the body cavity. The absence of a diaphragm in amphibians permits the expansion of the ovaries into the abdomino−thoracic cavity (Tyler, 2003).

In previtellogenic ovaries, the oocytes are light yellow in color. During vitellogenesis, in species that produce pigmented eggs, the animal hemisphere becomes dark brown with the deposit of melanin and the vegetal hemisphere remains an opaque, light yellow. Duellman and Trueb (1986) suggested that the presence of melanin in oviposited eggs exposed to sunlight is an important feature that protects the embryos during their development, both from ultraviolet radiation and through heat absorption.

Several authors have described the histological structure of the ovaries and oogenesis in amphibians (Adams, 1940; Hope et al., 1963; Dumont, 1972; Joly & Picheral, 1972; Spornitz & Kress, 1973; Dodd, 1977; Guraya, 1978; Wallace, 1978; Lofts, 1984; Pancharatna & Saidapur, 1985; Dodd, 1986; Iela et al., 1986; Berois & de Sa, 1988; Bement & Capco, 1990; Wallace & Selman, 1990; Sharon et al., 1997; Pawar & Pancharatna, 1999; Sretarugsa, Weerachatyanukul, Chavadej, Kruatrachue, & Sobhon, 2001; Uribe, 2001; Sánchez & Villecco, 2003; Uribe, 2003; Oliveira & Souza-Santos, 2004; Beyo et al., 2007a; Beyo, Sreejith, Divya, Oommen, & Akbarsha, 2007; Beyo, Divya, Oommen, & Akbarsha, 2008; Beyo, Divya, Smita, Oommen, & Akbarsha, 2008; Ogielska & Bartmanska, 2009; Uribe, 2009). The ovary is a saccular organ surrounded by the ovarian wall, and has a central cavity filled with lymph (Figure 4.2(a−b)). In ovaries composed of several lobes, each lobe is also sacular and contains a lumen. The sacular structure of the ovary provides space for the progressively growing oocytes, which are supported by a peduncle at the germinal epithelium and project into the lumen during their development.

As a consequence of the origin of the ovary, its wall is limited externally by the germinal epithelium (Figures 4.2 (b−d) and 4.3(a)). This epithelium contains oogonia, early oocytes, and somatic epithelial cells, and is supported by a basement membrane that separates the epithelium from the subjacent stroma. The oogonia (Figure 4.2(d)) and early

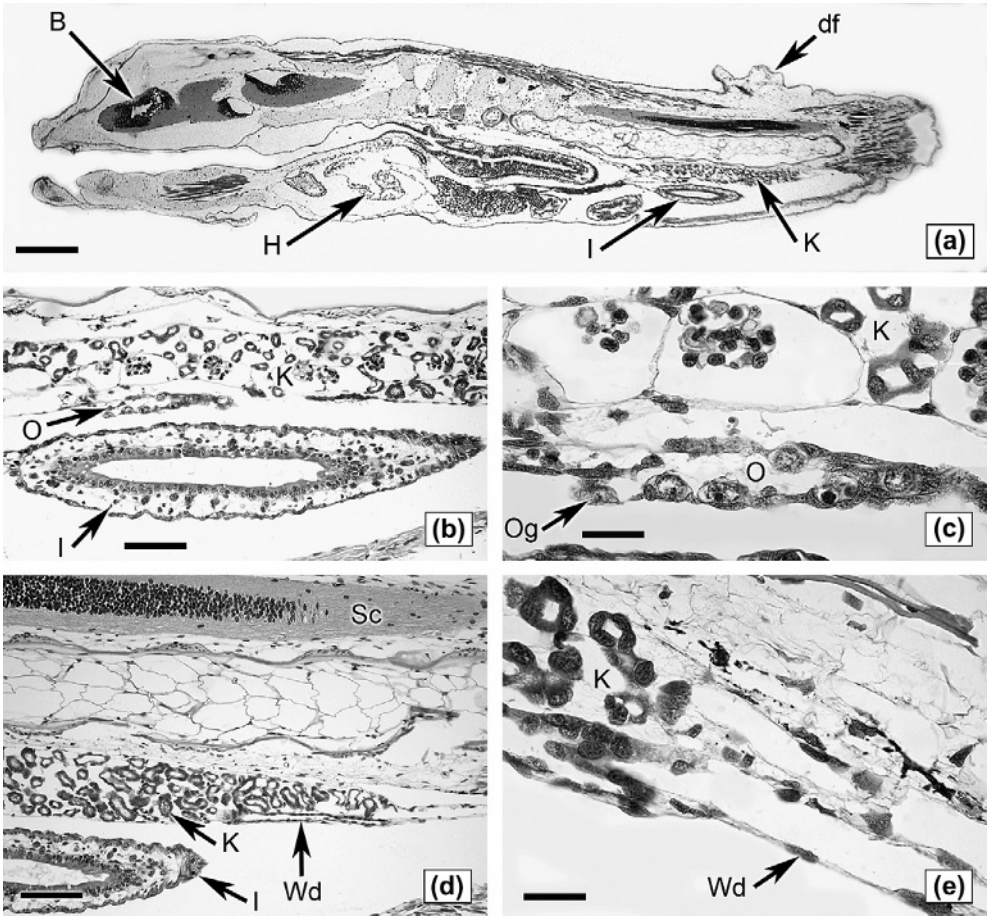

FIGURE 4.1 Longitudinal sections of a larva of *Ambystoma dumerilii*, three weeks after hatching. (a–c) Images of the ovary in three progressive magnifications. (a) Longitudinal section of a complete larva. Brain (B), heart (H), intestine (I), kidney (K), and dorsal fin (df) are seen. (b) Details of (a), in the region of the early ovary (O). The ovary is ventral to the kidney (K) and dorsal to the intestine (I). Oogonia (Og) at the periphery of the ovary (O) are seen. (d–e) The Wolffian duct (Wd) is seen ventral to the kidney (K). Also seen are the spinal cord (Sc) and intestine (I). Masson's trichrome staining. Bars: (a) 1 mm; (b) 50 µm; (c) 10 µm; (d) 50 µm; (e) 10 µm.

oocytes (Figure 4.2(b–d)) are located randomly among the somatic epithelial cells. The ovarian stroma contains vascularized, loose connective tissue with blood and lymphatic vessels, nerves, collagen fibers, and various types of somatic cells such as fibroblasts, macrophages, lymphocytes, and melanocytes. During the annual reproductive cycle, the ovary contains follicles in different stages of development (Figure 4.3(a–b)), including degenerating atretic follicles (Hope et al., 1963; Byskov, 1978; Lofts, 1984; Dodd, 1986; Uribe, 2001; 2003).

3. OOGENESIS

Oogenesis in amphibians, as in all other animals, is a complex process of cellular and molecular changes that occur during the formation, growth, and maturation of the female germinal cells. In every annual reproductive cycle, numerous oogonia divide mitotically (Figure 4.2(d)), becoming oocytes. These oocytes undertake primary and secondary growth following stimulation by GTHs, giving rise to full-grown oocytes that undergo final maturation and ovulate during the breeding season.

Oogenesis in amphibians is indeterminate because, during each reproductive cycle in the adult life of the female, the number of oocytes is renewed and typically increases. This proliferation is possible because oogonia remain in the germinal epithelium of adult ovaries, and these germ cells develop into successive generations of oocytes (Jørgensen, 1992; Uribe, 2003; Sánchez & Villecco, 2003; Norris & Lopez, 2005; Norris, 2007; Beyo et al., 2007b). The indeterminate type of reproductive cycle differentiates amphibians from birds and mammals, in which all oogonia initiate the meiotic process in young females. Consequently, in birds and mammalian females, proliferation of oocytes is not possible in adults (Dodd, 1977; Tokarz, 1978; Lofts, 1984; Jørgensen, 1992; Norris, 2007). However, in amphibians, ovulation is followed in the ovary by the proliferation of oogonia, which renew the

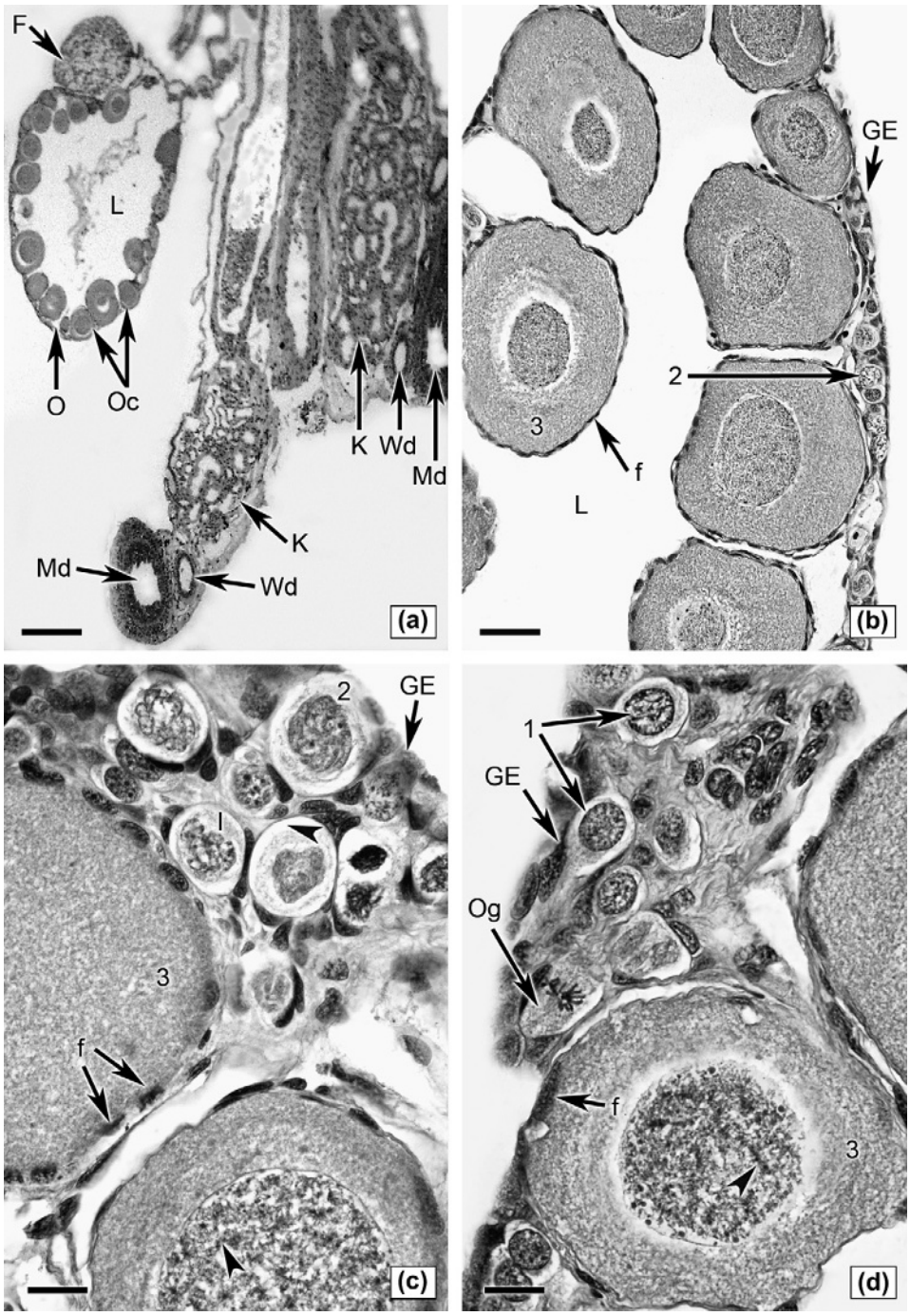

FIGURE 4.2 Ovary of a juvenile female *Ambystoma dumerilii* during primary growth. (a–d) Sequence of images in three progressive magnifications. (a) Anatomical relationships of the ovary in the abdominal cavity. The saccular structure of the ovary (O) is evident, with a central lumen (L). The ovarian wall contains oocytes at different stages of primary growth. The fat body (F) is adjacent to the ovary (O). Kidney (K), Wolffian duct (Wd), and Müllerian duct (Md) of both sides of the reproductive system are seen. (b–d) Ovarian wall surrounded by the germinal epithelium (GE), containing oocytes in stages 1 (1), 2 (2), and 3 (3). Oogonia (Og) during mitotic metaphase, abundant early meiotic oocytes in stages 1 (1) and 2 (2). Oocytes in stage 3 (3) with large germinal vesicle and lampbrush chromosomes (arrow head) are evident. Follicle cells (f) surround the oocytes. Ovarian stages are described in Table 1. Hematoxylin and eosin staining. Bars: (a) 0.2 mm; (b) 30 µm; (c) 10 µm; (d) 10 µm.

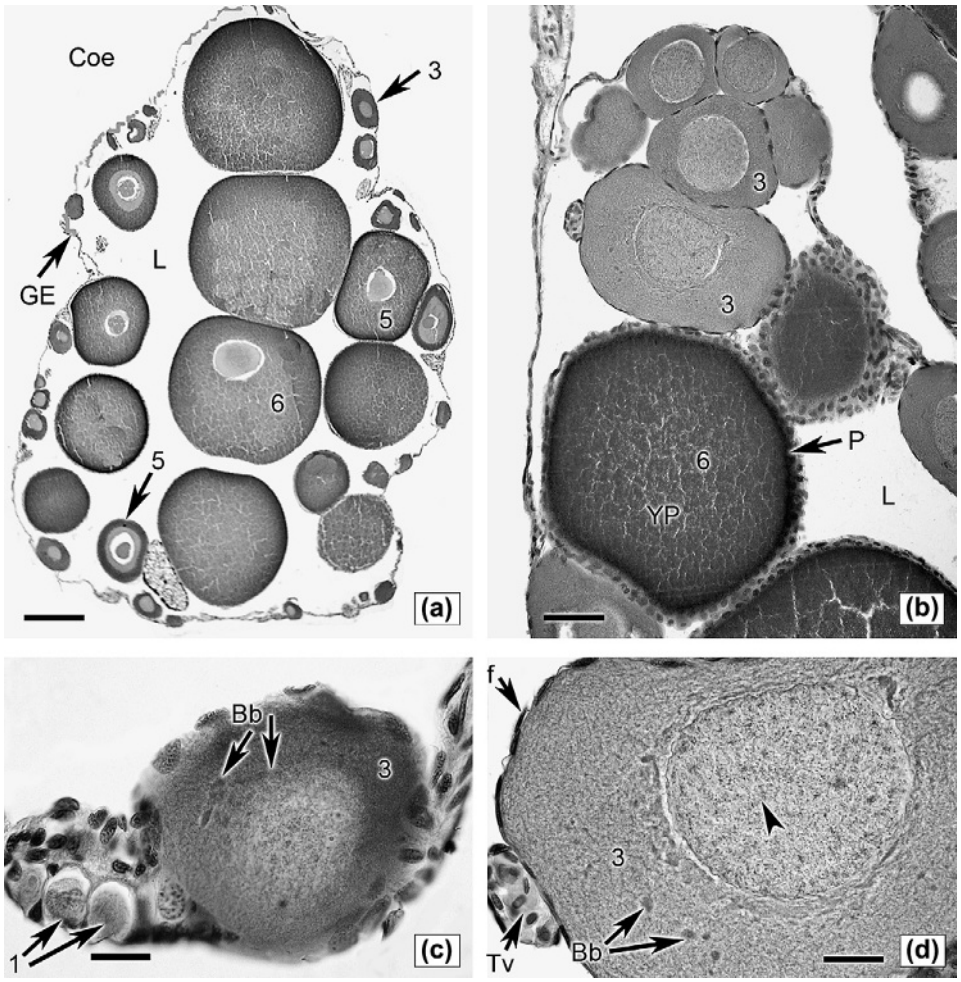

FIGURE 4.3 Ovarian structure of adult female *Ambystoma dumerilii*. (a–b) Ovaries surrounded by the coelom (Coe) with oocytes in various stages of primary and secondary growth: stages 3 (3), 5 (5), and 6 (6). Wide ovarian lumen (L) is seen. The ovarian wall is limited by the germinal epithelium (GE). Section of an oocyte in secondary growth, in stage 6 (6), containing abundant yolk platelets (YP) and pigment (P) at the periphery. (c) Nest of early oocytes in stage 1 (1) containing spherical nuclei with early diplotene chromosomes. Oocyte in stage 3 (3) with Balbiani bodies (Bb) near the germinal vesicle. (d) Oocyte during primary growth in stage 3 (3). The spherical germinal vesicle with lampbrush chromosomes (arrow head) is seen. The abundant ooplasm contains Balbiani bodies (Bb). Squamous follicle cells (f) and thecal blood vessels (Tv) are seen around the oocyte. Ovarian stages are described in Table 1. Hematoxylin and eosin staining. Bars: (a) 0.3 mm; (b) 20 µm; (c–d) 10 µm.

population of early oocytes, while previtellogenic oocytes, already present in the ovary, proceed to develop into full-grown oocytes (Jørgensen, 1992).

The number of eggs ovulated at any one time ranges from very few to numerous. Oviparous amphibians produce hundreds or thousands of eggs per clutch, whereas viviparous species produce few eggs (Duellman & Trueb, 1986; Norris & Lopez, 2005). As two extreme examples, in microhylid species, five or six eggs are ovulated during the reproductive cycle. In *Bufo marinus*, up to 30 000 eggs are ovulated (Tyler, 2003).

During oogenesis in the majority of described species, the oocytes increase greatly in volume, becoming full-grown oocytes at approximately 1.2–2.0 mm in diameter. The volume of the oocyte is variable between species. For example, the full-grown oocytes of *Pachymedusa dacnicolor* are 2.5 mm in diameter (Iela et al., 1986), but in *Gastrotheca riobambae* they become 3.3 mm in diameter (del Pino, 1989). In other species, the oocytes become even larger: 5–6 mm in diameter in the anurans *Pipa pipa*, *Hylactophryne augusti*, and *Flectonotus pygmaeus* and in the urodeles *Necturus maculosus*, *Bolitoglossa subpalmata*, and *Dicamptodon ensatus*. The caecilian *Ichthyophis glutinosus* has eggs that are 35 mm in diameter, these being the largest eggs described in amphibians (see the review by Duellman & Trueb (1986).

3.1. Oogonia

Oogonia are the source of a renewing stem germ cell population in the ovary. They divide mitotically, increasing in number. Oogonia are 8–12 µm in diameter and their

shape is spherical or ovoidal. Histologically, oogonia have a translucent ooplasm. The nucleus is ovoidal, containing a single nucleolus and fine granular chromatin (Figure 4.2 (d)). The oogonia are scattered in the germinal epithelium as individual cells or form small groups in cell nests (Figures 4.2(c–d) and 4.3(c)). Frequently, oogonia in a cell nest are connected by intercellular bridges and undergo synchronous development.

At the initiation of meiosis, oogonia become primary oocytes, progressing through leptotene, zygotene, pachytene, and early diplotene of the first meiotic prophase. Female fertility requires precise regulation of the meiotic process during the seasonal cycle (Hammes, 2004). Oocytes become arrested in late diplotene, during which growth occurs, ending when they become full-grown. At this time, a GTH signal from the pituitary causes meiosis to resume. Oocytes enter the maturation stage, and ovulation occurs (Tokarz, 1978; Bement & Capco, 1990; Jørgensen, 1992; Sánchez & Villecco, 2003; Hammes, 2004; Beyo et al., 2007a; Ogielska & Bartmanska, 2009).

3.2. Folliculogenesis

At the beginning of meiosis, folliculogenesis is initiated when oocytes, in cell nests, begin to become surrounded by a single layer of somatic epithelial cells, the prefollicle cells. Later, intercellular bridges between the oocytes are broken, as each oocyte progressively becomes surrounded by a single layer of squamous prefollicle cells. Folliculogenesis is completed when the developing follicle is encompassed by a basement membrane. Then, prefollicle cells become follicle cells in a primordial follicle.

Beyo et al. (2007a; 2007b) described the sequential changes during folliculogenesis in two species of caecilian, *Ichthyophis tricolor* and *Gegeneophis ramaswamii*, and these changes are quite similar to those described in fish. Observations in teleost fishes have examined oogonia, early oocytes, and prefollicle cells in the germinal epithelium and discussed the events of the folliculogenesis. The teleost follicle is a morphological unit composed of the oocyte and the surrounding follicle cells. During folliculogenesis, a delicate basement membrane completely encloses the follicle. Subsequently, a thin theca, derived from the stroma, develops around the basement membrane. The follicle (oocyte and follicle cells) and surrounding tissues (basement membrane and theca) become the ovarian follicle complex (Grier, Uribe, & Patiño, 2009).

The amphibian primordial follicle undergoes morphological and physiological changes throughout its development, including primary growth or previtellogenesis (Figures 4.2(b–d), 4.3, and 4.4(a–c)); secondary growth or vitellogenesis (Figures 4.3(a–b), 4.4(b–f), and 4.5) until full-grown (Figures 4.6 and 4.7(a)); and, finally, oocyte maturation, when meiotic arrest is broken and the oocyte is released into the coelom during ovulation.

3.3. Primary Growth

During primary growth (Figures 4.2(b–d), 4.3(b–d), and 4.4(a)), the size of the amphibian oocyte increases significantly. The nuclei of the oocytes, now called germinal vesicles, have distinctive morphology. Each germinal vesicle enlarges in volume and contains the paired, replicated, homologous, diplotene chromosomes developing multiple transcriptionally lateral loops forming the characteristic lampbrush chromosomes (Figures 4.2(c–d), 4.3 (d), 4.4(d), 4.5(c), and 4.6(b)). The lateral loops are extended portions of DNA that are capable of transcribing genes, which provide RNAs for early embryogenesis. During primary growth the oocytes are very active in the synthesis of RNAs. The germinal vesicle develops multiple nucleoli that synthesize ribosomal RNA (Figures 4.4(a–d) and 4.6(b)). The activity of the nucleoli permits the storage of numerous ribosomes in the ooplasm.

The ooplasm grows extensively, involving an enormous increase in cell organelles associated with synthetic activities, such as mitochondria, endoplasmic reticulum, Golgi apparatus, and ribosomes. In primary growth, the ooplasm becomes basophilic, due to the synthesis of abundant cellular machinery for DNA, RNAs (mRNA, tRNA, and rRNA), and protein synthesis (enzymes, precursors for molecular synthesis, and diverse proteins). Mitochondria replicate actively during primary growth and continue to do so during secondary growth. Oil droplets are formed in the peripheral ooplasm. Cortical granules begin to form, originally dispersed throughout the ooplasm but later moving to the peripheral ooplasm. The cortical granules have been described in several anuran species such as *Rana pipiens* (Ward & Ward, 1968), *Xenopus laevis* (Grey, Wolf, & Hendrick, 1974), *Bufo japonicus* (Yamazaki & Katagiri, 1991), *Bufo arenarum* (Oterino, Sánchez Toranzo, Zelarayán, Valz-Gianinet, & Bühler, 2001; Oterino et al., 2006), and *Rana ridibunda* (Ogielska & Bartmanska, 2009). The cortical granules are membrane-bounded vesicles of 1.5 to 2.5 μm in diameter (Grey et al., 1974). After fertilization, they undergo exocytosis and their contents (glycoproteins) are released outside the oolemma, resulting in a slow and permanent block to polyspermy (Yamazaki & Katagiri, 1991; Oterino et al., 2001; 2006; Ogielska & Bartmanska, 2009). The formation of most of these ooplasmic contents, which are used during early embryonic development, occurs or is initiated during primary growth of oogenesis (Gall et al., 2004; Schmidt-Zachmann, 2005).

Basophilic irregular structures of the ooplasm adjacent to the germinal vesicle are evident during primary growth. These structures are known as Balbiani bodies, vitelline bodies, or mitochondrial clouds (Figure 4.3(c–d)). Balbiani

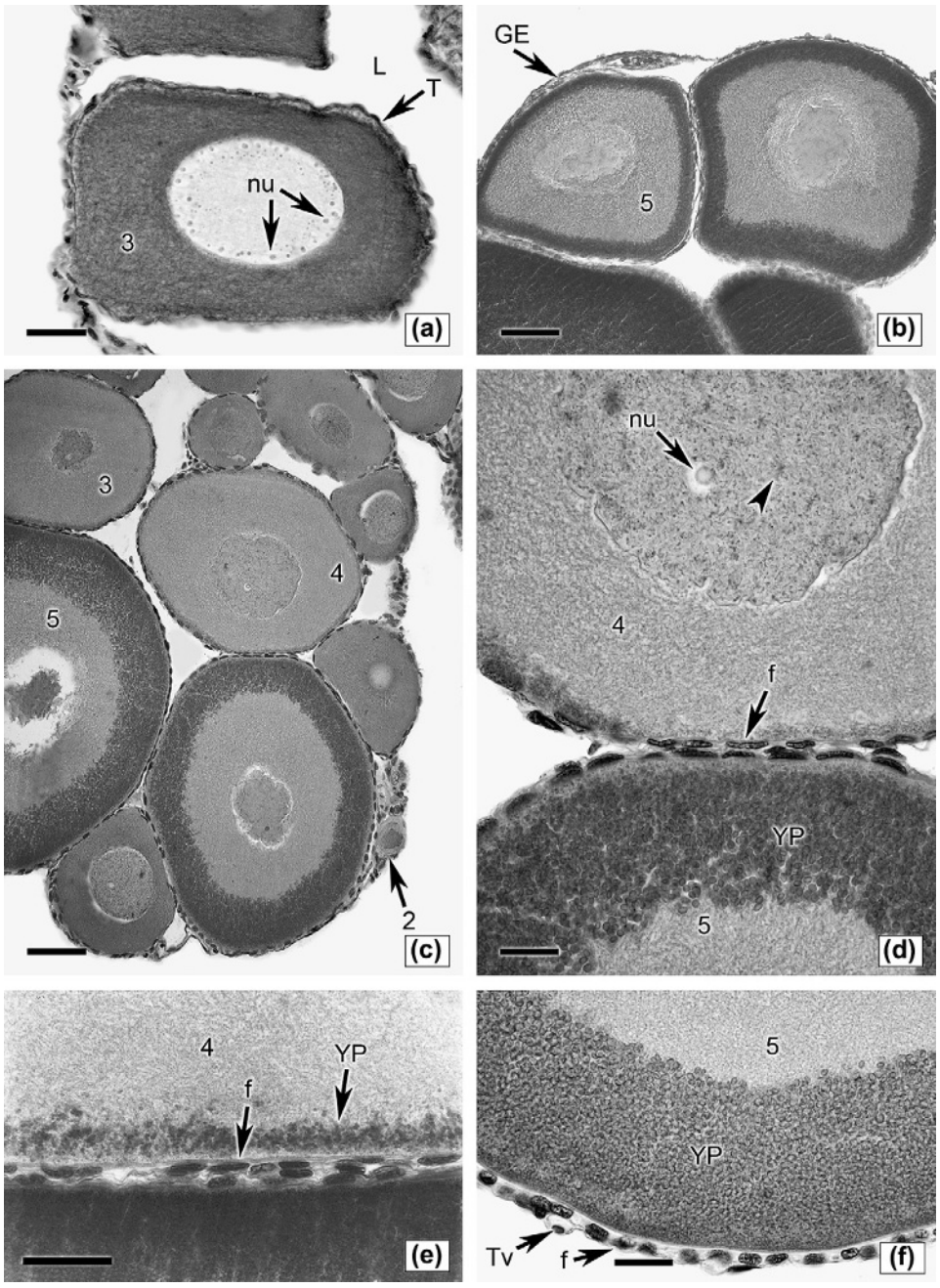

FIGURE 4.4 Ovaries of adult female *Hyla eximia* (a–c) and *Ambystoma dumerilii* (c–f). (a) Oocyte in primary growth, stage 3 (3). The ooplasm is basophilic. The germinal vesicle contains numerous nucleoli (nu), situated along the periphery. The follicle is surrounded by the theca (T). The ovarian lumen (L) is seen. (b) Oocytes in secondary growth, stage 5 (5). The ooplasm contains yolk platelets, increasing in number from the periphery. The germinal epithelium (GE) surrounds the ovary. (c) Oocytes during various stages of oogenesis: in primary growth, stages 2 (2) and 3 (3), and secondary growth, stages 4 (4) and 5 (5). Note the irregularly folded contour of the germinal vesicle in the oocytes in stages 4 (4) and 5 (5). (d–e) Details of (c). Oocytes in secondary growth, stages 4 (4) and 5 (5). The contour of the germinal vesicle is irregularly folded. The nucleoli (nu) and lampbrush chromosomes (arrow head) are clearly observed. The peripheral ooplasm of oocyte in stage 4 (4) contains the earliest acidophilic yolk platelets, seen as a few small acidophilic granules. The increase of yolk platelets is clear in the oocyte of stage 5 (5). The nuclei of follicle cells (f) are squamous. (f) Oocyte in stage 5 (5) with abundant yolk platelets. The theca (Tv) contains blood vessels. Ovarian stages are described in Table 1. (a) Masson's trichrome. (b–f) Hematoxylin and eosin staining. Bars: (a) 20 μm; (b–c) 50 μm; (d–f) 10 μm.

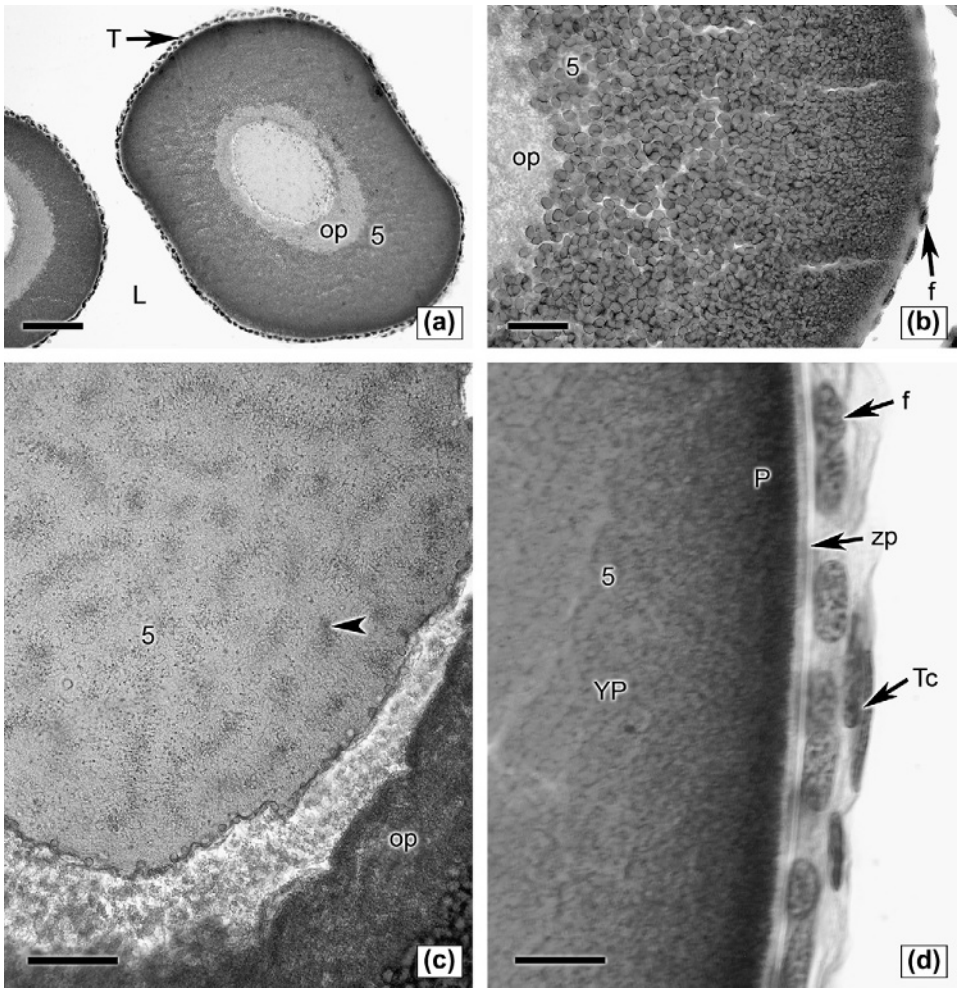

FIGURE 4.5 Ovary of adult female *Ambystoma mexicanum* during secondary growth. (a–d) Oocyte at stage 5 (5). Late deposition of yolk platelets (YP) located within the ooplasm. Only the ooplasm (op) surrounding the germinal vesicle lacks yolk platelets. The peripheral animal pole contains pigment (P). Germinal vesicle of the oocyte with folded membrane. Lampbrush chromosomes (arrow head) are well defined. The zona pellucida (zp), follicle cells (f) with flattened nuclei, theca (T) containing squamous cells (Tc), and ovarian lumen (L) are observed. Ovarian stages are described in Table 1. (a,c) Masson's trichrome. (b,d) Hematoxylin and eosin staining. Bars: (a) 20 µm; (b–d) 10 µm.

bodies are a temporary site of organelle accumulation and are composed of mitochondria, ribosomes, fibro-granular material, and Golgi apparatus (Guraya, 1978; Lofts, 1984; Palecek, Habrová, Nedvídek, & Romanovský, 1985; Wallace & Selman, 1990; Uribe, 2003; Oliveira & Souza-Santos, 2004; Beyo et al., 2007a; 2007b; Zelazowska, Kilarski, Bilinski, Podder, & Kloc, 2007; Ogielska & Bartmanska, 2009; Uribe, 2009). Palecek et al. (1985) observed in primary growth oocytes of *X. laevis* that Balbiani bodies contain a large amount of tubulin. In secondary growth oocytes, Balbiani bodies are dispersed at the periphery of the ooplasm where tubulin may be involved in the pathways for transport of the yolk proteins bound in endosomes. Zelazowska et al. (2007), comparing Balbiani bodies in *X. laevis* and in the sturgeon *Acipenser guelden-staedtii*, confirmed the role of these structures in the formation of the intracellular transport system.

The follicle cells form a single layer of squamous cells around the oocyte, which progressively becomes cuboidal (Figures 4.2(b–d) and 4.3(d)). The basement membrane and the vascularized theca completely surround the follicle (Figures 4.3(d) and 4.4(a)).

3.4. Secondary Growth

Secondary growth or vitellogenesis is a process of yolk accumulation (Figures 4.3(a–b), 4.4(b–f), 4.5, and 4.6). The yolk is the most abundant material stored during oogenesis in the ooplasm and is used for nutrition and metabolic activities during the development of the embryo. The yolk is stored in membrane-bound yolk platelets, which are large crystalline structures (Lange, 1985; Richter, 1987). Glycogen granules and oil droplets are also

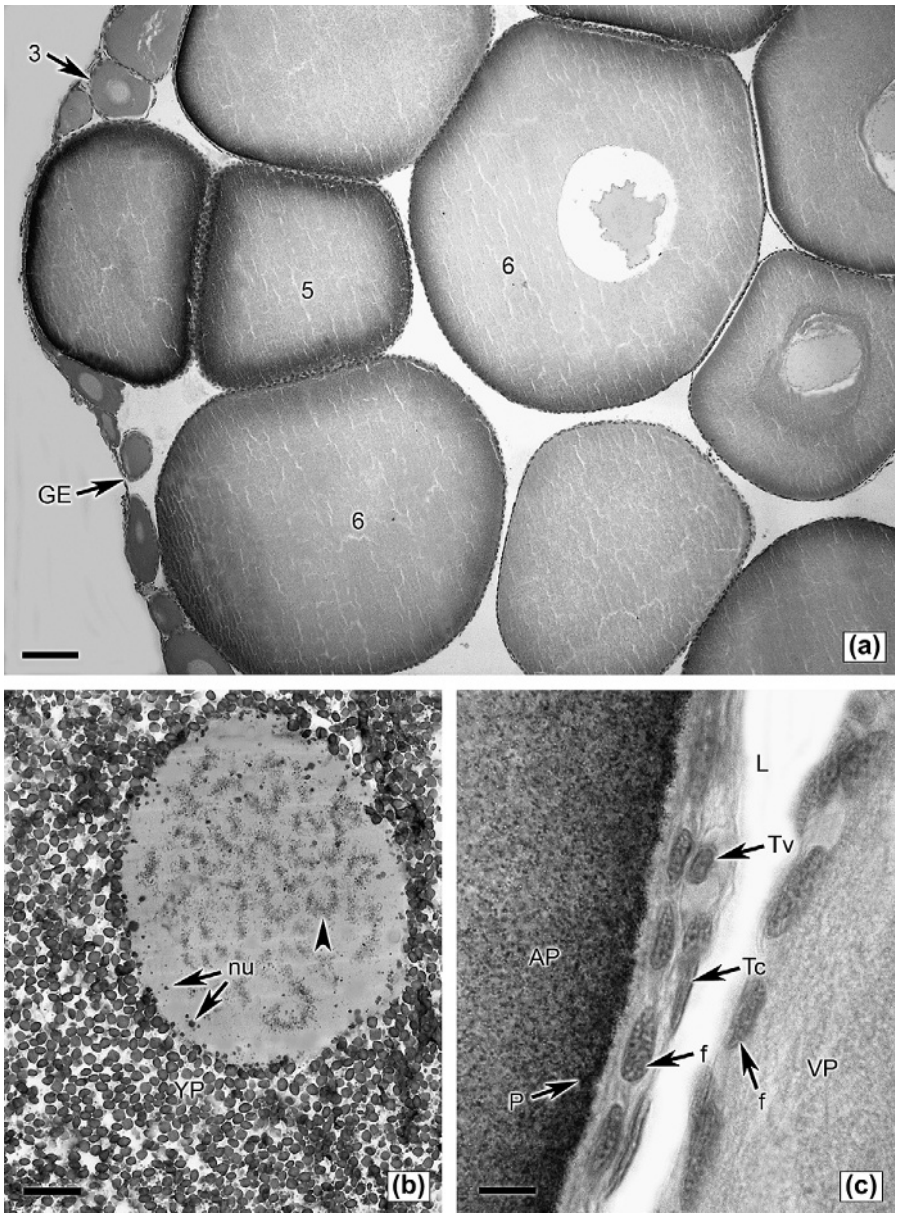

FIGURE 4.6 Ovary of adult female *Ambystoma dumerilii* during primary and secondary growth. (a) Oocytes during primary growth, stage 3 (3); secondary growth, in stage 5 (5); full-gown oocyte, stage 6 (6). The germinal epithelium (GE) surrounds the ovary. (b) Germinal vesicle of a full-grown oocyte with yolk platelets (YP) located in the ooplasm, surrounding the periphery of the membrane of the germinal vesicle. Lampbrush chromosomes (arrow head) and nucleoli (nu) are well defined. (c) Peripheral regions of two oocytes; in one of them the animal pole (AP) is seen containing pigment (P). The other section of an oocyte is at the vegetal pole (VP), without pigment. Follicle cells (f), thecal cells (Tc), and thecal blood vessels (Tv) are observed. The lumen (L) surrounds the theca. Ovarian stages are described in Table 1. (a,c) Alcian-blue staining. (b) Hematoxylin and eosin staining. Bars: (a) 50 μm; (b—c) 10 μm.

stored during secondary growth forming, in addition to yolk platelets, an essential complex for embryonic nutrition.

Redshaw (1972), Holland and Dumont (1975), Wallace (1978), Lange (1985), Richter (1987), and Nigel Finn (2007) summarized the sequence of events that occurs during the formation of yolk platelets. The precursor molecule of the major yolk component is a lipophosphoprotein known as vitellogenin (Vtg). This protein is synthesized in the liver, mediated by 17β-estradiol (E_2), and transported to the ovarian follicles through the circulatory system (Di Fiore, Assisi, & Botte, 1998). Vitellogenin penetrates into the oocytes by binding to specific receptors of the oolemma. Then, Vtg becomes internalized in vesicles by endocytosis. Ultrastructural studies prepared by Holland and Dumont (1975) in *X. laevis* complemented evidence that endocytosis is the means by which

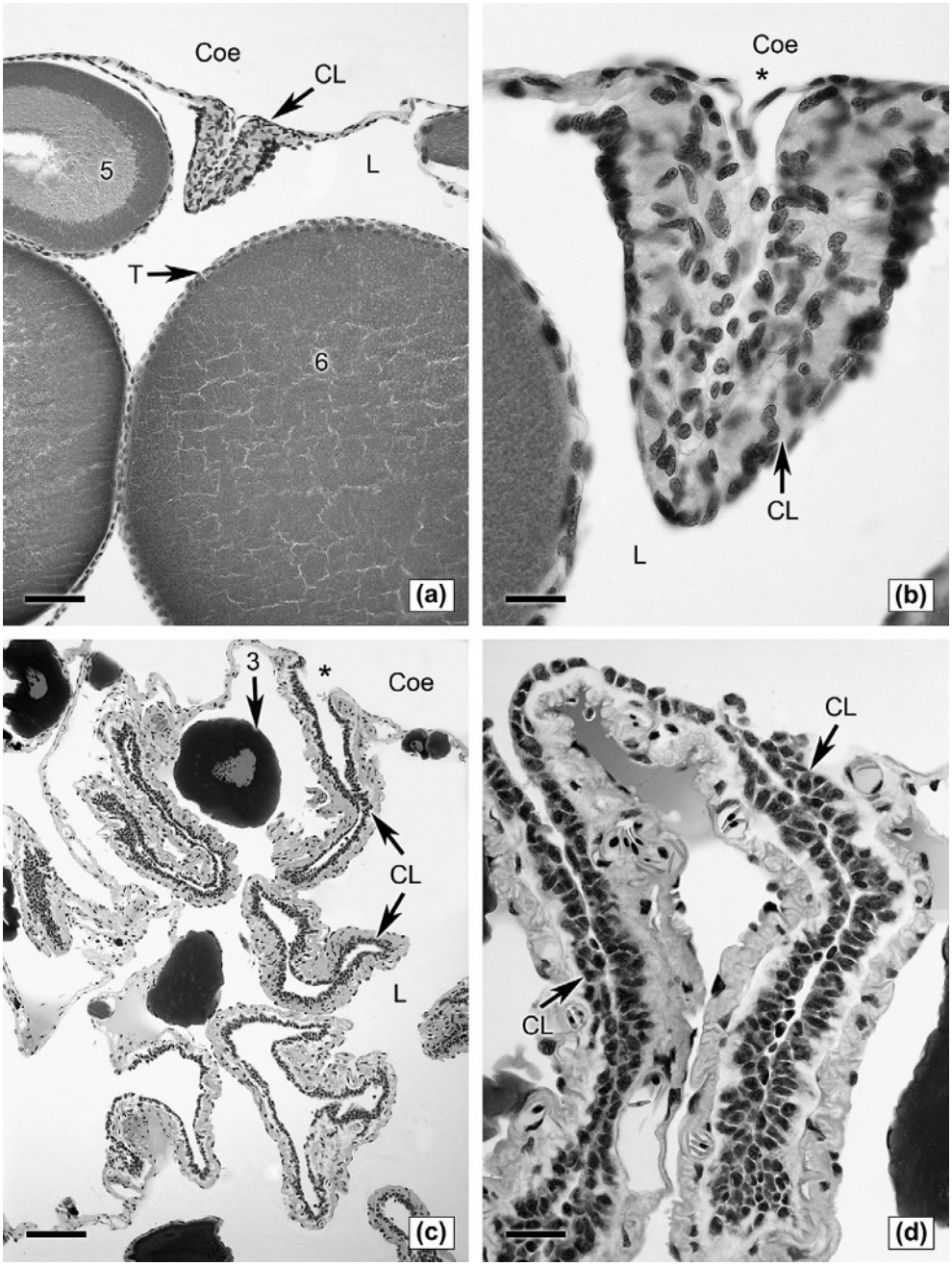

FIGURE 4.7 Ovaries of adult female *Ambystoma dumerilii* (a–c) and *Osteopilus septentrionalis* (c–d) with corpora lutea. (a–b) Ovary with oocytes in secondary growth, stages 5 (5) and 6 (6). The theca surrounds the follicles. A corpus luteum (CL) at the periphery of the ovary is seen. Ovarian lumen (L) is seen. Exterior to the ovarian wall is the coelom (Coe). The lumina of the corpora lutea (*) are continuous with the coelom (Coe). (c–d) Ovary after ovulation. Oocytes in primary growth, in stage 3 (3). Abundant corpora lutea are seen. The lumina of the corpora lutea (*) are continuous with the coelom (Coe). Hematoxylin and eosin staining. Bars: (a) 50 µm; (b) 20 µm; (c) 0.2 mm; (d) 20 µm.

developing oocytes acquire yolk precursors. These authors monitored labeled Vtg in the blood and ovary, observing that Vtg was rapidly removed from the net of capillaries of the theca and transported to the oocyte by channels between the follicle cells. Once in the ooplasm, Vtg is divided into two proteins, lipovitellin and phosvitin. The division of Vtg occurs by proteolitic degradation (Dumont, 1972; Holland & Dumont, 1975; Wallace, 1978; Di Fiore et al., 1998; Nigel Finn, 2007). Inside the oocyte, lipovitellin and the phosvitin are packaged as yolk platelets. Associated with yolk deposition, there are also diverse enzymes stored in the oocyte that are used for the fragmentation of the yolk,

providing nutrition to the embryos. Wiley and Wallace (1981), analyzing the composition of yolk in *X. laevis*, isolated other polypeptides, called phosvettes, and proposed that the various proteins found in yolk are diverse products resulting from the cleavage of Vtg molecules (see also Richter, 1987). An analysis of the exact sequential differences between the yolk proteins in vertebrates would provide additional understanding of the evolution of vitellogenesis, a widely conserved and distributed process for storing nutrients in oocytes of vertebrates.

At the end of secondary growth, vitellogenesis is completed. The growing oocytes have reached their maximal diameter and are full-grown (Figures 4.6(a) and 4.7(a)).

Numerous microvilli on the oocyte cell membrane and at the apical end of the follicle cells amplify to a great extent the surface of interchange between these two types of cells. This results in a glycoprotein layer deposited around the microvilli. The deposit of glycoprotein, the zona pellucida (also called the vitelline envelope in amphibians), around the oocyte is similar in all vertebrates (Spargo & Hope, 2003). After conducting phylogenetic analyses of zona pellucida genes in several vertebrates, Spargo and Hope (2003) stated that, despite different terms, its gross morphology and functions are similar. They proposed a unified nomenclature for the vertebrate zona pellucida gene family. Microvilli and surrounding glycoprotein form the zona pellucida, which then forms an inner layer, the zona radiata, and an outer homogeneous layer. The zona radiata is distinguished by numerous striations formed by the microvilli. The homogeneous layer is a thin layer of amorphous material composed of glycoproteins (Hope et al., 1963; Wischnitzer, 1964; Guraya, 1978; Spargo & Hope, 2003; Uribe, 2003). Many micropinocytotic vesicles can be seen in the peripheral ooplasm near the base of the microvilli, indicating the active uptake of Vtg (Spargo & Hope, 2003) and other ooplasmic constituents during oocyte growth.

During secondary growth, the animal−vegetal polarity of the oocyte is established, arising from diverse gradients of ooplasmic components. The yolk platelets are larger and more abundant in the vegetal pole. The animal pole contains the germinal vesicle and ooplasmic components such as the endoplasmic reticulum, cortical granules, and ribosomes. Glycogen granules and oil droplets are situated toward the animal pole. The RNAs exhibit a distinct pattern of localization in anuran oocytes, as described for *R. pipiens* and *Eleutherodactylus coqui* (Nath, Boorech, Beckham, Burns, & Elinson, 2004) and in *X. laevis* (King, Messitt, & Mowry, 2005). Further, King et al. (2005) extensively revised the diversity of RNAs in oocytes of *X. laevis*, indicating the polarized localization of the RNAs according to animal or vegetal polarity and confirming the important role of this polarized distribution in embryonic development. The pigment melanin is also situated at the animal pole in the peripheral ooplasm (Figures 4.3(b), 4.5(d), and 4.6(c)). The irregular distribution and final development of ooplasmic components gives the full-grown oocyte a well-defined animal−vegetal axis of polarity.

The follicle cells, which surround the oocyte and are closely apposed to the oocyte membrane, remain as a single layer of cells throughout oocyte growth but become cuboidal and thicker than the squamous epithelium described in early primary growth oocytes (Figures 4.4(d−f), 4.5(b) and (d), and 4.6(c)). Ultrastructural analysis and histochemical techniques on follicle cells reveal the presence of abundant RNA, endoplasmic reticulum, ribosomes, Golgi apparatus, phospholipids, glycogen granules, and oil droplets—features that suggest active synthesis of proteins, lipids, and carbohydrates (Joly & Picherald, 1972; Guraya, 1976; Dodd, 1986). Guraya (1978) mentioned the active role in steroidogenesis of follicle cells, describing the presence of steroidogenic enzymes in these cells. Externally to the follicle cells, the theca is formed by vascularized and fibrous connective tissue, with fibroblasts and bundles of collagen fibers (Figures 4.4(f), 4.5(a) and (d), and 4.6(c)).

As in all vertebrates, the development of sequential stages of the follicles of amphibians is related to endocrine regulation. The pituitary secretes two glycoprotein GTHs, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). The GTH levels increase during oogenesis and reach a peak just before ovulation (Fernández & Ramos, 2003). Both GTHs are involved in the control of ovarian steroidogenesis. A positive correspondence between FSH and E_2 and between LH and progesterone (P_4) secretions has been described (Fernández & Ramos, 2003). Follicle cells are the primary source of several steroids, namely E_2, P_4, 17α hydroxyprogesterone, androstenedione (AND), and T.

The levels of ovarian steroids change in correlation with the morphology of the reproductive system and are associated with seasonal variations. 17β-estradiol has an essential role in vitellogenesis, activating the liver for the liberation of Vtg into the blood vessels. After ovulation, when there are only primary growth oocytes, the ovary secretes low levels of steroids. These levels increase in secondary growth oocytes.

Cyclic changes in hormonal peak levels are described in *Pachymedusa dacnicolor* (Iela et al., 1986), *B. japonicus* (Itoh & Ishii, 1990), *X. laevis*, and *Rana nigromaculata* (Fernández & Ramos, 2003). 17β-estradiol is low at the beginning of oogenesis and increases progressively until the periovulatory period. The peak of P_4 occurs during the preovulatory period. Progesterone and testosterone (T) reach their maximal levels when oocytes are full-grown (Redshaw, 1972; Fernández & Ramos, 2003).

Kwon, Choi, Ahn, and Yoon (1995) described the differences in the production of E_2, T, and P_4 by the ovarian follicles of *R. nigromaculata* as a response to a pituitary homogenate. The smaller follicles produce very low levels of the three steroids during primary growth. The medium-sized vitellogenic follicles, with oocytes in early secondary growth, actively secrete all three steroids. In large vitellogenic follicles, secretion of all three steroids increases during late secondary growth. In contrast, large preovulatory follicles secrete more P_4, less T, and very low levels of E_2.

3.5. Oocyte Maturation

At the end of vitellogenesis, the full-grown oocyte enters into final oocyte maturation, including ooplasmic and nuclear events associated with the resumption of meiosis. A full-grown oocyte cannot be fertilized until it has undergone maturation (Yamashita, Mita, Yoshida, & Kondo, 2000).

During oocyte maturation, several nuclear and ooplasmic changes occur in the oocyte: (1) cortical granules become aligned in one layer beneath the oolemma, (2) the cytoskeleton is rearranged, (3) the oocyte is hydrated, becoming more voluminous, (4) meiotic arrest in the diplotene stage is broken and the oocyte resumes meiosis, (5) the germinal vesicle migrates to the animal pole, (6) the germinal vesicle membrane breaks down and nucleoli disappear, (7) the communication between follicle cells and oocyte, mediated by the microvilli of oocyte and follicle cells, is interrupted, and (8) the chromosomes complete the first division of meiosis.

Oocyte maturation requires the activation of maturation-promoting factor (MPF), an ooplasmic protein involved in nuclear membrane breakdown, chromosome condensation, and spindle formation (Maller, 1985; Schmitt & Nebreda, 2002; Hammes, 2004). The activation of MPF is the key that catalyzes the end of meiotic arrest in diplotene and the continuation of meiosis though metaphase I, anaphase I, and telophase I (division of the primary oocyte, forming a secondary oocyte and the first polar body) until metaphase II of meiosis, when a second meiotic arrest occurs. The secondary oocyte remains in metaphase II until fertilization. Ferrell (1999), Yamashita et al. (2000), and Schmitt and Nebreda (2002) described the sequence of changes during maturation as a cascade, since MPF is involved in oocyte maturation throughout the sequential pathway (Di Fiore et al., 1998; Lutz et al., 2001; Fernández & Ramos, 2003).

Oocyte maturation is initiated by LH (Nagahama, 1987; Yamashita et al., 2000). As a result of the LH stimulation, the follicle cells synthesize and secrete P_4, which activates MPF, resulting in oocyte maturation and ensuing ovulation (Redshaw, 1972; Masui & Clarke, 1979; Iela et al., 1986; Bement & Capco, 1990; Jørgensen, 1992; Ferrell, 1999; Yamashita et al., 2000; Sánchez & Villecco, 2003).

Steroidogenesis during oocyte maturation in *X. laevis* has been analyzed by Hammes (2004) and Ogielska and Bartmanska (2009), who described the activity of both oocyte and follicle cells in this process. The oocyte plays an important role in the production of steroids, used to promote its own maturation. The follicle cells produce pregnenolone and P_4, which enter the oocyte. The oocyte contains high levels of CYP17, an enzyme that converts pregnenolone and P_4 to androgens. Pregnenolone is metabolized to dehydroepiandrosterone (DHEA), and P_4 is metabolized to AND. From the oocyte, DHEA enters follicle cells, where it is metabolized to AND and T by 3β-hydroxysteroid dehydrogenase (3β-HSD). Besides, the AND synthesized in the oocyte from the P_4 is transferred to the follicle cells where it is converted to T. Later, both AND and T from the follicle cells re-enter the oocyte to promote maturation. Thecal cells are also an important source of T secretion. A characteristic aspect of ovarian steroids in female anurans is the presence of high concentrations of T associated with the promotion of oocyte maturation (Iela et al., 1986; Di Fiore et al., 1998; Lutz et al., 2001; Fernández & Ramos, 2003). This hormone remains low in the rest of the reproductive cycle. However, the physiological role of androgens during oocyte maturation in amphibians has not been elucidated.

After ovulation, the mature oocytes (now termed eggs) are released to the coelom and enter the oviduct. The ovary now contains only small, primary growth oocytes that will become mature during the next reproductive season (Xavier, 1987; Norris, 2007).

3.6. Oogenesis Stages

For histological description, amphibian oogenesis can be divided into seven stages. These stages are similar to those previously reported by several authors, such as Dumont (1972), Berois and de Sa (1988), del Pino (1989), Sharon et al. (1997), Sretarugsa et al. (2001), Uribe (2003), Beyo et al. (2007b, 2008a), Ogielska and Bartmanska (2009), and Uribe (2009). Three stages occur during primary growth (stages 1−3), three more stages during secondary growth (stages 4−6), and the last stage occurs when the germinal vesicle moves to the animal pole and breaks down (stage 7). The morphological changes of the ovarian follicles during the seven stages are described and illustrated here in ovaries of the anurans *Hyla eximia* and *Osteopilus septentrionalis* and of the urodeles *Ambystoma dumerilii* and *Ambystoma mexicanum*. See Table 4.1 for a summary of the seven stages.

TABLE 4.1 Stages of oogenesis in the anurans *Hyla eximia* and *Osteopilus septentrionalis*, and in the urodeles *Ambystoma dumerilii* and *Ambystoma mexicanum*

STAGES	Primary Growth. Stage 1	Primary Growth. Stage 2	Primary Growth. Stage 3	Secondary Growth. Stage 4	Secondary Growth. Stage 5	Secondary Growth. Stage 6	Oocyte Maturation. Stage 7
Oocyte approximate diameter	30 µm	100 µm	300 µm	700 µm	1000 µm	1800 µm	1800 µm
Germinal vesicle	One nucleolus	Two or more nucleoli	Lampbrush chromosomes and numerous nucleoli	Lampbrush chromosomes and numerous peripheral nucleoli	Lampbrush chromosomes and numerous peripheral nucleoli	Lampbrush chromosomes and numerous peripheral nucleoli	Moving to the animal pole and breaks down
Ooplasm	Hyaline	Initial basophilia, Balbiani bodies, and cortical granules	Basophilic, Balbiani bodies, cortical granules, and oil droplets	Small and scarce yolk platelets at the periphery	Progressive accumulation of yolk platelets	Yolk platelets distributed since the germinal vesicle to the periphery	Yolk platelets distributed since the germinal vesicle to the periphery
Follicle cells	Squamous follicle cells form an incomplete layer	Squamous follicle cells form a complete layer	Squamous follicle cells	Cuboidal follicle cells	Cuboidal follicle cells	Squamous follicle cells	Squamous follicle cells

3.6.1. Primary growth: stage 1

See Figure 4.2(c–d). The oocytes are frequently located in cell nests. There are intercellular bridges between oocytes in the cell nests. Oocytes grow to 30 µm in diameter. The germinal vesicle is central, is spherical in shape, and has a single nucleolus. The chromosomes are in early meiotic prophase, from leptotene to early diplotene when lampbrush chromosomes form. They are observed as fine fibers of chromatin. The ooplasm has a hyaline appearance. Prefollicle cell processes begin to surround each oocyte, marking the beginning of folliculogenesis.

3.6.2. Primary growth: stage 2

See Figure 4.2(b–c). The diplotene oocyte attains a diameter of 100 µm. The germinal vesicle is central and spherical, and has one, two, or more nucleoli. The pairs of duplicated homologous chromosomes in the diplotene stage form the lampbrush chromosomes, which gradually develop lateral loops. The ooplasm becomes basophilic. The Balbiani body begins to aggregate into clumps adjacent to the germinal vesicle; they are irregular in shape. The cortical granules begin to form throughout the ooplasm. The oocyte is totally surrounded by a single layer of squamous follicle cells, forming the follicle. At this time, folliculogenesis is completed. The basement membrane separates follicle cells from thecal cells.

3.6.3. Primary growth: stage 3

See Figures 4.2(b–d), 4.3, 4.4(a) and (c), and 4.6(a). The oocyte diameter increases, reaching 300 µm. The germinal vesicle is large and spherical; its contour becomes irregularly folded. The lampbrush chromosomes present multiple and irregular loops of DNA extended laterally. The germinal vesicle contains numerous nucleoli, situated mainly along the periphery of the nuclear membrane. The ooplasm is homogeneous and finely granular, becoming basophilic because of the enormous increment of ribosomes. Several irregular clumps of Balbiani bodies occur near the germinal vesicle. The cortical granules are at the periphery of the oocyte. Oil droplets are also seen in the peripheral ooplasm. The follicle cells are squamous in shape. The follicle is limited by the basement membrane. The theca contains capillaries and fine collagen fibers.

3.6.4. Secondary growth: stage 4

See Figures 4.2(c–e). The oocyte diameter attains 700 µm. The contour of the germinal vesicle is irregularly folded, in a similar manner to that seen in the previous stage. The nucleoli are located at the periphery of the germinal vesicle. The lampbrush chromosomes are clearly observed within the germinal vesicle. The peripheral ooplasm contains the earliest acidophilic yolk platelets, seen as a few small granules. Balbiani bodies migrate to the oocyte periphery, where they disperse. The zona pellucida is thin and well-defined. Follicle cells increase their association with the oocyte by numerous interdigitated microvilli from both types of cells extending into the zona pellucida. The cuboidal follicle cells form a layer lined by the basement membrane. The connective tissue adjacent to the basement membrane forms a thin vascularized and fibrous theca, similar to that seen in the previous stage.

3.6.5. Secondary growth: stage 5

See Figures 4.3(a), 4.4(b–d) and (f), 4.5, 4.6(a), and 4.7 (a)). The oocyte attains a diameter of 800–1000 µm. Similarly to the previous stage, the germinal vesicle shows lampbrush chromosomes, nucleoli situated at the periphery, and a folded membrane. The ooplasm exhibits a progressive accumulation of acidophilic, ovoid yolk platelets, but the ooplasm around the germinal vesicle lacks yolk platelets. The animal hemisphere is distinguished by the deposition of melanin at the peripheral ooplasm. The follicle cells are slightly thicker than those seen in the previous stage as cuboidal cells, and microvilli from both the follicle cells and the oocyte penetrate into the zona pellucida. The zona pellucida is evident maintaining the development of the microvilli from the surfaces of the oocyte and follicle cells. The microvilli are extended into the amorphous material, the homogeneous layer.

3.6.6. Secondary growth; full-grown: stage 6

See Figures 4.3(a–b), 4.6, 4.7(a). The oocyte reaches its maximal diameter, attaining 1800 µm. The oocyte is full-grown and filled with yolk platelets, extending from the periphery to the ooplasm adjacent to the germinal vesicle. The yolk platelets increase in size and number and the majority are situated in the vegetal pole. The animal pole contains smaller yolk platelets than those seen in the vegetal hemisphere. Melanin deposition at the periphery of the animal hemisphere increases. The follicles are squamous. The thecal cells are similar to those seen in the previous stage.

3.6.7. Oocyte maturation: stage 7

At the end of vitellogenesis, when the deposition of yolk is completed, the full-grown oocyte enters into oocyte maturation and meiosis resumes. The oocyte germinal vesicle moves to the periphery at the animal pole. Breakdown of the germinal vesicle membrane, disintegration of the nucleoli, and formation of spindle fibers precede the first meiotic division and formation of a secondary oocyte and the first polar body. Subsequently, ovulation occurs.

3.7. Corpus Luteum

After ovulation, the ruptured follicle collapses and most of the follicle cells remain in the ovary. These follicle cells hypertrophy and become a postovulatory follicle or corpus luteum. The corpus luteum is a transitory organ that has a hormonal regulatory role in the reproductive cycle. The luteal cells are continuous with the epithelial cells of the germinal epithelium. Consequently, the lumen of the corpus luteum is continuous with the coelom (Figure 4.7). The corpus luteum is surrounded by a basement membrane and theca. The theca becomes fibrous, multilayered, and vascularized (Dodd, 1977; 1986; Xavier, 1987). However, extensive vascularization of the corpus luteum has been reported only for some species of caecilian (Exbrayat & Collenot, 1983; Norris & Lopez, 2005). After ovulation, the corpora lutea are abundant (Figure 4.7(c−d)).

The role in steroidogenesis of the luteal cells has been analyzed by several authors (Joly & Picheral, 1972; Saidapur & Nadkarni, 1974; Guraya, 1976). Luteal cells exhibit the histochemical and ultrastructural characteristics of active steroid-secreting cells, based on the presence of steroidogenic enzymes. Corpora lutea in amphibians are capable of converting pregnenolone to P_4 (Lofts, 1984; Xavier, 1987). The presence of an intense 3β-HSD activity has been demonstrated histochemically (Saidapur, 1982; Chieffi & Pierantoni, 1987; Norris, 2007). During resorption of the corpus luteum, the following are observed: a decrease in the size of the cells, pyknosis of their nuclei, and an accumulation of oil droplets in the ooplasm (Xavier, 1987; Exbrayat & Collenot, 1983).

The function of the corpus luteum changes in viviparous urodeles, such as *S. salamandra* and *S. atra*, during gestation (Saidapur, 1982; Lofts, 1984; Xavier, 1987; see also Chapter 7, this volume). Well-organized secretory corpora lutea remain functional throughout two-thirds of gestation in these viviparous species (Xavier, 1987). Wake (1993) mentioned that all viviparous urodeles have corpora lutea that appear to be involved in the maintenance of the pregnancy. The involvement of corpora lutea in viviparous species is also considered by Joly et al. (1994), who observed that the P_4 content in the ovary is high at the beginning of gestation and decreases during gestation. As was mentioned for oviparous species, in viviparous species the corpora lutea are competent in converting pregnenolone to P_4 (Lofts, 1984; Xavier, 1987).

Development of new oocytes may be arrested by the presence of corpora lutea in both oviparous and viviparous species, and new oocytes begin their development only after the degeneration of the corpora lutea (Norris, 2007), as observed in the viviparous *S. atra* (Xavier, 1987). Thus, there is a probable relationship between the inhibition of folliculogenesis and the presence of corpora lutea during gestation in amphibians.

3.8. Follicular Atresia

Follicular atresia is the degeneration and resorption of follicles (Figure 4.8(a−f)). Atresia occurs during various stages of follicular development, at any stage of primary or secondary growth (Adams, 1940; Guraya, 1969; Byskov, 1978; Saidapur, 1978; Pancharatna & Saidapur, 1985; Dood, 1986; Uribe, 2009). Atretic follicles are rare in ovaries of amphibians, compared to the extensive atresia found in mammalian ovaries (Norris & Lopez, 2005).

During atresia, several morphological changes occur, as summarized by Saidapur (1978). (1) The oocyte becomes shrunken, suggesting a relative loss of water. (2) The ooplasm and the germinal vesicle are gradually resorbed by follicle cells, which become phagocytic, while the yolk platelets and pigment granules fuse, forming aggregations. (3) The zona pellucida becomes broken and diffuse and the follicle cells hypertrophy. Byskov (1978) described the retraction of the oocyte and follicle cell microvilli from the zona pellucida when these cells are disconnected during atresia. Later on, the oocyte is digested and removed by the follicle cells, leaving an irregular mass surrounded by the theca. In vitellogenic oocytes, both lipid and protein yolk coalesce to form a mass of triglycerides, which is gradually digested by the follicle cells, resulting in aggregations of pigmented bodies. The final part of atresia consists of follicle cells filling with the phagocytized remains of oocyte pigment and becoming surrounded by hypertrophied thecal cells. Adams (1940) observed atretic follicles in the ovaries of *Notophthsalmud (Triturus) viridescens*, and described progressive morphological changes during the hypertrophy of follicle cells, phagocytosis of yolk by follicle cells and leucocytes, deposition of pigment, and finally the gradual disappearance of the follicle. Saidapur and Nadkarni (1973) summarized oocyte atresia in *Rana cyanophlyctis* and described four stages. (1) Hypertrophy of the follicle cells, signs of the coalescence of yolk platelets, and breakdown of the zona pellucida. (2) The hypertrophied follicle cells move into the ooplasm and the theca becomes thickened and more vascularized. (3) The ooplasm is phagocytized by follicle cells, leaving a remaining mass of pigmented material. (4) There is a gradual decrease in the number of the follicle cells until only pigment remains, which decreases slowly during an unknown period of time.

It is not understood why some oocytes are able to continue development until ovulation whereas others undergo atresia (Saidapur, 1978). The causes of atresia need to be determined more precisely. It has been suggested that atretic follicles contribute to steroid secretion and regulation of the number of ovulated oocytes (Guraya, 1969; Byskov, 1978; Saidapur, 1978). Consequently, atresia plays an important role in fecundity by influencing the number of ovulated oocytes.

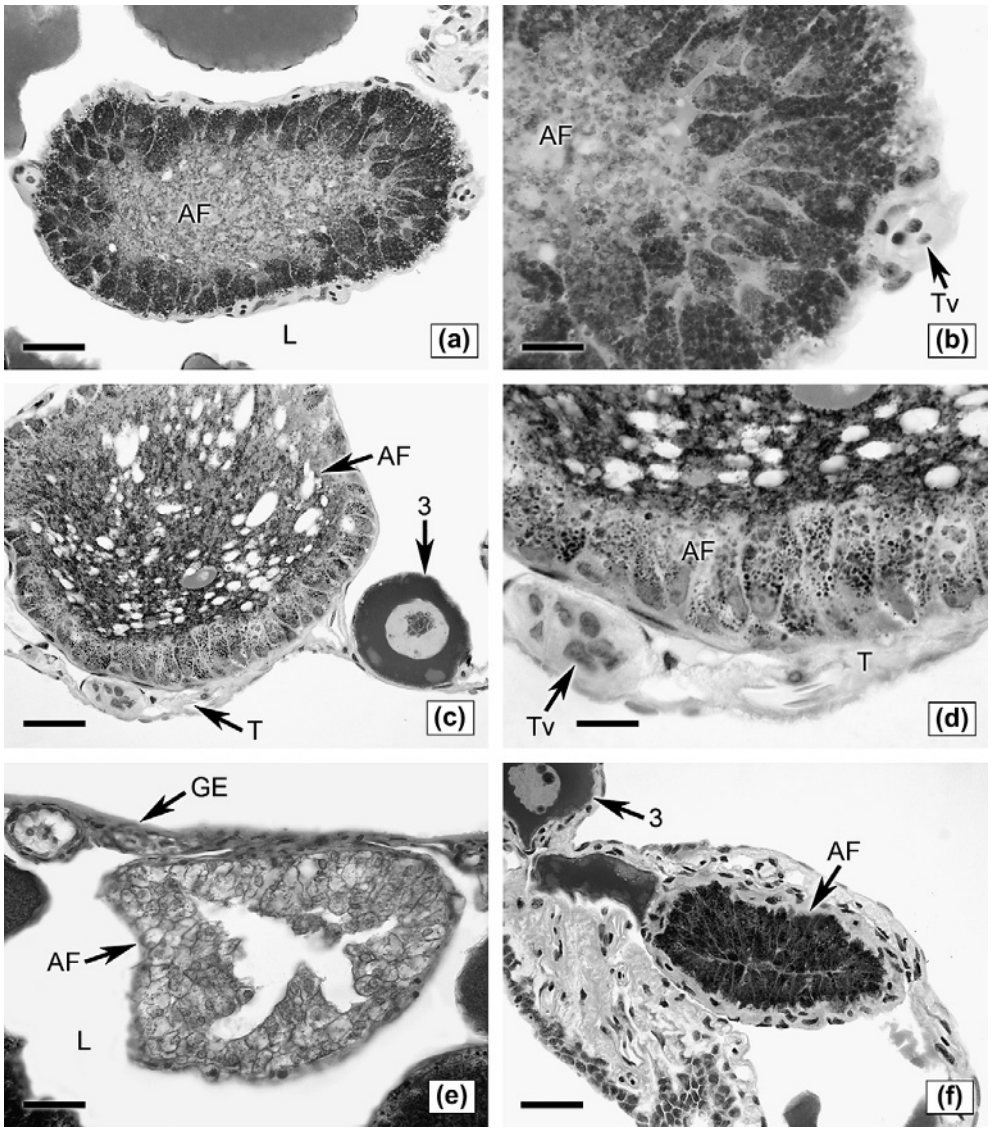

FIGURE 4.8 Ovaries of adult female *Osteopilus septentrionalis* (a–d,f) and *Ambystoma mexicanum* (e) with atretic follicles (AF). The follicle cells are abundant, hypertrophied, and irregular in shape. Theca (T) with blood vessels (Tv) surrounds the atretic follicles. Germinal epithelium (GE), oocytes in primary growth, stage 3 (3), and ovarian lumen (L) are seen. (a–b) Atretic follicle with the oocyte in early deposition of yolk. (c–d) Atretic follicle with the oocyte in late deposition of yolk. (e) The follicle cells occupy the center of the follicle. Several layers of follicle cells are seen. (f) The oocyte in an advanced stage of disintegration with follicle cells remaining. (a–d,f) Hematoxylin and eosin staining. (e) Masson's trichrome staining. Bars: (a,c,e–f) 50 μm; (b,d) 20 μm.

According to the phase of the annual reproductive cycle, and unfavorable environmental conditions, the abundance of atretic follicles can vary (Saidapur, 1978; Pancharatna & Saidapur, 1985). According to these authors, the ovary of seasonal breeders contains more atretic follicles during the non-breeding season.

4. OVIDUCTAL STRUCTURE

At ovulation, the ovary releases eggs into the body cavity, where they are propelled to the infundibulum by abundant and large cilia of the coelomic epithelium. Then, the eggs enter the oviducts and continue their transit through the caudal portions of the oviducts to the exterior. During the passage of eggs through the oviduct, they are surrounded by concentric jelly capsules secreted by oviductal glands. These capsules are composed of mucopolysaccharides and muco-proteins (Loft, 1984; Duellman & Trueb, 1986; Morelle & Strecker, 1998; Coppin, Maes, Morelle, & Strecker, 1999; Delplace, Maes, Lemoine, & Strecker, 2002).

The oviducts are paired, elongated ducts, extending on each side of the midline of the body cavity. They are

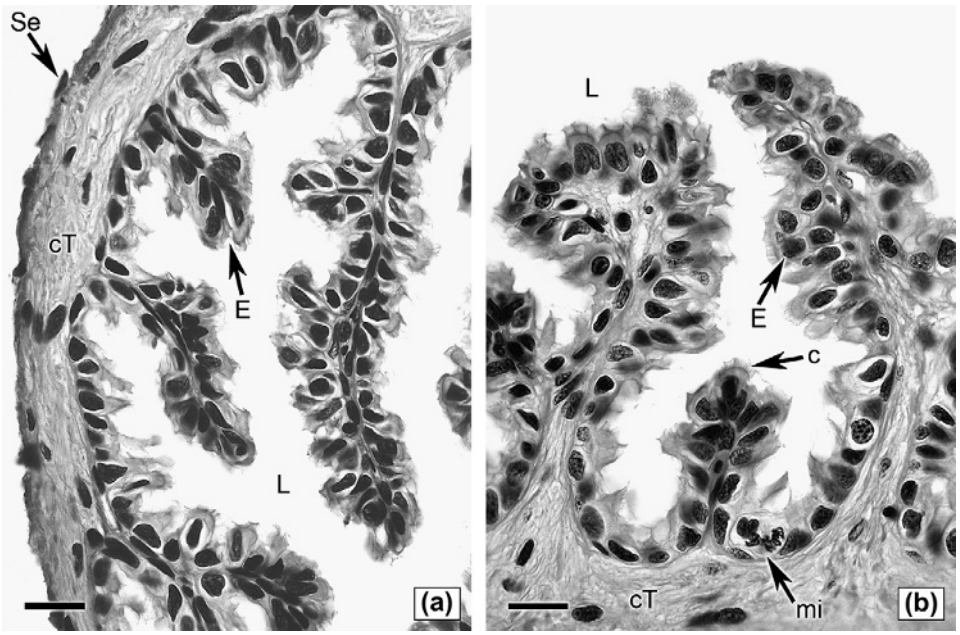

FIGURE 4.9 Oviduct of adult female *Ambystoma mexicanum*. (a—b) Cross sections of the infundibulum. The wall shows large and irregular folds projected into the lumen (L). The folds are bordered by columnar epithelium (E). The epithelium contains ciliated cells (c). Epithelial cell in mitosis (mi), connective tissue (cT), and serosa (S). Hematoxylin and eosin staining. Bars: (a) 50 µm; (b) 20 µm.

suspended dorsally from the body wall by the mesotubaria. The embryonic origin of the oviducts is the Müllerian ducts, under the influence of ovarian estrogens. The Müllerian ducts develop as paired invaginations of coelomic genital ridge epithelium, near the mesonephric ducts. These invaginations grow caudally until they reach the cloaca, by which they connect to the exterior (Adams, 1940; Potemkina, 1967; Lofts, 1984; Sever et al., 1996; Wake & Dickie, 1998; Pawar & Pancharatna, 1999; Greven, 2003; Tyler, 2003).

The reproductive tracts of female amphibians serve several roles in reproduction: (1) they transport eggs to the exterior, (2) they prepare eggs to accept sperm, (3) they provide the eggs with jelly coats, and (4) in viviparous species, they maintain the best conditions of temperature and hydration for the embryos. Viviparous species have morphological modifications of the oviduct in order to maintain developing embryos (Wake, 1970; 1977; Pancharatna & Saidapur, 1985; Wake, 1985; 1993; Greven & Guex, 1994; Wake, 1998; Wake & Dickie, 1998; Greven 1998, Greven 2003; see also Chapter 7, this volume).

The seasonal reproductive activity of amphibians is not only observed in the cyclical changes of the ovary but also in the oviduct (Lofts, 1984; Pawar & Pancharatna, 1999). Oviductal features change according to hormonal regulation during the reproductive cycle. During non-breeding seasons, the oviducts are less folded, narrower, have little secretory activity, and the diameter is reduced in the entire oviduct (Adams, 1940; Brandon, 1970; Duellman & Trueb, 1986; Uribe et al., 1989; Pawar & Pancharatna, 1999; Norris, 2007). During the breeding season, the oviducts become larger and thicker, and have an intense secretory activity. Guillette, Norris, and Norman (1985) observed that E_2 or T alone did not have an effect on the oviducts of *Ambystoma tigrinum*, whereas a combination of these hormones produces significantly hypertrophy. Dubowsky and Smalley (1993) concluded that ovariectomized *R. pipiens* treated with T or E_2 plus T had the largest oviducts compared with smaller oviducts in animals treated with E_2 alone. These observations suggest that oviduct growth with T may be due to the aromatization of T to E_2. The presence of aromatase in the oviduct of *R. pipiens* was demonstrated by Kobayashi, Zimniski, and Smalley (1996). Norris, Carr, Summers, and Featherston (1997) noted that the epithelium and connective tissue of the oviducts of *A. tigrinum* were also more stimulated by combined E_2 and T treatment than by treatment with E_2 alone.

The walls of amphibian oviducts are composed of three tissue layers (McCurdy, 1931; Kambara, 1956; Vilter, 1967; Uribe et al., 1989; Sever et al., 1996; Greven, 1998; Pawar & Pancharatna, 1999; Uribe, 2001; Greven, 2003; Uribe, 2009). The inner layer is the mucosa, which is composed of epithelium and connective tissue. The epithelium consists of one layer of luminal, columnar cells, and abundant glands. The subjacent connective tissue contains blood vessels. The middle layer of the oviducts is the myometrium, formed by smooth muscle fibers, in circular orientation. The outer layer is the serosa, composed of thin connective tissue and a squamous mesothelial epithelium.

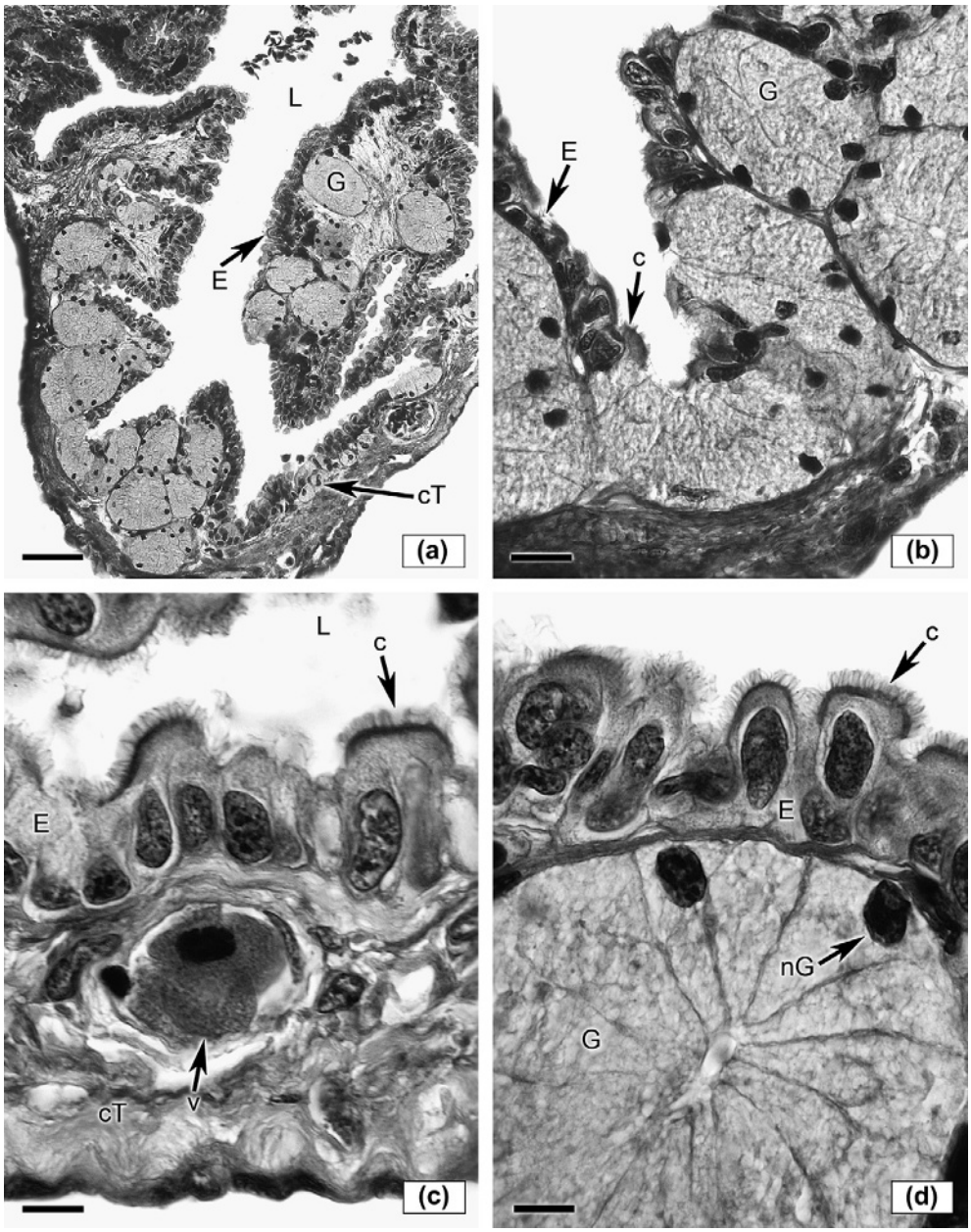

FIGURE 4.10 Oviduct of adult female *Ambystoma dumerilii*. (a–d) Cross section of the pars recta. The wall shows large and irregular folds projected into the lumen (L). The folds are limited by columnar epithelium (E) containing abundant ciliated cells (c) and gland cells (G). The gland cells are columnar, with basal nuclei (nG). The ciliated columnar cells (c) are smaller compared to the glandular cells (G). Connective tissue (cT) and blood vessels (v) are seen. Masson's trichrome. Bars: (a) 0.2 mm; (b) 20 µm; (c–d) 10 µm.

According to Uribe et al. (1989), Sever et al. (1996), Wake and Dickie (1998), Greven (2003), Fernández and Ramos (2003), Tyler (2003), and Uribe (2009), the oviducts of amphibians can be divided into four morpho-physiological regions. (1) The infundibulum: the expanded cephalic end of the oviduct, which is an ample opening that receives the eggs after ovulation. (2) The pars recta or atrium: a short and straight region narrower than the infundibulum. (3) The pars convoluta or ampulla: the largest region of the oviduct with large convolutions. (4) The ovisac of oviparous species: a short and straight region; plus caudal portions of both oviduct joining and opening together into the cloaca. In viviparous species, the ovisac is elongated to form the pars uterine (see also Chapter 7, this volume).

4.1. Infundibulum

The infundibulum (Figure 4.9(a–b)) consists of a thin wall that possesses a highly folded mucosa. The mucosal folds

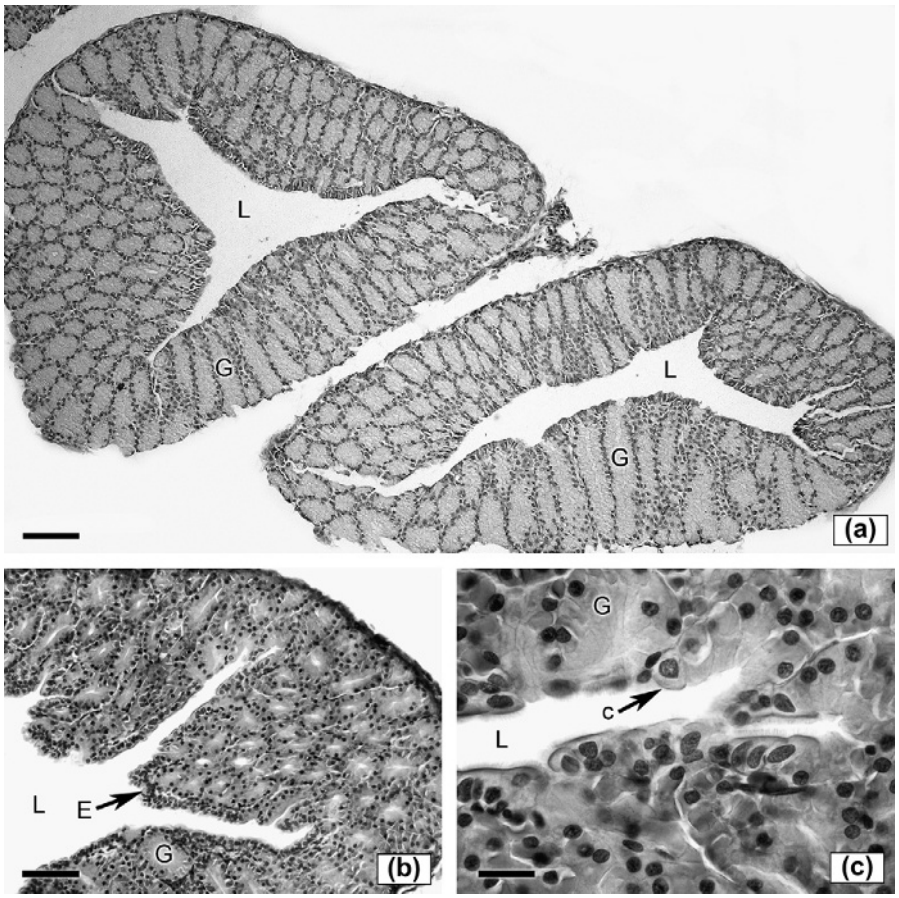

FIGURE 4.11 Oviduct of adult female *Ambystoma mexicanum*. Cross sections of the pars convoluta during primary growth of oocytes in the ovary. (a–c) The glands (G) form the thicker part of the wall of the oviduct. The epithelium (E) contains ciliated cells (c). Lumen (L). (a) Hematoxylin and eosin staining. (b–c) Masson's trichrome. Bars: (a) 0.3 mm; (b) 50 µm; (c) 20 µm.

are irregular in shape and size, and project into the lumen. The mucosa of the infundibulum is bordered by a cuboidal or columnar epithelium with both ciliated and secretory cells. The epithelium is separated from the stroma by a basement membrane. The stroma is formed of loose connective tissue that contains blood vessels. There is a thin myometrium formed of circular smooth muscle fibers. The serosa surrounds the outer wall of the infundibulum (Uribe et al., 1989; Uribe, 2009).

4.2. Pars Recta or Atrium

The pars recta (Figure 4.10) is a region of transition between the aglandular infundibulum and the highly glandular pars convoluta. The wall of the pars recta is folded, as in the infundibulum, but the folds are shorter and thicker. The epithelium shows few glandular cells, occurring singly or in groups and interspersed among the cuboidal epithelial cells. The cuboidal epithelial cells have a well-defined, ciliated apical end. The glandular cells increase gradually in number along the pars recta. The secretory cells become larger than the non-secretory cells of the epithelium. Their nuclei are basal, and the cytoplasm shows abundant, hyaline globules. The myometrium and the serosa are similar to those seen in the previous region (Uribe et al., 1989).

The pars recta produces a gelatinous secretion that contains a proteolytic enzyme, called oviductin, described in *B. arenarum* (Hardy & Hedrick, 1992). Oviductin surrounds the zona pellucida of the oocyte during its transit through the pars recta. The presence of oviductin is indispensable for sperm penetration (Hardy & Hedrick, 1992; Fernández & Ramos, 2003).

Grey, Working, and Hendrick (1977) found that bundles of fibers contained in the zona pellucida are transformed during transit through the pars recta. The authors compared the structure of the bundles of fibers in coelomic and oviposited eggs, describing changes in the structure of these bundles in oviposited eggs. The oviposited eggs with changed zona pellucida were competent for fertilization.

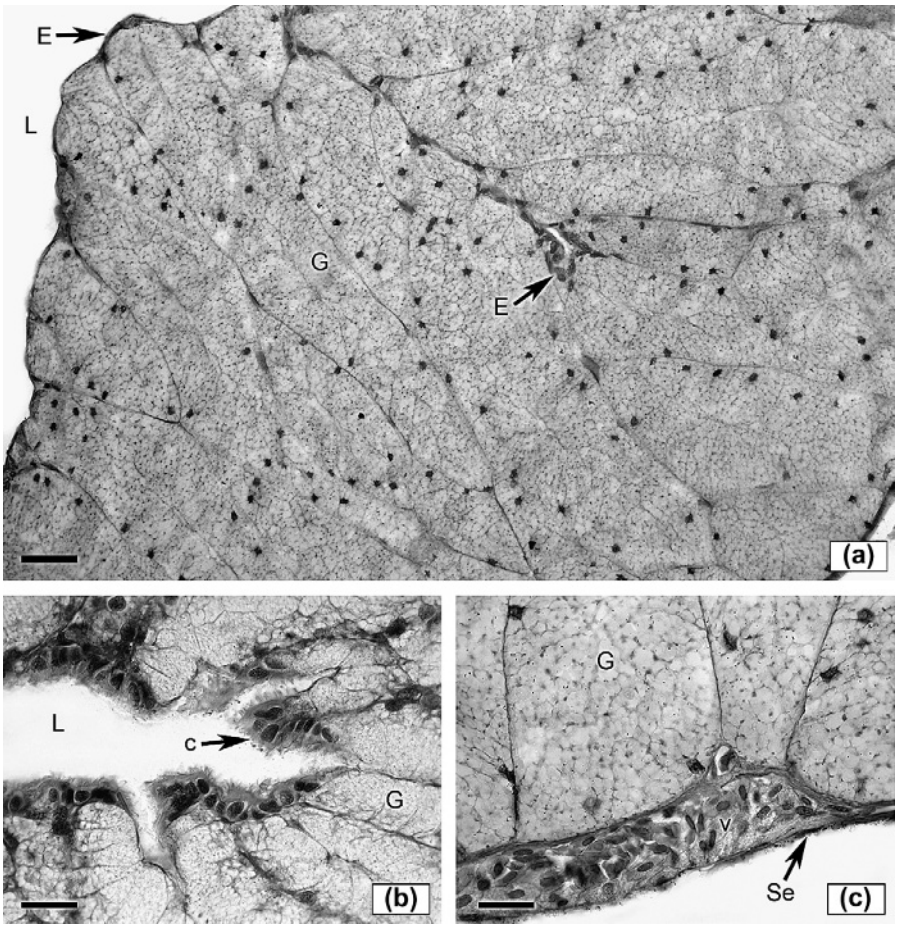

FIGURE 4.12 Oviduct of adult female *Ambystoma dumerilii*. (a–c) Cross sections of the pars convoluta during secondary growth of oocytes in the ovary. Note the hypertrophy of the glands (G). Compare the thickness of the wall of the oviduct of (a) with the previous stage in Figure 4.11(a–b). The thickness of the wall attains the maximal size here. Epithelium (E) with ciliated cells (c). The connective tissue contains blood vessels (v). The exterior of the oviductal wall is limited by the serosa (Se). Lumen (L). Hematoxylin and eosin staining. Bars: (a) 0.3 mm; (b) 50 µm; (c) 20 µm.

4.3. Pars Convoluta or Ampulla

The pars convoluta (Figures 4.11 and 4.12) is the longest and thickest part of the oviduct. The luminal epithelium contains only a few ciliated, cuboidal, or irregular cells among numerous glandular cells. The cilia promote the transport of eggs through the oviduct (Figure 4.13). This region shows a progressive increase in the number of exocrine tubular glands that form the thick wall of the oviduct (Uribe et al., 1989) (Figures 4.11(a) and 4.12(a)). Pawar and Pancharatna (1999) described the changes of the oviduct of *R. cyanophlyctis* during the annual cycle, observing the increase in weight of the pars convoluta specifically connected with the development of the glands, which become filled with secretions. The glandular cells are large, prismatic in shape, and have basal nuclei and hyaline vacuolated cytoplasm (Figures 4.11 and 4.12). The glands are greatly hypertrophied during the reproductive season (Figure 4.12(a)), and are surrounded by thin layers of vascularized connective tissue (Figure 4.12(c)). After the passage of the eggs, the glands become reduced in size and secretory activity (Yoshizaki & Katagiri, 1981). The myometrium consists of two or three layers of muscle cells in circular arrangement (Uribe, 2009). The size and extent of the epithelial glands diminishes gradually in a caudal direction (Figure 4.14). These glands open into the oviductal lumen, secreting abundant components of the jelly coats that are added to the eggs during their passage through this region. Progesterone, synthesized by the ovary, stimulates the oviductal glands to produce jelly coats (Saidapur & Nadkarni, 1974).

The jelly coats are composed of a species-specific variety of oviductal mucins involved in molecular recognition between eggs and sperm during fertilization (Morelle & Strecker, 1998; Coppin et al., 1999; Pawar & Pancharatna, 1999; Delplace et al., 2002; Sasaki, Kamimura, Takai, Watanabe, & Otinake, 2002; Watanabe & Onitake, 2002; Ogielska & Bartmanska, 2009). Jelly coats, complemented

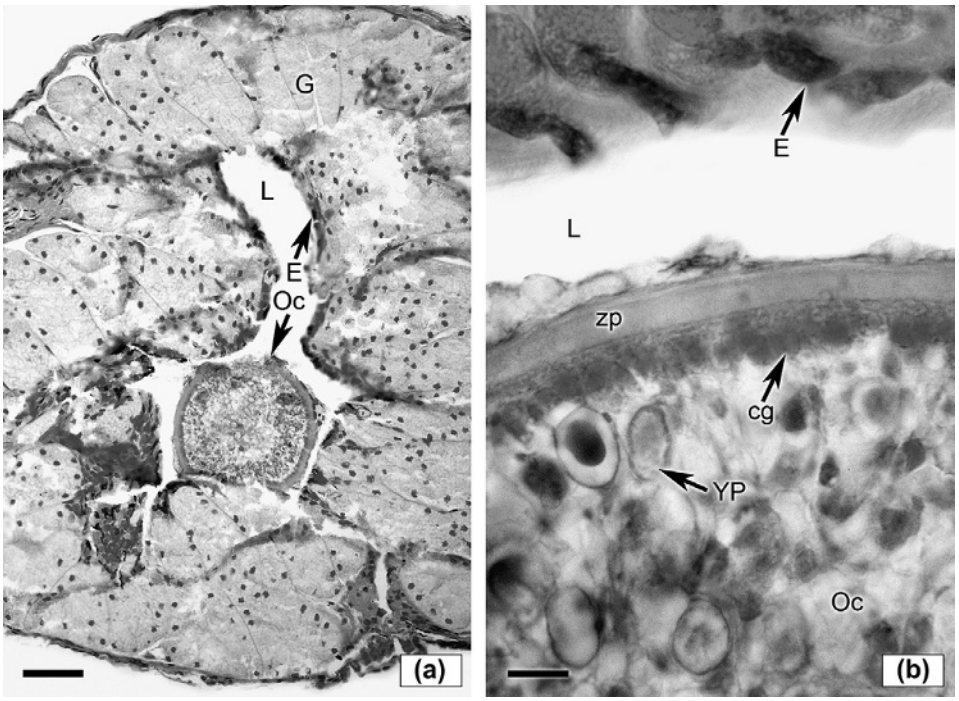

FIGURE 4.13 Oviduct of adult female *Ambystoma dumerilii*. (a–b) Cross sections of the caudal region of the pars convoluta containing an oocyte in transit. The glands (G) and the epithelium (E) of the pars convoluta are seen. Note the oocyte (Oc) in the lumen (L) of the oviduct. The oocyte contains yolk platelets (YP) and cortical granules (cg) at the periphery. The zona pellucida (zp) surrounds the oocyte. Alcian blue staining. Bars: (a) 50 µm; (b) 10 µm.

with the secretions of pars recta, are essential for fertilization, as documented by Katagiri (1965), Duellman and Trueb (1986), Watanabe and Onitake (2002), and Fernández and Ramos (2003). These authors described positive reactions of the sperm to substances from the jelly coats that diffuse into the water. Watanabe and Onitake (2003) suggested that, in the mechanics of internal fertilization in urodeles, the egg–sperm interaction is induced in the jelly coat, suggesting that the signal for the initiation of sperm motility is present in the jelly coat. The jelly coats also play a role in blocking polyspermy during fertilization (Fernández & Ramos, 2003). In salamanders, sperm did not fertilize eggs lacking jelly coats (Greven, 1998). Itoh, Kamimura, Watanabe, and Onitake (2002) examined fertilization efficiency in eggs of the urodele *Cynops pyrrhogaster* in the presence or absence of jelly coats, observing that the presence of jelly coats results in considerably higher fertilization efficiency.

After oviposition, when the eggs are released into the environment, the sticky jelly coats have important and diverse functions for the developing embryos, for example anchorage of the embryos to the substrate, protection against mechanical damage, thermal insulation (Salthe, 1963), prevention of desiccation (Heatwole, 1961), and protection against ingestion by predators because of their toxic properties (Ward & Sexton, 1981).

The pars convoluta of some species may be subdivided into several sub-regions, according to functional properties. These subdivisions correspond to different types of secretion that contribute to the jelly coats (as summarized by Salthe, 1963; Greven, 1998; Wake & Dickie, 1998). Other authors subdivide the urodele pars convoluta into two (Kambara, 1956), three (Vilter, 1967), or four (Adams, 1940) portions, based on differences in their secretory products.

The jelly coats may contain several layers. Each layer has specific carbohydrate components that accumulate while eggs pass through the oviduct (Salthe, 1963; Wake & Dickie, 1998; Coppin et al., 1999; Watanabe & Onitake, 2003). Morelle and Strecker (1998) illustrated a detailed biochemical analysis of jelly coats in *Rana utricularia*, identifying ten different oligosaccharides. Coppin et al. (1999) recognized 13 oligosaccharides in *Rana temporaria* eggs. The number of layers composing jelly coats also varies between species. For example, *Rana catesbiana* has one layer (Duellman & Trueb, 1986) and numerous species have two layers, for example *R. temporaria* (Duellman & Trueb, 1986) and *Lepidobatrachus laevis* (Carroll, Wei, Nagel, & Ruibal, 1991). There are three jelly coat layers surrounding the eggs of *X. laevis* (del Pino, 1973) and *Discoglossus pictus* (Denis-Donini & Campanella, 1977). There are four layers surrounding the urodele egg of *A.*

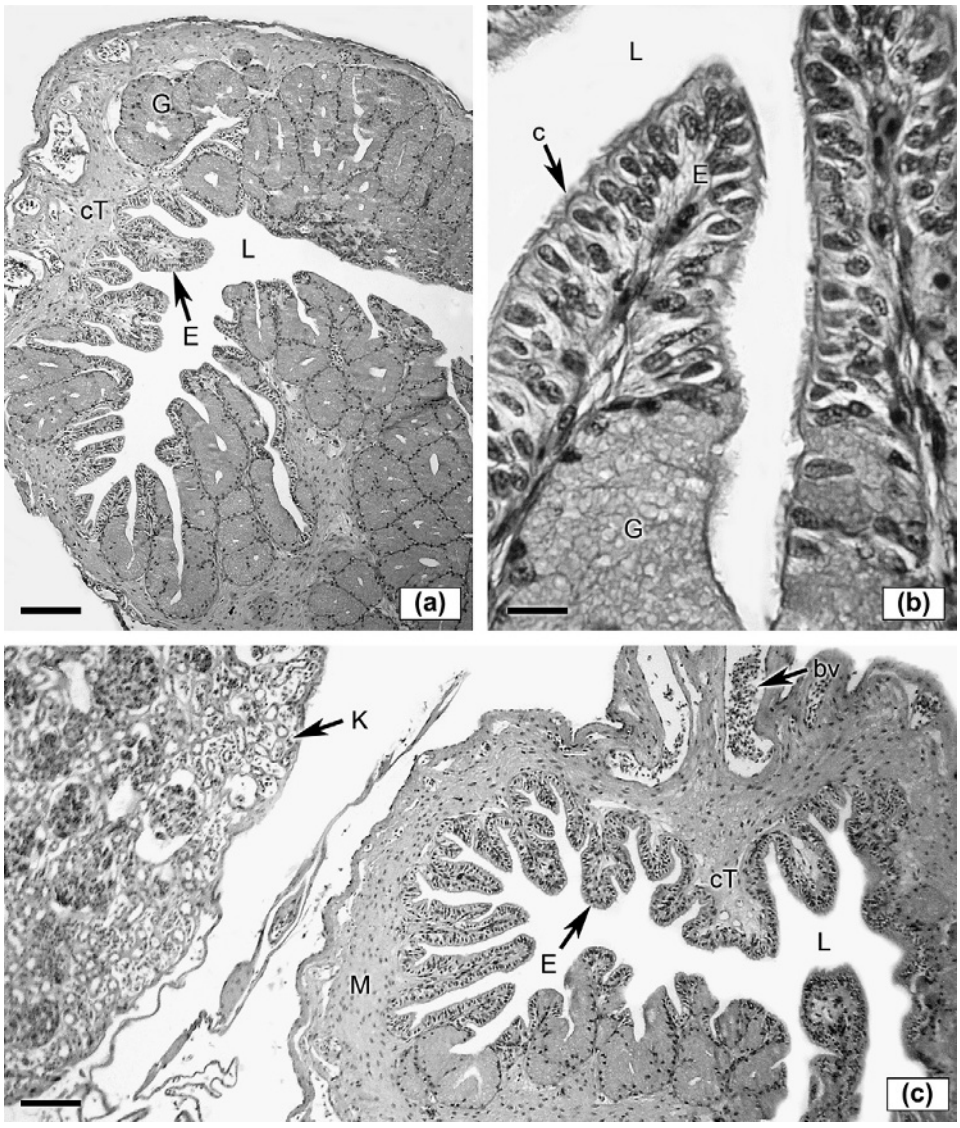

FIGURE 4.14 Oviduct of adult female *Ambystoma dumerilii*. (a—b) Cross sections of the caudal portion of the pars convoluta. The wall of the oviduct has folds projected into the lumen (L). The folds contain epithelium (E) with abundant ciliated cells (c) and scarce gland cells (G). The connective tissue (cT) and muscle (M) form a thicker layer compared with the anterior portions of the pars convoluta. Compare this figure with the pars convoluta in Figure 4.12(c). (c) More caudal portion of the pars convoluta, bordering on the ovisac region. The adjacent kidney (K) is seen. The reduction of glands (G) is evident when comparing (a) and (c). Connective tissue (cT) and muscle (M) are increased in thickness. Epithelium (E) with ciliated cells (c), and the lumen (L). Hematoxylin and eosin staining. Bars: (a,c) 0.2 mm; (cb) 20 μm.

mexicanum (Carroll, Palmer, & Ruibal, 1992) and the eggs of *Bufo bufo formosus* (Katagiri, 1965). Sasaki et al. (2002) identified six jelly coat layers surrounding the newt *C. pyrrhogaster*'s egg. Salthe (1963) described the morphology of jelly coats in an extensive group of amphibians.

4.4. Ovisac

The ovisac (Figure 4.15) forms a short and straight aglandular caudal region in oviparous species. Both ovisacs fuse at their caudal ends, forming only one opening to the cloaca (Uribe et al., 1989; Uribe, 2009). The mucosa is bordered by a columnar epithelium with ciliated cells. The cilia are large and abundant. In contrast to the more anterior regions, the ovisac contains thick layers of connective tissue and circular myometrium (Figure 4.15(c)). The increase in the number of muscle fibers in this region supports the intense contractions during the oviposition. Scattered, irregular, stellate-shaped melanocytes are seen at the periphery of this region. They possess long and thin cytoplasmic processes that extend into the connective

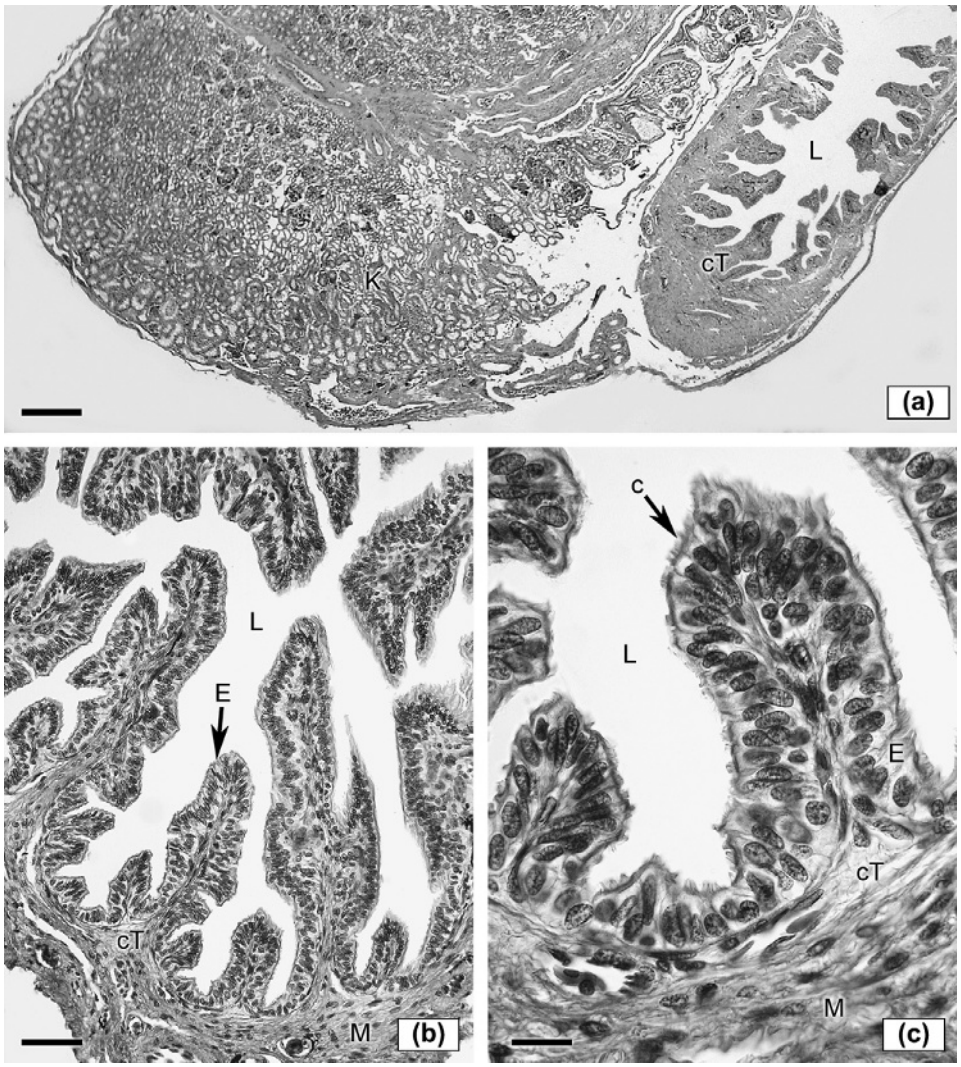

FIGURE 4.15 Oviduct of adult female *Ambystoma dumerilii*. Cross sections of the ovisac. (a–c) The ovisac and adjacent kidney (K). The wall contains irregular folds projected in the lumen (L), thick layers of connective tissue (cT), and muscle (M). The epithelium (E) is formed by columnar ciliated cells (c). The muscle layer is the thickest part of the oviduct wall. The muscle cells present a circular arrangement. Hematoxylin and eosin staining. Bars: (a) 0.3 mm; (b) 50 μm; (c) 20 μm.

tissue. The cytoplasm of these cells contains dark brown, fine granules of melanin. When the eggs reach the ovisac, they are stored until the amplexus occurs and, subsequently, the oviposition and fertilization form the embryos.

Relatively little is known about the endocrine factors affecting oviposition, and considerable research is needed. As in other vertebrates, neuro-hypophysial peptides, such as arginine, vasotocin (AVT), and mesotocin, are known to stimulate oviductal contractions during oviposition (Heller, Ferreri, & Leathers, 1970). In *A. tigrinum*, the responsiveness of the myometrium to AVT appears to be primed by prior exposure to P_4 (Guillette et al., 1985).

As described in this chapter, the reproductive system of amphibians is similar to those observed in other vertebrates regarding morphological and physiological seasonal changes. These changes are defined by the annual fluctuations in environmental conditions, such as light, temperature, rainfall, and humidity. The environmental conditions regulate the physiology of the neuro-endocrinological system, which controls the reproductive process. This control occurs via hypothalamic releasing factors and hypophyseal gonadotropins, elements that stimulate the follicles during oogenesis. In addition to describing the essential elements of the morphology and regulatory endocrinology of the reproductive system, it is indispensable to articulate these elements with ecological and evolutionary aspects that may contribute, in a wide perspective, to the understanding of the amphibian reproductive process.

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ABBREVIATIONS

3β-HSD	3β-hydroxysteroid dehydrogenase
AND	Androstenedione
AVT	Arginine vasotocin
DHEA	Dehydroepiandrosterone
E_2	17β-estradiol
FSH	Follicle-stimulating hormone
GTH	Gonadotropin
LH	Luteinizing hormone
MPF	Maturation promoting factor
P_4	Progesterone
T	Testosterone
Vtg	Vitellogenin

REFERENCES

Adams, A. E. (1940). Sexual conditions in *Triturus viridescens*. *Am. J. Anat., 66*, 235−275.

Bement, W. M., & Capco, D. G. (1990). Transformation of the amphibian oocyte into the egg: structural and biochemical events. *J. Electron Micros. Tech., 16*, 202−234.

Berois, N., & de Sa, R. (1988). Histology of the ovaries and fat bodies of *Chthonerpeton indistinctum*. *J. Herpetol., 22*(2), 146−151.

Beyo, R. S., Divya, L., Oommen, O. V., & Akbarsha, M. A. (2008a). Accumulation of yolk in a caecilian (*Gegeneophis ramaswamii*) oocyte: A light and transmission electron microscopic study. *J. Morphol., 269*, 1412−1424.

Beyo, R. S., Divya, L., Smita, M., Oommen, O. V., & Akbarsha, M. A. (2008b). Stages in follicle cell/oocyte interface during vitellogenesis in caecilians *Ichthyophis tricolor* and *Gegeneophis ramaswamii*: a transmission electron-microscopic study. *Cell. Tissue. Res., 331*(2), 519−528.

Beyo, R. S., Sreejith, P., Divya, L., Oommen, O. V., & Akbarsha, M. A. (2007a). Assembly of ovarian follicles in the caecilians *Ichthyophis tricolor* and *Gegeneophis ramaswamii*: light and transmission electron microscopic study. *Zygote, 15*(3), 199−213.

Beyo, R. S., Sreejith, P., Divya, L., Oommen, O. V., & Akbarsha, M. A. (2007b). Ultrastructural observations of previtellogenic ovarian follicles of the caecilians *Ichthyophis tricolor* and *Gegeneophis ramaswamii*. *J. Morphol., 268*, 329−342.

Brandon, R. A. (1970). Courtship, spermatophores and eggs of the Mexican achoque, *Ambystoma (Bathysiredon) dumerilii* (Duges). *Zool. J. Linn. Soc., 49*, 247−254.

Brizzi, R., Delfino, G., & Jantra, S. (2003). An overview of breeding glands. In B. G. M. Jamieson (Ed.), *"Reproductive Biology and Phylogeny of Anura" Chapter 6. Series: Reproductive Biology and Phylogeny* (pp. 253−317). NH, USA: Science Publishers, Inc. Enfield.

Brizzi, R., Delfino, G., Selmi, M. G., & Sever, D. M. (1995). Spermathecae of *Salamandrina terdigitata* (Amphibia: Salamandridae): patterns of sperm storage and degradation. *J. Morphol., 223*, 21−33.

Bruce, R. C. (1975). Reproductive biology of the mud salamander, *Pseudotriton montanus*, in western South Carolina. *Copeia, 1975*, 129−137.

Byskov, A. G. (1978). Follicular atresia. In R. E. Jones (Ed.), *"The vertebrate ovary"* (pp. 533−555). New York: Plenum Press.

Carroll, E. J., Jr., Palmer, R., & Ruibal, R. (1992). Structure and macromolecular composition of the jelly-coats of the urodele *Ambystoma mexicanum*. *Dev. Growth, Differen., 34*(5), 501−508.

Carroll, E. J., Jr., Wei, S. H., Nagel, G. M., & Ruibal, R. (1991). Structure and macromolecular composition of the egg and embryo jelly coats of the anuran *Lepidobatrachus laevis*. *Dev. Growth, Differen., 33*, 37−43.

Chieffi, G., & Pierantoni, R. (1987). Regulation of ovarian steroidogenesis. In D. O. Norris, & R. E. Jones (Eds.), *"Hormones and Reproduction in Fishes, Amphibians and Reptiles"* (pp. 117−144). New York: Plenum Press.

Coppin, A., Maes, E., Morelle, W., & Strecker, G. (1999). Structural analysis of 13 neutral oligosaccharide-alditols released by reductive β-elimination from oviducal mucins of *Rana temporaria*. *Euro. J. Biochem., 266*(1), 94−104.

del Pino, E. M. (1973). Interactions between gametes and environment in the toad *Xenopus laevis* (Daudin) and their relationship to fertilization. *J. Exp. Zool., 185*, 121−132.

del Pino, E. M. (1989). Modifications of oogenesis and development in marsupial frogs. *Development, 107*, 169−187.

Delplace, F., Maes, E., Lemoine, J., & Strecker, G. (2002). Species-specificity of O-linked carbohydrate chains of the oviducal mucins in amphibians: structural analysis of neutral oligosaccharide alditols released by reductive beta-elimination from the egg-jelly coats of *Rana clamitans*. *Biochem. J., 363*(3), 457−471.

Denis-Donini, S., & Campanella, C. (1977). Ultrastructural and lectin binding changes during the formation of the animal dimple in oocytes of *Discoglossus pictus*. *Dev. Biol., 61*, 149−152.

Di Fiore, M. M., Assisi, L., & Botte, V. (1998). Aromatase and testosterone receptor in the liver of the female green frog, *Rana esculenta*. *Life. Sci., 62*(21), 1949−1958.

Dodd, J. M. (1977). The structure of the ovary of nonmammalian vertebrates. In S. Zuckerman, & B. J. Weir (Eds.), *"The Ovary", Vol 1* (pp. 219−263). New York: Acadamic Press.

Dodd, J. M. (1986). Vertebrate Endocrinology: Fundamentals and Biomedical Implications. In P. K. T. Pang, & M. P. Schreibman (Eds.), *"The ovary", Vol. 1* (pp. 351−397). New York: Academic Press.

Dubowsky, S., & Smalley, K. N. (1993). Testosterone induced growth of the oviduct in the frog, *Rana pipiens*: Evidence for local aromatization. *Gen. Comp. Endocrinol., 89*, 276−282.

Duellman, W. E., & Trueb, L. (1986). *"Biology of Amphibians"*. New York: McGraw-Hill.

Dumont, J. N. (1972). Oogenesis in *Xenopus laevis* (Daudin). 1. Stages of oocyte development in laboratory maintained animals. *J. Morphol., 136*, 153−179.

Exbrayat, J. M., & Collenot, G. (1983). Quelque aspects de l'evolution de l'ovaire de *Typhlonectes compressicaudus* (Dumeril et Bibron, 1841), Batracien Apode vivipare. Etude quantitative et histochimique des corps jaunes. *Reprod. Nutr. Develop., 23*, 889−898.

Fernández, S. N., & Ramos, I. (2003). Endocrinology of Reproduction. In B. G. M. Jamieson (Ed.), *"Reproductive Biology and Phylogeny of Anura"* (pp. 73–117). NH, USA: Science Publishers, Inc. Enfield, Volume 2 of Series: Reproductive Biology and Phylogeny. Chapter 4.

Ferrell, J. E., Jr. (1999). *Xenopus* oocyte maturation: new lessons from a good egg. *BioEssays, 21*, 833–842.

Gall, J. G., Wu, Z., Murphy, C., & Gao, H. (2004). Structure in the amphibian germinal vesicle. *Exp. Cell. Res., 296*(1), 28–34.

Greven, H. (1998). Survey of the oviduct of Salamandrids with special reference to the viviparous species. *J. Exp. Zool., 282*, 507–525.

Greven, H. (2003). Oviduct and egg-jelly. In D. Sever (Ed.), *"Reproductive Biology and Phylogeny of Urodela"* (pp. 151–181). NH, USA: Science Publishers, Inc. Enfield, Volume 1 of Series: Reproductive Biology and Phylogeny.

Greven, H., & Guex, G. D. (1994). Structural and physiological aspects of viviparity in *Salamandra salamandra*. In H. Greven, & B. Thiesmeier (Eds.), *" Mertensiella, Biology of Salamandra and Mertensiella"* (pp. 139–160). Bonn: Deutsche Gesellschaft für Herpetologie und Terrarienkunde.

Grey, R. D., Wolf, D. P., & Hedrick, J. L. (1974). Formation and structure of the fertilization envelope in *Xenopus laevis*. *Dev. Biol., 54*, 52–60.

Grey, R. D., Working, P. K., & Hedrick, J. L. (1977). Alteration of structure and penetrability of the vitelline envelope after passage of eggs from coelom to oviduct in *Xenopus laevis*. *J. Exp. Zool., 201*(1), 73–83.

Grier, H. J., Uribe, M. C., & Patiño, R. (2009). Chapter 2. The Ovary, Folliculogenesis, and Oogenesis in Teleosts. In B. Jamieson (Ed.), *"Reproductive Biology and Phylogeny of Fish (Agnatha and Osteichthyes)"* (pp. 25–84). NH, USA; Plymouth, UK: Science Publishers, Inc. Enfield.

Guillette, L. J., Jr., Norris, D. O., & Norman, M. F. (1985). Response of amphibian (*Ambystoma tigrinum*) oviduct to arginine vasotocin and acetylcholine *in vitro*: Influence of steroid hormone pretreatment *in vivo*. *Comp. Biochem. Physiol. C, 80*, 151–154.

Guraya, S. S. (1969). Histochemical study of follicular atresia in the amphibian ovary. *Acta. Biol. Acad. Sci. Hung., 20*(1), 43–56.

Guraya, S. S. (1976). Recent advances in the morphology, histochemistry, and biochemistry of steroid-synthesizing celllular sites in the non-mammalian vertebrate ovary. *Rev. Cytol., 4*, 365–409.

Guraya, S. S. (1978). Maturation of the follicular wall of nonmammalian vertebrates. In R. E. Jones (Ed.), *"The Vertebrate Ovary. Comparative Biology and Evolution"* (pp. 261–329). New York: Plenum Press.

Hammes, S. R. (2004). Steroids and oocyte maturation—A new look at an old story. *Mol. Endocrinol., 18*(4), 769–775.

Hardy, D. M., & Hedrick, J. L. (1992). Isolation and characterization of oviductin from *Xenopus laevis* pars recta oviduct. *Biochemistry, 203*, 4466–4472.

Heatwole, H. (1961). Rates of desiccation and rehydration of eggs in a terrestrial salamander, *Plethodon cinereus*. *Copeia, 1961*, 110–112.

Heller, H. E., Ferreri, E., & Leathers, D. H. G. (1970). The effect of neurohypophysial hormones on the amphibian oviduct *in vitro*, with some remarks on the histology of this organ. *J. Endocrinol., 47*, 495–508.

Holland, C. A., & Dumont, J. N. (1975). Oogenesis in *Xenopus laevis* (Daudin). IV. Effects of gonadotropin, estrogen and starvation on endocytosis in developing oocytes. *Cell. Tissue Res., 162*, 177–184.

Hope, J., Humphries, A. A., Jr., & Bourne, G. H. (1963). Ultrastructural studies on developing oocytes of the salamander *Triturus viridescens*. I. The relationship between follicle cells and developing oocytes. *J. Ultrastruct. Res., 9*, 302–324.

Iela, L., Rastogi, R. K., Delrio, G., & Bagnara, J. T. (1986). Reproduction in the Mexican leaf frog, *Pachymedusa dacnicolor* III. The female. *Gen. Comp. Endocrinol., 63*, 381–392.

Itoh, M., & Ishii, S. (1990). Changes in plasma levels of gonadotropins and sex steroids in the toad, *Bufo japonicus*, in association with behavior during the breeding season. *Gen. Comp. Endocrinol., 80*(3), 451–464.

Itoh, T., Kamimura, S., Watanabe, A., & Onitake, K. (2002). Egg-jelly structure promotes efficiency of internal fertilization in the newt, *Cynops pyrrhogaster*. *J. Exp. Zool., 292*, 314–322.

Joly, J., & Picheral, B. (1972). Ultrastructure, histochimie et physiologie du follicle pre-ovulatoire et du corps jaune de l'urodele ovo-vivipare *Salamandra salamandra* L. *Gen. Comp. Endocrinol., 18*, 235–259.

Joly, J., Chesnel, F., & Boujard, D. (1994). Biological adaptations and reproductive strategies in the genus *Salamandra*. In H. Greven, & B. Thiesmeier (Eds.), *"Mertensiella, Biology of Salamandra and Mertensiella"* (pp. 255–269). Bonn: Deutsche Gesellschaft für Herpetologie und Terrarienkunde.

Jørgensen, C. B. (1992). Growth and Reproduction. In M. E. Feder, & W. W. Burggren (Eds.), *"Environmental physiology of the Amphibians"* (pp. 439–466). Chicago: The University of Chicago Press.

Kambara, S. (1956). Histochemical studies on the distribution of phosphatases in the oviduct of the newt, *Triturus pirrhogaster*. *Annot. Zool. Jap., 29*, 86–90.

Katagiri, C. (1965). The fertilizability of coelomic and oviductal eggs of the toad *Bufo bufo formosus*. *J. Fac. Sci. Hokkaido. Univ. Ser. VI. Zool., 15*, 633–643.

King, M. L., Messitt, T. J., & Mowry, K. L. (2005). Putting RNAs in the right place at the right time: RNA localization in the frog oocyte. *Biol. Cell, 97*, 19–33.

Kobayashi, F., Zimniski, S. J., & Smalley, K. N. (1996). Characterization of oviductal aromatase in the northern leopard frog, *Rana pipiens*. *Comp. Biochem. Physiol. B, 113*, 653–657.

Kwon, H. B., Choi, H. H., Ahn, R. S., & Yoon, Y. D. (1995). Steroid production by amphibian (*Rana nigromaculata*) ovarian follicles at different developmental stages. *J. Exp. Zool., 260*(1), 66–73.

Lange, R. H. (1985). The vertebrate yolk-platelet cristal: comparative analysis of an *in vivo* crystalline aggregate. *Int. Rev. Cytol., 97*, 133–181.

Licht, P., & Porter, D. A. (1987). Role of gonadotropin-realising hormone in regulation of gonadotropin secretion from amphibian and reptilian pituitaries. In D. O. Norris, & R. E. Jones (Eds.), *"Hormones and Reproduction in Fishes, Amphibians and Reptiles"* (pp. 61–85). New York: Plenum Press.

Loader, S. P., Wilkinson, M., Gower, D. J., & Msuya, C. A. (2003). A remarkable young *Scolecomorphus vittatus* (Amphibia: Gymnophiona: Scolecomorphidae) from the North Pare Mountain, Tanzania. *J. Zoology, 259*(1), 93–101.

Lofts, B. (1984). Amphibians. In G. E. Lamming (Ed.), *"Marshall's Physiology of Reproduction"* (pp. 127–205). Edinburgh: Churchill Livingstone.

Lutz, L. B., Cole, L. M., Gupta, M. K., Kwist, K. W., Auchus, R. J., & Hammes, S. R. (2001). Evidence that androgens are the primary steroids produced by *Xenopus laevis* ovaries and may signal

through the classical androgen receptor to promote oocyte maturation. *Proceedings of the Natl. Acad. of Sci. of the U.S.A, 24*, 13728−13733.

Maller, J. L. (1985). Regulation of amphibian oocyte maturation. *Cell Diff., 16*(4), 211−221.

Masui, Y., & Clarke, H. J. (1979). Oocyte maturation. *Int. Rev. Cytol., 57*, 185−282.

McCurdy, H. M. (1931). Development of the sex organs in *Triturus torosus*. *Am. J. Anat., 47*, 367−403.

Morelle, W., & Strecker, G. (1998). Structural analysis of a new series of oligosaccharide-alditols released by reductive β-elimination from oviducal mucins of *Rana utricularia*. *Biochem. J., 330*, 469−478.

Nagahama, Y. (1987). Endocrine control of oocyte maturation. In D. O. Norris, & R. E. Jones (Eds.), *"Hormones and Reproduction in Fishes, Amphibians and Reptiles"* (pp. 171−202). New York: Plenum Press.

Nath, K., Boorech, J. L., Beckham, Y. M., Burns, M. M., & Elinson, R. P. (2004). Status of RNAs, localized in *Xenopus laevis* oocytes, in the frogs *Rana pipiens* and *Eleutherodactylus coqui*. *J. Exp. Zool., 304B* (1), 28−39.

Nigel Finn, R. (2007). Vertebrate yolk complexes and the functional implications of phosvitins and other subdomains in vitellogenins. *Biol. Reprod., 76*, 926−935.

Norris, D. O. (2007). Reproduction in Amphibians. *"Vertebrate Endocrinology"* (4th ed.). New York: Acadamic Press.

Norris, D. O., & Lopez, K. H. (2005). Anatomy of the amphibian endocrine system. In H. Heatwole (Ed.), *"Amphibian Biology. Chapter 1. Vol. 6, Endocrinology"* (pp. 2021−2044). Chipping Norton, NSW, Australia: Surrey Beatty & Sons.

Norris, D. O., Carr, J. A., Summers, C. H., & Featherston, R. (1997). Interactions of androgens and estradiol on sex accessory ducts of larval tiger salamanders, *Ambystoma tigrinum*. *Gen. Comp. Endocrinol., 106*, 348−355.

Ogielska, M., & Bartmanska, J. (2009). Oogenesis and female reproductive system in Amphibia-Anura. Chapter 5. In M. Ogielska (Ed.), *"Reproduction of Amphibians", Series: Biological Systems in Vertebrates*. Enfield, NH, USA. Plymouth, U.K.: Science Publishers, Inc. in press.

Oliveira de, C., & Souza-Santos, L. R. (2004). Histological characterization of cellular types during *Scinax fuscovarius* oogenesis (Lutz) (Anura, Hylidae). *Rev. Bras. Zool., 21*(4), 919−923.

Oterino, J., Sánchez Toranzo, G., Zelarayán, L., Ajmat, M. T., Bonilla, F., & Bühler, M. I. (2006). Behavior of the vitelline envelope in *Bufo arenarum* oocytes matured *in vitro* in blockade to polyspermy. *Zygote, 14*, 97−206.

Oterino, J., Sánchez Toranzo, G., Zelarayán, L., Valz-Gianinet, D. N., & Bühler, M. I. (2001). Cortical granule exocitosis in *Bufo arenarum* oocytes matured *in vitro*. *Zygote, 9*(3), 251−259.

Palecek, J., Habrová, V., Nedvídek, J., & Romanovský, A. (1985). Dynamics of tubulin structures in *Xenopus laevis* oogenesis J. *Embryol. Exp. Morph., 87*, 75−86.

Pancharatna, M., & Saidapur, S. K. (1985). Ovarian cycle in the frog *Rana cyanophlyctis*: A quantitative study of follicular kinetics in relation to body mass, oviduct, and fat body cycles. *J. Morphol., 186*, 135−147.

Pawar, V. G., & Pancharatna, K. (1999). Annual oviduct cycle in the Indian skipper frog, *Rana cyanophlyctis* (Schneider 1799): a morphological study. *Trop. Zoo. 12*, 79−88.

Potemkina, D. A. (1967). Formation of the Müllerian ducts in the urodele (*Ambystoma mexicanum*). *Dokl. Biol. Sci., 174*(1), 305−308.

Redshaw, M. R. (1972). The hormonal control of the amphibian ovary. *Am. Zool., 12*, 289−306.

Richter, H. P. (1987). Membranes during yolk-platelet development in oocytes of the toad *Bufo marinus*. *Roux's Arch. Dev. Biol., 196*, 367−371.

Saidapur, S. K. (1978). Follicular atresia in the ovaries of nonmammalian vertebrates. *Inter. Rev. Cytol., 54*, 225−244.

Saidapur, S. K. (1982). Structure and function of postovulatory follicles (corpora lutea) in the ovaries of nonmammalian vertebrates. *Inter. Rev. Cytol., 75*, 243−285.

Saidapur, S. K., & Nadkarni, V. B. (1973). Follicular atresia in the ovary of the frog *Rana cyanophlyctis* (Schneider). *Acta. Anat., 86*, 559−564.

Saidapur, S. K., & Nadkarni, V. B. (1974). Steroid-synthesizing cellular sites in amphibian ovary. A histochemical study. *Gen. Comp. Endocrinol., 22*, 459−462.

Salthe, S. N. (1963). The egg capsules in the Amphibia. *J. Morphol., 113*, 161−171.

Sánchez, S. S., & Villecco, E. I. (2003). Oogenesis. Chapter 3. In B. G. M. Jamieson (Ed.), *"Reproductive Biology and Phylogeny of Anura" Volume 2 of Series: Reproductive Biology and Phylogeny* (pp. 27−71). NH, USA: Science Publishers, Inc. Enfield.

Sasaki, T., Kamimura, S., Takai, H., Watanabe, A., & Otinake, K. (2002). The activity for the induction of the sperm acrosome reaction localizes in the outer layers and exists in the high-molecular-weight components of the egg-jelly of the newt, *Cynops pyrrhogaster. Zygote, 10*(1), 1−9.

Schmidt-Zachmann, M. S. (2005). The nucleolus—a multifunctional nuclear body. *Zellbiologie aktuell., 2*, 2−25.

Schmitt, A., & Nebreda, A. R. (2002). Signalling pathways in oocyte meiotic maturation. *J. Cell Sci., 115*, 2457−2459.

Sever, D. M. (1994). Observations on regionalization of secretory activity in the spermathecae of salamanders and comments on phylogeny of sperm storage in female salamanders. *Herpetologica., 50*, 383−397.

Sever, D. M. (2003). Courtship and mating glands. Chapter 9. In D. Sever (Ed.), *"Reproductive Biology and Phylogeny of Urodela" Volume 1 of Series: Reproductive Biology and Phylogeny* (pp. 323−381). NH, USA: Science Publishers, Inc. Enfield.

Sever, D. M., Rania, L. C., & Krenz, J. D. (1996). Reproduction of the salamander *Siren intermedia* Le Conte with especial reference to oviducal anatomy and mode of fertilization. *J. Morphol., 227*, 335−348.

Sharon, R., Degani, G., & Warburg, M. R. (1997). Oogenesis and the ovarian cycle in *Salamandra salamandra infraimmaculata* Mertens (Amphibia; Urodela; Salamandridae) in fringe areas of the taxon's Distribution. *J. Morphol., 231*, 149−160.

Spargo, S. C., & Hope, R. M. (2003). Evolution and nomenclature of the zona pellucida gene family. *Biol. Reprod., 68*, 358−362.

Spornitz, U. M., & Kress, A. (1973). Ultrastructural studies of oogenesis in some European amphibians. II. *Triturus vulgaris. Z. Zellforsch., 143*, 387−407.

Sretarugsa, P., Weerachatyanukul, W., Chavadej, J., Kruatrachue, M., & Sobhon, P. (2001). Classification of developing oocytes, ovarian development and seasonal variation in *Rana tigerina*. *Sci. Asia, 27*, 1−14.

Subtelny, S., & Penkala, J. E. (1984). Experimental evidence for a morphogenetic role in the emergence of PGCs from the endoderm of *Rana pipiens*. *Differentiation, 26*, 211−219.

Tanimura, A., & Iwasawa, H. (1988). Ultrastructural observations on the origin and differentiation of somatic cells during gonadal development in the frog *Rana nigromaculata*. *Dev. Growth and Different, 30* (6), 681−691.

Tanimura, A., & Iwasawa, H. (1989). Origin of somatic cells and histogenesis in the primordial gonad of the Japanese tree frog *Rhacophorus arboreus*. *Anat. Embryol., 180*(2), 165−173.

Tokarz, R. R. (1978). Oogonial proliferation, oogenesis, and folliculogenesis in nonmammalian vertebrates. In R. E. Jones (Ed.), *"The Vertebrate Ovary. Comparative Biology and Evolution"* (pp. 145−179). New York: Plenum Press.

Trauth, S. E. (1983). Reproductive biology and spermathecal anatomy of the dwarf salamander (*Eurycea quadridigitata*) in Alabama. *Herpetologica., 39*(1), 9−15.

Trauth, S. E. (1984). Spermathecal anatomy and the onset of mating in the slimy salamander (*Plethodon glutinosus*) in Alabama. *Herpetologica., 40*(3), 314−321.

Tyler, M. J. (2003). The gross anatomy of the reproductive system. In B. G. M. Jamieson (Ed.), *Reproductive Biology and Phylogeny Series: Vol. 2. "Reproductive Biology and Phylogeny of Anura"* (pp. 19−26) NH, USA: Science Publishers, Inc. Enfield.

Uribe, M. C. A. (2001). Reproductive systems of Caudata Amphibia. In H. M. Dutta, & J. S. D. Munshi (Eds.), *"Vertebrate Functional Morphology"* (pp. 267−293) Plymouth, UK: Science Publishers, Inc. Enfield (NH) USA.

Uribe, M. C. A. (2003). The ovary and oogenesis. In D. Sever (Ed.), *Reproductive Biology and Phylogeny series: Vol. 1 "Reproductive Biology and Phylogeny of Urodela"* (pp. 135−150) Plymouth, U.K: Science Publishers, Inc. Enfield. NH, USA.

Uribe, M. C. A. (2009). Oogenesis and female reproductive system in Amphibia-Urodela. In M. Ogielska (Ed.), *Biological Systems in Vertebrates Series: "Reproduction of Amphibians"* (pp. 273−304) Plymouth, U.K: Science Publishers, Inc. Enfield. NH, USA.

Uribe, M. C. A., Mena, L. F., Carrubba, M. R. C., Estrada, E. F., Palmer, B. D., & Guillette, L. J., Jr. (1989). Oviductal histology of the urodele, *Ambystoma mexicanum*. *J. Herpetol., 23*(3), 230−237.

Vilter, V. (1967). Histologie de l'oviducte chez *Salamandra atra* mature, Urodele totalment vivipare de haute montagne. *C.R. Soc. Biol. Paris, 161*, 260−264.

Wake, M. (1970). Evolutionary morphology of the caecilian urogenital system. Part II. The kidneys and urogenital ducts. *Acta. Anat., 75*, 321−358.

Wake, M. (1977). Fetal maintenance and its evolutionary significance in the Amphibia: Gymnophiona. *J. Herpetol., 11*(4), 379−386.

Wake, M. (1985). Oviduct structure and function in non-mammalian vertebrates. In H. Duncker, & G. Fleisher (Eds.). *"Vertebrate Morphology", vol. 30* (pp. 427−435). Stuttgart: Fortschr. Zool. Gustav Fischer.

Wake, M. (1993). Evolution of oviductal gestation in Amphibians. *J. Exp. Zool., 266*, 394−413.

Wake, M., & Dickie, R. (1998). Oviduct structure and function and reproductive modes in amphibians. *J. Exp. Zool., 282*, 477−506.

Wallace, R. A. (1978). Oocyte growth in non-mammalian vertebrates. In R. E. Jones (Ed.), *"The Vertebrate Ovary. Comparative Biology and Evolution"* (pp. 469−502). New York: Plenum Press.

Wallace, R. A., & Selman, K. (1990). Ultrastructural aspects of oogenesis and oocyte growth in fish and amphibians. *J. Electron. Microsc. Tech., 16*, 175−201.

Ward, D., & Sexton, O. J. (1981). Anti-predator role of salamander egg membranes. *Copeia, 1981*, 724−726.

Ward, R. T., & Ward, E. (1968). The origin and growth of cortical granules in the oocytes of *Rana pipiens*. *J. Micros., 7*, 1021−1030.

Watanabe, A., & Onitake, K. (2002). The urodele egg-coat as the apparatus adapted for the internal fertilization. *Zool. Sci., 19*(12), 1341−1347.

Watanabe, A., & Onitake, K. (2003). Sperm activation. In D. Sever (Ed.), *Reproductive Biology and Phylogeny Series: Vol. 1. "Reproductive Biology and Phylogeny of Urodela"* (pp. 425−445). NH, USA: Science Publishers, Inc. Enfield.

Whittier, J. M., & Crews, D. (1987). Seasonal reproduction: patterns and control. In D. O. Norris, & R. E. Jones (Eds.), *"Hormones and Reproduction in Fishes, Amphibians and Reptiles"* (pp. 385−409). New York: Plenum Press.

Wiley, H. S., & Wallace, R. A. (1981). The structure of vitellogenin. Multiple vitellogenins in *Xenopus laevis* give rise to multiple forms of the yolk proteins. *J. Biol. Chemistry, 256*, 8626−8634.

Wischnitzer, S. (1964). An electron microscope study of the formation of the zona pellucida in oocytes from *Triturus viridescens*. *Cell Tissue Res., 64*(2), 196−209.

Xavier, F. (1987). Functional morphology and regulation of the corpus luteum. In D. O. Norris, & R. E. Jones (Eds.), *"Hormones and Reproduction in Fishes, Amphibians and Reptiles"* (pp. 241−282). New York: Plenum Press.

Yamasaki, H., & Katagiri, C. (1991). Egg exudate-induced reduction of sperm lysin sensitivity in the vitelline coat fertilization of *Bufo japonicus* and its participation in polyspermy block. *J. Exp. Zool., 258*(3), 404−413.

Yamashita, M., Mita, K., Yoshida, N., & Kondo, T. (2000). Molecular mechanisms of initiation of oocyte maturation: general and species-specific aspects. In L. Meijer, A. Jézéquel, & B. Ducommun (Eds.), *"Progress in Cell Cycle Research", Volume 4* (pp. 115−129). New York: Kluwer Academic/Plenum Publishers.

Yoshizaki, N., & Katagiri, C. H. (1981). Oviductal contribution to alteration of the vitelline coat in the frog, *Rana japonica*. An electron microscope study. *Dev. Growth and Different., 23*, 495−506.

Zelazowska, M., Kilarski, W., Bilinski, S., Podder, D., & Kloc, M. (2007). Balbiani cytoplasm in oocytes of a primitive fish, the sturgeon *Acipenser gueldenstaedtii*, and its potential homology to the Balbiani body (mitochondrial cloud) of *Xenopus laevis* oocytes. *Cell Tissue Res., 329*(1), 137−145.

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Chapter 5

Hormones, Sex Accessory Structures, and Secondary Sexual Characteristics in Amphibians

David M. Sever* and Nancy L. Staub†

* *Southeastern Louisiana University, Hammond, LA, USA,* † *Gonzaga University, Spokane, WA, USA*

SUMMARY

Gonadal steroid hormones, particularly testosterone (T) and related androgens, are important in the development and seasonal variation of sexually dimorphic organs. Other hormones, such as prolactin, have been found to be necessary in conjunction with gonadal steroids for the full structural development and function of some sex accessory structures (e.g., oviductal and cloacal gland secretions) and secondary sexual characteristics (e.g., genial glands and skin glands of newts). Thyroid hormones work synergistically with prolactin and T in hypertrophy of the tail fin and nuptial pads of American newts (e.g., *Notophthalmus viridescens*), whereas oxytocin antagonizes the influence of prolactin. In the Japanese newt (*Cynops pyrrhogaster*), however, estrogens block the action of prolactin on increasing tail height, explaining the sexual differences in tail morphology. Arginine vasotocin (AVT) has been shown to stimulate labor in the viviparous salamandrid *Salamandra salamandra*, corticosterone influences the development of salamander cloacal glands, and prostaglandin $PGF_{2\alpha}$ causes the release of sperm from the salamander spermatheca by triggering contraction of the myoepithelium.

1. INTRODUCTION

The sex accessory structures of amphibians are the genital ducts and derivatives of these structures. The secondary sexual characteristics are all of the differences between the sexes due to sexual maturation other than those connected with the gonads and their ducts. In this chapter, we review the effects of hormones on development, anatomy, function, and seasonal variation of the sex accessory structures and secondary sexual characteristics of amphibians. We introduce the more general term, secondary characteristics, to refer to non-sexually dimorphic traits that arise at sexual maturity. A list of the structures considered is presented in Table 5.1 and we will report on representative studies related to these organs.

The role of gonadal steroid hormones in causing seasonal changes in sex accessory structures and secondary sexual characteristics has been well-documented (see, for example the reviews by Norris (1987), Fernández and Ramos (2003), Kikuyama, Tanaka, and Moore (2003), and Exbrayat (2006)). In males, the primary testicular steroids are testosterone (T) and 5α-dihydrotestosterone, although others have been detected; i.e., 11-ketotestosterone, 11β-hydroxytestosterone, androstenedione, progesterone (P_4), 17β-estradiol (E_2), and estrone. In females, the primary steroids are T, androstenedione, E_2, estrone, and P_4 (Moore & Deviche, 1988). Gonadal steroids are also important in the behaviors associated with these characteristics, but other hormones such as the gonadotropins (GTHs) follicle-stimulating hormone and luteinizing hormone, and the neuropeptide arginine vasotocin (AVT), are also critical in mating behaviors and show changes in concentrations that correlate with gonadal activity and changes in sex accessory structures and secondary sexual characteristics (Deviche, Propper, & Moore, 1990). This chapter, however, is not concerned with behavior, which is covered elsewhere (Chapter 8, this volume). Gonadotropin-releasing hormone (GnRH) stimulates the release of GTHs, and GTHs stimulate development of the gonads and thus production of gonadal steroids. The feedback relationships in these cycles have been examined in a number of amphibians but are not a concern of this chapter (see Chapter 2, this volume).

2. SEX ACCESSORY STRUCTURES

2.1. Wolffian Ducts/Vas Deferens

From each testis, sperm pass through longitudinal tubules into the Wolffian (archinephric, mesonephric) duct; no epididymis exists. The Wolffian duct is both a sperm and

TABLE 5.1 Sex accessory structures and secondary sexual characteristics of amphibians covered in this chapter, and hormones that have been implicated in their development and function

Organ	Hormones
Sex Accessory Structures	
Wolffian ducts/Vas deferens	DHT, E_2, T
Müllerian ducts	DHT, E_2, MIH, PRL, T
Oviduct of *Ascaphus*	
Uterus of viviparous anurans	
Uterus of *Salamandra*	AVT
Uterus of caecilians	progesterone
Penis of *Ascaphus*	
Cloacal glands of salamanders	B, DHEA, E_2, progesterone, T
Phallodeum of caecilians	
Secondary Sexual Characteristics	
Amphibian skin	[^{3}H]-T, [^{3}H]-estradiol
Anuran breeding glands	E_2, T
Adhesive glands	T
Nuptial excrescences of anurans	T
Tail fin and nuptial pads of newts	OXY, PRL, TSH, T
Teeth of plethodontids	T
Tusks/spines	
Muscles	estrogen, T
Vomeronasal organ	
Color	E_2, T
Salamander courtship glands	
Caudal glands in the middorsal tail base	
Caudal glands in the midventral tail base	
Cloacal glands that secrete onto epidermis	
Genial glands	PRL, T
Mental glands	T
Nasolabial glands (cirri)	T
Skin glands of *Taricha*	PRL

urine duct, but, in plethodontid salamanders, the anterior extension of the Wolffian duct is strictly a sperm duct and the name 'vas deferens' is appropriate for this portion (Uribe Aranzábal, 2003). Development of the Wolffian ducts in larval and postmetamorphic amphibians is stimulated by androgens (Norris, 1987) and, in *Rana japonicus*, the effects of T were enhanced by treatment with E_2 (Tojio & Iwasawa, 1977).

The caudal portion of the Wolffian duct stores sperm prior to the mating season and becomes secretory in *Notophthalmus viridescens*, *Taricha torosa*, *Ambystoma tigrinum* (Norris, 1987), and A*mbystoma dumerillii* (Uribe, 2001). Norris, Norman, Pancak, and Duvall (1985) reported that hypertrophy of the vasa deferentia epithelium and secretory activity of the cloacal gland complex in neotenic *A. tigrinum* were closely tied to high levels of plasma androgens (T and DHT). The diameter of the vasa deferentia was associated with the presence of stored sperm and not with plasma androgen levels.

2.2. Müllerian Ducts

Müllerian ducts develop into functional oviducts under the influence of ovarian estrogens during ontogeny, and this has been documented in many studies (Norris, Carr, Summers, & Featherston, 1997; Greven, 2003). Boisseau (1975) showed that exposure to E_2 hastens morphogenesis of ciliated and secretory cells in the salamandrid *Pleurodeles waltl*. Jego (1977), also in *P. waltl*, found that E_2 had different effects on fucosyltransferase and galactosyltransferase, enzymes involved in the formation of glycoproteins. In the newt *Cynops pyrrhogaster*, both prolactin (PRL) and sex steroids (E_2, T) were needed for production of oviductal mucopolysaccharides (Kikuyama, Seshimo, Shirama, Kato, & Noumura, 1986; Polzonetti-Magni, Carnevali, Yamamoto, & Kikuyama, 1995). Without PRL, E_2 could neither induce full structural development nor stimulate egg jelly synthesis in *C. pyrrhogaster* (Kikuyama et al., 2000).

Clark, Norris, & Jones (1995) studied the interactions of E_2 or dihydrotestosterone (DHT) and the pesticides dichlorodiphenyltrichloroethane (technical grade, 80% p,p^1-DDT and 20% o,p^1-DDT) and dichlorodiphenyldichloroethylene (p,p^1-DDE) on the development of Wolffian ducts and Müllerian ducts in larval male and female *A. tigrinum*. 17β-estradiol and DHT stimulated Müllerian duct development in both sexes, whereas the Wolffian duct was stimulated only by DHT. Dichlorodiphenyltrichloroethane had an antiestrogenic effect on gonaduct development, and DDE had an estrogenic effect on the Müllerian ducts of females. The Müllerian ducts of males and the Wolffian ducts of both sexes were unaffected by DDT or DDE alone. Their results contradicted the expected estrogenic actions of DDT and the antiandrogenic actions of DDE.

The anuran oviduct is divided into three parts (Fernández & Ramos, 2003). The most anterior region is the pars recta, which collects ovulated eggs through the ostium. The pars recta secretes oviductin, which modifies the egg vitelline envelope to render it susceptible for sperm penetration. The middle region of the anuran oviduct is the pars convoluta, which secretes the highly viscous jelly layers onto the eggs. The distal portion of the oviduct is the ovisac, where eggs are held prior to ovulation. In the preovulatory period of the ovarian cycle of *Bufo arenarum*,

serum T, DHT, and P_4 reach the highest circulating levels whereas E_2 shows the lowest values detected during the cycle (Fernández, Mansilla, & Miceli, 1984). Under these hormonal conditions, the entire oviduct reaches maximal development of secretory cells. A marked decrease in androgen and P_4 circulating levels and a steady increase in E_2 are associated with ovulation (Fernández et al., 1984; Fernández and Ramos, 2003).

The oviduct, in at least some species, may secrete a substance that alerts males to female receptivity. Water that held ovariectomized female newts *C. pyrrhogaster* treated with PRL and E_2 was significantly more attractive to males than water that held ovariectomized females treated with saline (Toyoda, Tanaka, Matsuda, & Kikuyama, 1994).

In most male anurans and salamanders, the Müllerian duct regresses in the embryo in response to Müllerian-inhibiting hormone (MIH), which is secreted by the Sertoli cells of the developing testis (Akbarsha, Jancy, Smita, & Oommen, 2006). Vestiges of the Müllerian duct persist in some male anurans and salamanders, but they have no function (Duellman & Trueb, 1987). A rudimentary Müllerian duct is retained in male bufonid toads (e.g., *Bufo woodhousei*) and, if the testes are removed, leaving the ovarian-like Bidder's organs, the Müllerian ducts may develop into oviducts (Witschi, 1942). The Müllerian duct is retained in male caecilians as a functional glandular structure, the Müllerian gland. The role of MIH in male caecilians is unknown. The caecilian Müllerian gland is involved in the production of seminal fluid and is hypothesized to be homologous to the mammalian prostate gland (Wake, 1981).

Some specializations of the amphibian oviduct are discussed below.

2.2.1. Oviduct of Ascaphus

Sperm storage tubules occur in the oviducts of females of *Ascaphus truei* and *Ascaphus montanus*, just distal to the region where egg jelly is applied to the eggs (Sever, Moriarty, Rania, & Hamlett, 2001). These two species of frog, found in the northwestern USA and southwestern Canada, have traditionally been the sole members of the family Ascaphidae, usually considered the basal family of frogs. Recently, however, they have been placed in the family Leiopelmatidae, which now is the most basal family (Frost et al., 2006). The two species of *Ascaphus* are the only frogs out of over 5200 described species in which females are known to store sperm.

The sperm storage tubules are simple tubular glands lined with simple columnar ciliated and secretory cells, and the secretory cells secrete a neutral carbohydrate. The sperm storage tubules do not seem to be modified for sperm maintenance and appear to be sites of temporary sperm residence (Sever, Hamlett, Slabach, Stephenson, & Verrell, 2003). No studies exist on the hormonal influences on sperm storage in *Ascaphus*.

2.2.2. Uterus of viviparous anurans

Viviparity is known in several species of African bufonid (including at least *Nectophrynoides tornieri*, *Nectophrynoides viviparus*, *Nimbaphrynoides occidentalis*, and *Nimbaphrynoides liberiensis* (Wake, 1980; 1993)), and in the leptodactylid frog *Eleurodactylus jasperi* from Puerto Rico (Wake, 1978). After the bufonids exhaust the yolk, they are nourished by ingesting 'uterine milk,' a muco-protein secreted by the oviductal epithelial glands (Vilter & Lugand, 1959). Corpora lutea are present in pregnant *N. occidentalis* and apparently regulate oviductal nutrient secretion (Lamotte & Xavier, 1972). Intra-oviductal nutrition in *E. jasperi* is dependent upon yolk throughout uterine development, and no corpora lutea are present in the ovaries (Wake, 1978). Other knowledge about the hormonal influences on gravidity in these species is lacking. For *E. jasperi*, such information may never be gained as the species may be extinct (Wake & Dickie, 1998).

2.2.3. Uterus of Salamandra

Greven (2003) recognized two types of viviparity in salamanders: larviparity, in which the female gives birth to true larvae, and pueriparity, in which the species gives birth to transformed young. The salamanders concerned are all in the European/Asian genus *Salamandra* (Salamandridae) and, depending upon one's preference in taxonomy, four to eight species (or more) are involved. Some of these are variably larviparous or pueriparous. Nutritive support after consumption of the initial yolk stores includes oophagy, adelphophagy, and epitheliophagy (Greven, 2003). Oophagy and adelphophagy are both forms of embryonic cannibalism, with the former involving ingestion of degrading, unfertilized eggs and the latter the consumption of smaller siblings. Epitheliophagy is the ingestion of the uterine epithelial lining from a proliferating zone called the zona trophica (Greven, 2003). Gestation time varies depending upon species and climatic conditions and ranges from five to fourteen months in *S. salamandra* and up to four years in *Salamandra atra* and *Salamandra lanzai* (Greven, 2003).

Knowledge of the hormonal influences on pregnancy in salamanders is limited. Greven and Guex (1994) reported that application of an estrogen increased secretory activity in the oviduct of *S. atra*. Histology, histochemistry, and ultrastructure have provided evidence for corpora lutea during the gestation period, but these may become reduced late in pregnancy, and embryos remain

alive and develop after ovariectomy (Greven, 2003). Pregnant females of *S. salamandra* injected intramuscularly with large doses of AVT gave birth to their young but this was difficult to achieve in *S. atra* (Heller, Ferreri, & Leathers, 1970). Birth of young in *S. salamandra*, however, occurs over several days whereas two weeks may pass before parturition of the second of two offspring by *S. atra*. Much remains to be learned about viviparity in salamanders.

2.2.4. Uterus of caecilians

Wake (1977) estimated that 50% of the 170 known caecilian species are viviparous, and, in two families (Scolecomorphidae (six species) from east Africa and Typhlonectidae (twelve species) from South America), all species are viviparous. All live-bearing caecilians provide maternal nutrition to the developing young via a secretory epithelium that is orally ingested by the fetuses (Wake, 2006). In addition, the highly elaborated gills of embryonic *Typhlonectes compressicauda* form placental-like attachments with the uterine epithelium for nutrient uptake (Exbrayat & Hraoui-Bloquet, 2006). Finally, intra-uterine cannibalism of eggs and/or embryos has been reported for *T. compressicauda* (Exbrayat & Hraoui-Bloquet, 2006). Exbryat and Delsol (1988) presented morphological evidence that corpora lutea persist through the pregnancy of *T. compressicauda*, and proposed that P_4 was involved in maintaining gestation. Otherwise, little is known about hormonal influences on viviparity in caecilians.

2.3. Penis of *Ascaphus*

As indicated in Section 2.3.1, *Ascaphus truei* and its sibling species *A. montanus* are phylogenetically basal frogs that practice internal fertilization. These frogs are the only anurans that truly can be said to engage in copulation while in amplexus (termed 'copulexus' by Sever et al. (2001)), and the only anamniotes to possess a 'true penis;' i.e., a copulatory organ in which cavernous tissue becomes engorged with blood during erection (Sever et al., 2003). The penis has been traditionally referred to as a 'tail,' but it has no relationship to the caudal vertebrae and is a fleshy extension of the cloaca. When engorged, the penis forms a sulcus for passage of sperm and is inserted in the cloaca of the female (Noble, 1925; Noble & Putnam, 1931; Slater, 1931). Nothing is known about hormonal influences on the penis of *Ascaphus*.

2.4. Cloacal Glands of Salamanders

The seven families of salamander comprising the traditional suborder Salamandroidea are unique among vertebrates in the possession of a distinct set of male cloacal glands that make spermatophores as well as female cloacal glands that store sperm (Sever, 1991a; 2003). The result is internal fertilization either in the oviduct (some salamandrids) or as eggs pass through the cloaca (rest of the species). The remaining three families of salamander reproduce by external fertilization and either lack cloacal glands (Sirenidae (Sever et al., 1996)) or generally possess just one type of cloacal gland that is similar in both males and females (Hynobiidae, Cryptobranchidae). These are the three basal families of urodeles (Larson, Weisrock, & Kozak, 2003; Wiens, Bonett, & Chippindale, 2005). Sever (1991b) proposed that cloacal glands of hynobiids and cryptobranchids function in pheromone production and that this is the ancestral function of cloacal glands.

Development of cloacal glands is controlled by sex hormones associated with maturation and sexual activity, and the glands are most hypertrophied during the breeding season (Norris & Moore, 1975; Norris, 1987; Iwata, Toyoda, Yamamoto, & Kikuyama, 2000; Kikuyama et al., 2000). Individual gland clusters are virtually unrecognizable prior to sexual maturity or in individuals collected outside the breeding season (Wilder, 1925; Williams, Martan, & Brandon, 1985; Sever 1994). Mature neotenic salamanders have cloacal gland clusters similar to those of metamorphosed salamanders (Licht & Sever 1991; Trauth, Sever, & Semlitsch, 1994; Krenz & Sever, 1995), except for neotenic hemidactyliines in which, the pheromone-producing glands are absent or reduced (Sever, 1985). Some relevant papers on the hormonal control of cloacal glands are presented below.

Benson (1965) in an unpublished dissertation studied the effects of various hormone treatments on the cloacal glands of *N. viridescens*. Evidence from castrates injected with androgens (T and dehydroepiandrosterone) and GTHs (chorionic gonadotropin (CG) and luteinizing hormone (LH)) indicates that the cloacal glands are stimulated directly by male hormones and only indirectly by GTHs. In addition, cloacal glands of castrates were found to be directly stimulated by three other steroidal hormones (estriol, P_4, and corticosterone), whereas a fourth, deoxycorticosterone acetate (DOCA), had no effect. In intact animals, the corticosteroid DOCA induced massive involution of the cloacal glands whereas estriol, P_4, and corticosterone caused only mild inhibition.

Norris and Moore (1975) conducted a study in which they injected immature male and female *A. tigrinum* larvae with T, E_2, P_4, $T + E_2$, $T + P_4$, or $E_2 + P_4$ on alternate days for 40 days. Only T treatment induced development of male-type cloacal glands. 17β-estradiol significantly reduced the response to T, and P_4 had no effect.

Sever (1980) injected the paedomorphic plethodontid *Eurycea tynerensis* with *l*-thyroxin pentahydrate to induce metamorphosis to study the effects on male cloacal glands. Individuals were also injected with T because they were collected while in a sexually inactive condition. Paedomorphic *E. tynerensis* lack one type of cloacal gland, called the vent gland, which arises from the cornified epidermis lining the posterior angle of the cloacal orifice. Larval salamanders lack the cornified epidermis found in metamorphosed salamanders. Although the epidermis changed to the metamorphosed condition in the *E. tynerensis* induced to metamorphose, vent glands did not appear, suggesting that *E. tynerensis* has lost the genetic ability for the development of vent glands. Other cloacal glands were similar in both paedomorphic and metamorphosed *E. tynerensis*, and similar to those of other species of *Eurycea*.

Sodefrin is a pheromone produced by the dorsal gland of the newt *C. pyrrhogaster* (Kikuyama et al., 1995; Toyoda et al., 1995). Sodefrin synthesis appears to be under the control of PRL and androgens because the amount of the pheromone increases following treatment with both and less markedly with androgens alone (Yamamoto, Toyoda, Tanaka, Hayashi, & Kikuyama, 1996). These hormones also affect the structural development of the dorsal gland and ventral gland. In hypophysectomized and/or castrated animals treated with prolactin and androgens and those injected with saline, the dorsal glands were similar to those of breeding and non-breeding males, respectively (Kikuyama et al., 1995). The same conditions probably apply to production of a similar pheromone, silefrin, from the dorsal gland of *Cynops ensicauda* (Yamamoto et al., 2000).

Little work has been done on hormonal influences on sperm storage in the female spermathecae, which can occur through periods when the ovary is quiescent and gonadal steroids are presumably at low levels. Hardy and Dent (1987) studied the effects of some neurotransmitters and hormones on the release of sperm from the spermatheca. They injected into the spermathecal region of females with either saline, saline plus acetylcholine, norepinephrine, AVT, or the prostaglandin $PGF_{2\alpha}$. The number of sperm present in the cloacae was highest in those females injected with $PGF_{2\alpha}$. Hardy and Dent proposed an active role for the spermathecal myoepithelium in the discharge of stored sperm and a role for $PGF_{2\alpha}$ in triggering that discharge.

2.5. Phallodeum of Caecilians

The phallodeum comprises the posterior wall of the male cloaca and is contained in a connective tissue capsule from which it is separated by a periphallodeal space (Exbryat & Estabel, 2006). Extrusion of the phallodeum during sexual activity involves eversion of the tubular structure so that the inner spinous epithelium becomes situated on the external face of the phallodeum (Tonutti, 1931). The linings of the phallodeum vary with the sexual cycle, but the hormonal influences on these changes have not been described.

3. SECONDARY SEXUAL CHARACTERISTICS

Secondary sexual characteristics are common in amphibians. Darwin reviewed secondary sexual characteristics in amphibians and noted that many develop during the breeding season (Darwin, 1871), thus implicating the role of sex steroids in their development. Shine (1979) summarized the patterns of sexual dimorphism in amphibians, particularly those associated with sexual selection. In this section we review the hormonal basis of these traits, which represent a small subset of all dimorphic characteristics.

There are other traits, however, that arise at sexual maturity and are not primary or secondary sexual characteristics. We introduce the term 'secondary characteristics' to refer to traits that arise at sexual maturity, yet that are not necessarily sexually dimorphic. Secondary characteristics include, as a subset, secondary sexual characteristics, which are traits that arise at sexual maturity and are sexually dimorphic. An example of a monomorphic secondary characteristic is the adult coloration of males and females of the African frog, *Hyperolius viridiflavis*. The adult phenotype is different from the juvenile color pattern, yet is not sexually dimorphic. In one of the few studies to address the hormonal basis of a monomorphic secondary characteristic, Hayes (1997) showed that both T and E_2 induce the adult color pattern in this sexually monochromatic frog. The Harderian gland in frogs is another example of a secondary characteristic; it is sexually dimorphic in mammals but is not dimorphic in frogs. The presence of androgen receptors in both males and females explains the lack of dimorphism in frogs (D'Istria et al., 1991; Chieffi-Baccari et al., 1993). Some secondary characteristics are behavioral; in some species of ranid frog, females as well as males produce mating vocalizations (Emerson & Boyd, 1999).

Although the examples above are not traditional secondary sexual characteristics because they are not sexually dimorphic, they do develop as a consequence of sexual maturation. By identifying these traits with a specific term, we acknowledge their derived status from the juvenile condition. Because of sex steroid differences in males and females, dimorphism or secondary sexual traits are predicted to develop at sexual maturity. Thus, the intriguing question with secondary characteristics is, 'Why are they *not* dimorphic?'. One potential hormonal mechanism for monomorphic secondary characteristics is high levels of androgens in females (Staub & De Beer, 1997).

3.1. Amphibian Skin

Because sex steroid receptors are present in the integument, D'Istria, Delrio, and Chieffi (1975) suggested that the entire integument of amphibians be considered a secondary characteristic. They collected adult males of *Rana esculenta* and *Triturus cristatus* in breeding condition, castrated them, and treated them with [^{3}H]-T, [^{3}H] E_2, [^{3}H]-estrone, or [^{3}H]-P_4. In *R. esculenta*, they found receptors for [^{3}H]-T whereas, in *T. cristatus*, only receptor sites for [^{3}H]-E_2 were observed. No morphological effects were described. In a later study, the thickness of the epidermis, height of granular cells, and diameter of mucous glands were strongly correlated with androgen levels in both males and females (D'Istria, Picilli, Basile, Delrio, & Chieffi, 1982).

The presence of multicellular mucous and serous (granular) exocrine glands in the dermis of metamorphosed skin is a synapomorphy for extant Amphibia (Sever & Houck, 1985). In addition, many amphibians have lipid glands and mixed mucous-serous glands in the dermis, plus specialized glands representing modified mucous or serous glands (Staub & Paladin, 1997; Brizzi, Delfino, & Jantra, 2003). These specialized glands include poison glands used in defense and breeding glands used in social communication and reproduction (Thomas, Tsang, & Licht, 1993; Brizzi et al., 2003; Sever, 2003).

3.2. Anuran Breeding Glands

Thomas et al. (1993) found sexually dimorphic skin glands in 14 frog species representing six families, and described the histology and histochemistry of these glands. The glands were apparently found only in males (although only female *Rana pipiens* were examined). In 12 species these breeding glands were multicellular alveolar glands that stained for neutral mucoproteins. In the other two species, the glands were similar to the protein-secreting granular glands. Specialized mucous glands on the dorsal surface of males only in several species of *Rana* are hypothesized to be courtship glands (Brizzi, Delfino, & Pellegrini, 2002). Thomas and Licht (1993) studied the effects of castration and T treatment on the dorsal skin glands of male *Xenopus laevis* and *R. pipiens* to see whether the dorsal breeding glands or any other dorsal skin glands were androgen-dependent. The dorsal skin glands of *X. laevis* were unaffected by T whereas breeding, mucous, and seromucous glands of *R. pipiens* all responded to androgen injections. T induced a significant increase in epithelial height in breeding glands but not in overall size. In castrated frogs, seromucous glands were abundant and breeding glands virtually absent whereas, in T-treated frogs, breeding glands were abundant and seromucous glands less common. Thomas and Licht (1993) suggested that seromucous glands may be the regressed form of breeding glands in the dorsal skin of *R. pipiens* and that the dorsal skin is overall a secondary sex characteristic, as suggested by D'Istria et al. (1975) for amphibians in general.

Brizzi et al. (2003) provide an excellent review of anuran breeding glands and report their occurrence in a wide variety of taxa, leading to the possibility that a close examination of virtually any species could reveal their presence. Except for nuptial excrescences ('thumb pads' or 'nuptial pads'), discussed below, these glandular areas often do not exhibit an obvious external manifestation, so no equally obvious androgen-dependent change can be determined without correlated histological studies. In those that have been done, however, androgens have been implicated in glandular hypertrophy. The extensive literature on the location of specialized male breeding glands includes abdominal, femoral, humeral, mental, pectoral, postaxillary, and ventrolateral glands as well as numerous 'not specifically defined' breeding glands (Brizzi et al., 2003). However, when females are studied as well, they also respond to seasonal variation in androgen levels (e.g., D'Istria et al., 1982). Research is needed to better understand the normal role of androgens in skin gland development in female amphibians.

3.2.1. Adhesive glands

Among the more unique types of breeding glands are adhesive glands in the pectoral region of certain male frogs that cause the venter of the male to adhere to the skin of the dorsum of the female during amplexus. Such glands were first reported in the Microhylidae for *Kaloula conjuncta* from the Philippines by Taylor (1920), who stated that males adhere to females by virtue of a slimy secretion from the belly. Inger (1954) later reported 'belly glands' from *Kaloula picta* and *Kaloula rigida*, and noted that they were absent in other members of the genus. Fitch (1956) described adhesion during amplexus of the North American microhylid *Gastrophryne olivacea* and, subsequently, Conaway and Metter (1967) described adhesive glands in *Gastrophryne carolinensis*. Adhesive glands are also known from male *Breviceps* in the south African family Brevicipitidae (Poynton, 1964; Visser, Cei, & Gutierrez, 1982). Because *Breviceps* is not closely related to microhylids, the adhesive glands in that taxon must have evolved independently (Siegel, Sever, Schriever, & Chabarria, 2008). Ultrastructurally, the adhesive glands of *Gastrophryne* appear to be modified mucous glands.

The adhesion of bisexual pairs during amplexus in these species has been proposed as an adaptation for protecting a female's backside from a rival male (Fitch, 1956), keeping pairs together in case of a mating disturbance (Fitch, 1956), helping a male with short arms to stay amplexed to his potential mate (Wager, 1965; Conaway &

Metter, 1967), or causing strong adherence for burrowing into a nesting chamber (Visser et al., 1982).

Metter and Conaway (1969) studied the effects of sex hormones on the development of adhesive glands in both *G. carolinensis* and *G. olivacea*. Pellets of 95% T proprionate and 5% petroleum jelly and pellets of estradiol benzoate (E_2-B) were inserted into dorsal lymph sacs of adult females and juveniles of both sexes, and injections of human CG (hCG) were given to adult females and juvenile males. In addition, adult males were castrated, and their breeding glands underwent full regression in 28 days, whereas mucous and granular glands were unaffected. Adhesive glands developed to an apparently functional state in females and juveniles treated with T after 28 days. 17β-estradiol-B caused no change in glands in any animal tested. Human CG caused some development of adhesive glands in juvenile males, but no treated juvenile males lived longer than 14 days. Human CG produced no effects on females. Metter and Conaway (1969) proposed that the adhesive glands are derived from mucous rather than granular glands, based upon the similarity of regressed and undeveloped adhesive glands to immature mucous glands.

3.3. Nuptial Excrescences of Anurans

A good review of hormonal influences on the glandular nuptial excrescences ('pads') occurring in many anurans is presented by Brizzi et al. (2003). These pads are modified patches of skin occurring on the fingers ('thumb pads'), hands, and/or forearms ('nuptial pads') of males in breeding condition. The pads are keratinizations of the skin superficial to large dermal mucous glands. Pads have been implicated in aiding the male to clasp the female during mating (Duellman & Trueb, 1986). Hypertrophy of the pads is associated with annual variation in testicular hormones, and the pads can be induced by treatment with androgens in adult males, adult females, and even tadpoles. For example, testosterone cypionate implants in *R. pipiens* stimulated thickening of the epidermis and dermis, formation of keratinized papillae, hypertrophy of gland epithelium, and accumulation of secretory product (Lynch & Blackburn, 1995). Androgen receptors in the nuptial pad of *Rana chensinensis* are positively correlated with development of these secondary sexual characteristics (Yang, Zhang, & Cui, 2005). Androgen receptor mRNA increases in the thumb pad (*R. esculenta*) following treatment with T (Varriale & Serino, 1994). An extensive literature exists on the effect of androgens on anuran nuptial pads, and Brizzi et al. (2003) give references for 15 species from six families. Duellman and Trueb (1987) state that nuptial excrescences are 'nearly universal' in frogs that have amplexus in water and are best developed in stream breeders. The normal role of estrogens in the development and maintenance of nuptial pads and other secondary sexual characteristics is less studied. One study does show that the combination of E_2 and T results in greater development of the thumb pad in *Rana nigromaculata* than treatment with T alone; injection with only E_2 had no effect on thumb pad development (Iwasawa & Kobayashi, 1974).

3.4. Tusks/Spines

Duellman and Trueb (1986) provide a review of spines and tusks present in males, and in some females, of many species of anuran. Spines can be prepollical or humeral and are used in male−male combat, although, in some species with spines, no male−male combat has been observed. Tusks on the lower jaw are also a secondary sexual characteristic and are believed to be used for defense of foam nests, for example. Relative to studies on nuptial excrescences, the hormonal basis of these traits is largely unknown.

3.5. Tail Fin and Nuptial Pads of Newts

During the breeding season, males of *N. viridescens* have increased tail fin height and possess black, keratinized nuptial pads on the hind legs. Females lack these characteristics, and a decline in tail fin height and loss of nuptial pads occurs in males at the end of the mating season or in breeding males conditioned to a laboratory environment of room temperature and long day lengths (Singhas & Dent, 1975).

Singhas and Dent (1975) performed a number of experiments on hormonal control of these characteristics. For tail height, decline was unaffected by thyroidectomy or administration of T on alternate days, but decline was slowed by autografting the pituitary gland and was halted by administration of PRL on alternate days. Tail height of laboratory-conditioned males was restored by administration of PRL over a period of 2−3 weeks; this response was reduced in hypophysectomized males but subsequently restored by additional treatment with thyroid hormone or thyroid-stimulating hormone (TSH). For nuptial pads, decline as in the tail fin was delayed by autografting of the pituitary and administration of prolactin, but in addition by thyroidectomy. T alone did not affect the loss of nuptial pads, but loss was prevented by the combination of T and PRL, and this combination caused restoration of pads in laboratory-conditioned males. In hypophysectomized, laboratory-conditioned males, thyroxine (T_4) or TSH was needed in addition to T and PRL to restore nuptial pads. Luteinizing hormone but not P_4 effectively substituted for T.

Thus, thyroid hormones play an important role in both tail height and nuptial pad presence, and seem to work synergistically with PRL and T. Singhas and Dent (1975) proposed that the function of thyroid hormone is a passive one and that it acts in a general way to promote the

wellbeing of the animal, perhaps through stimulating sloughing of the skin.

In a later study, Dent (1982) studied the interactions between T_4 and oxytocin (OXY) with PRL on the growth of tail fins in *N. viridescens*. Again, he found that physiological levels of T_4 did not antagonize the stimulatory effect of PRL on growth of the adult tail fin. Oxytocin, however, in doses of 100 μU antagonized the stimulatory action of 0.3 U of prolactin. Dent (1982) proposed that the antagonistic interaction between PRL and T_4 seen in larvae persists in adult urodeles when osmoregulatory action predominates and is lacking in situations in which the primary action of PRL is the stimulation of growth.

Adult male *Cynops* also possess a broader tail with a well-developed fin as compared to the female. Kikuyama et al. (1986) found that PRL increased tail height and that estrogens blocked the action of PRL in *C. pyrrhogaster* and *C. ensicauda*. The antagonism by estrogens may explain the sex difference in the tail morphology of *Cynops*.

3.6. Teeth of Plethodontids

Noble and Pope (1929) noted that the males of 'most' plethodontids have premaxillary teeth that are elongate, monocuspid, and directed more or less forward. These teeth are used in the 'vaccination' mode of pheromone delivery during courtship (Houck & Arnold, 2003). The male abrades the female's skin with his premaxillary teeth and rubs secretions from his mental gland into the scraped site. Because this mode of pheromone delivery exists in some members in all lineages of plethodontids, vaccination is considered the ancestral condition in plethodontids. In *Desmognathus* spp., such teeth can develop in an adult female if a testis is transplanted into the body (Noble & Pope, 1929). Sever (unpublished) induced development of enlarged premaxillary teeth, mental glands, and cirri in females of *Eurycea quadridigitata* injected with T enanthate.

Molecular techniques allow a more mechanistic description of how these derived monocuspid premaxillary teeth in plethodontids arise. Immunohistological studies on the plethodontid salamander *Bolitoglossa schizodactyla* show that different dental lamina have differential expression of androgen receptors; androgen receptor expression is limited to the premaxillary lamina (Ehmcke, Wistuba, Clemen, & Schlatt, 2003). The distribution of androgen receptors explains the variation in tooth shape and size among the different tooth-bearing bones as well as explaining the sexual dimorphism in tooth type (Ehmcke et al., 2003).

3.7. Muscles

A wide body of literature demonstrates the androgenic basis of sexually dimorphic forelimb (e.g., Regnier & Herrera, 1993) and laryngeal muscles of anurans (e.g., Tobias et al., 1993). Kelley's pioneering work on the larynx of African clawed frogs (*X. laevis*) (see Kelley (1986) for a review of early work) has led to an understanding of the androgenic basis of dimorphism in behavior (e.g., Fischer & Kelley, 1991), axon number (e.g., Kelley & Dennison, 1990), and muscle tension, twitch type, and fiber recruitment (e.g., Sassoon et al., 1987; Tobias et al., 1993). Recent work on this model system shows that T can also explain sexual dimorphism in neuron size. When female *X. laevis* are treated with T, masculinization of laryngeal muscle and motoneuron size is complete after four weeks (Potter et al., 2005).

Another sexually dimorphic characteristic in *X. laevis* is the synapse strength at laryngeal muscles. Juveniles of both sexes have weak synapses and the strong synapse in laryngeal muscles of mature females is controlled by 17β-estradiol (Tobias & Kelley, 1995). Weak synapses are those in which the pre-synaptic neuron does not typically activate the post-synaptic neuron, and strong synapses are those in which the pre-synaptic neuron typically activates the post-synaptic neuron. 17β-estradiol exerts this effect via receptors in the laryngeal muscles (Wu, Tobias, & Kelley, 2003).

Forelimb muscles are often used by anurans during the breeding season to defend a territory and to grasp a female during amplexus, and some urodeles grasp females during amplexus as well. Sexual dimorphism in forelimb muscle mass is expected with these behaviors. For example, studies on the explosive breeder *Rana temporaria* show that many aspects of strength and stamina of the extensor carpi radialis muscle (used in clasping) show sexual dimorphism. Muscle length and mass were larger, muscle force was greater, and relaxation times slower in males than in females (Navas & James, 2007). With T injections, forelimb muscles hypertrophied independently of muscle innervation (Thibert, 1986). In *R. pipiens*, T increased muscle mass in the 22 different muscles of the frog forelimb but did not change the water or protein content (Sidor & Blackburn, 1998). Immunocytochemistry on the flexor carpi radialis muscle in male *X. laevis* revealed that androgen receptors are present in all forelimb muscles and particularly in those muscles innervated by spinal nerve 2 (Dorlochter et al., 1994). These muscle fibers hypertrophied in response to T administration, whereas muscles innervated by different spinal nerves did not (Dorlochter et al., 1994). Further, studies of the effects of T on synapse efficiency found that T enhances characteristics critical for successful amplectic behavior, such as fatigue resistance (Nagaya & Herrera, 1995).

T can also affect the contractile properties of muscle. When trunk muscles (used for sound production in males) of post-breeding season male *Hyla chrysoscelis* were treated with T, muscle mass and contractile speed increased to levels found during the breeding season. In females, T

administration had the same effect, increasing the mass and contractile speed of the trunk muscles (Girgenrath & Marsh, 2003).

3.8. Vomeronasal Organ

A growing body of literature exists on the vomeronasal organ (VNO) in plethodontid salamanders, which is sexually dimorphic (e.g., Dawley, 1992). The VNO lies in the nasal capsule and detects chemical signals such as pheromones. In male *Plethodon shermani*, the VNO is 1.7 times larger in volume than that of females, despite males being smaller than females (Woodley, 2007). The hormonal basis of this sexual dimorphism is not yet understood. Androgen production at sexual maturity does not explain the sexual dimorphism because subadults show dimorphism as well (Woodley, 2007). In contrast, no dimorphism occurs in the volume of the main olfactory epithelium or in the muscles associated with the naris in *P. shermani* (Woodley, 2007). Little is known about the VNO of frogs and caecilians.

3.9. Color

Thirty-two species of anuran are known to be dimorphic in color and relatively little is known about the hormonal basis of color changes (Hoffman & Blouin, 2000). The handful of studies that do address this question show that coloration can be controlled by steroids. In the cricket frog, *Acris gryllus*, T implants caused male secondary sexual characteristics, such as yellow coloration and darkening of the vocal sac, to develop (Greenberg, 1942). T and P_4 administration to *R. pipiens* darkened the skin by dispersing melanosomes within melanophores (Himes & Hadley, 1971). In the reedfrog *Hyperolius argus*, mature males retain the solid green color of juveniles and develop a vocal sac, whereas mature females develop a reddish brown dorsum with white spots (Hayes & Menendez, 1999). In a study examining the effects of steroids in both primary and secondary sexual differentiation, T induced vocal sac development and E_2-induced dorsal color change in this species (Hayes & Menendez, 1999). In contrast, both T and E_2 induced the adult color pattern in the sexually monochromatic frog *H. viridiflavus* (Hayes, 1997).

3.10. Salamander Courtship Glands

Courtship glands in salamanders are sexually dimorphic glands of males that secrete pheromones to elicit a mating response from females. The glands may be modified mucous or granular glands or cloacal glands. Selected studies on courtship glands in the skin are discussed below.

3.10.1. Caudal glands in the middorsal tail base

Hypertrophy of granular and/or mucous glands in the middorsal tail base has been reported in a number of salamanders. In *Ambystoma gracile* (Ambystomatidae), enlarged granular glands along the tail ridge are not sexually dimorphic and their toxic secretions are used in defense (Brodie & Gibson, 1969). In *Ambystoma macodactylum*, however, glands in this area become hypertrophied in both males and females when used for nutrient storage (Williams & Larsen, 1986).

Hypertrophied granular glands in the middorsal tail base have been reported in both males and females of European plethodontid *Hydromantes* spp. (Brizzi, Calloni, & Delfino, 1991). These caudal glands in *Hydromantes* may be defensive because they do not vary seasonally (Brizzi et al., 1991). In some other plethodontids, glands in this area that hypertrophy during the breeding season have been reported only in males. These caudal courtship glands have been reported in species of *Desmognathus*, *Eurycea*, and *Plethodon* (Noble, 1929; 1931; Newman, 1954; Sever, 1989; Trauth, Smith, Cheng, & Daniel, 1993; Houck & Sever, 1994; Mary & Trauth, 2006) and probably occur widely in the Plethodontidae. Histochemically, the staining characteristics of caudal courtship glands are similar to mental glands, indicating the presence of a mucoprotein or glycoprotein (Sever, 1989; Trauth et al., 1993). However, caudal courtship glands cytologically resemble granular glands whereas mental glands are modified mucous glands (Sever 1976b; 1989).

The caudal courtship glands apparently function during courtship in the tail straddle walk, a synapomorphy for the Plethodontidae (Houck & Sever, 1994). During the tail straddle walk, the female's chin is placed directly over the caudal courtship glands, so gland secretions are received by the distal ends of the female's nasolabial grooves (Noble, 1929; Arnold, 1977). Caudal courtship pheromones presumably increase the likelihood that the female will remain with the male during tail straddling, and therefore increase the chances for insemination (Houck & Sever, 1994).

Males of the west Asian salamandrids *Mertensiella caucasica* and *Salamandra luschani* possess a dorsal tail tubercle that may be 3.5 mm long and 2.3 mm high in an animal of 65–70 mm snout-to-vent length (Sever, Sparreboom, & Schultschik, 1997). Thus, the dorsal tubercle is a grossly distinct structure and remains so independently of breeding condition (Klewen, 1988). Histological and molecular evidence suggest that the dorsal tail tubercles are not homologous in *M. caucasica* and *S. luschani* and result from convergent evolution. In *M. caucasica*, the tubercle consists primarily of elongate mucous glands, with granular glands occurring only at the base. In *S. luschani*, mucous glands and granular glands occur

throughout the tubercle. Female *S. luschani* have a slight tubercle as well (Staub, Palmer, Carnes, Quitiquit, & Susantio, 2005).

The role of the dorsal tubercle in courtship in the two species is similar. The dorsal tubercle acts as a pseudospermatophore that is inserted and then withdrawn when the female is about ready to pick up the real spermatophore (Rehberg, 1981; Klewen, 1988; Schultschik, 1994). Apart from a mechanical stimulus and maneuvering device, the caudal tubercle may additionally provide a specific chemical stimulus, since it contains glands with specific types of secretion (Sever et al., 1997).

Work on the hormonal control of the diverse caudal glands described above is sorely lacking.

3.10.2. Caudal glands in the midventral tail base

In the plethodontid *Aneides lugubris*, modified granular glands in this area are more hypertrophied in males than females (Staub & Paladin, 1997). The enlarged glands of males seem similar in appearance and histochemistry (e.g., positive with the periodic acid and Schiff's reagent procedure, PAS+) to middorsal caudal courtship glands, but their function is unknown (Staub & Paladin, 1997). In *Plethodon cinereus*, enlarged granular glands (PAS−) in this area are used in scent-marking and are not sexually dimorphic (Simons & Felgenhauer, 1992; Jaeger & Gabor, 1993). Once again, hormonal studies on these glands are needed.

3.10.3. Cloacal glands that secrete onto epidermal areas

The cloacal gland complexes were discussed in Section 2 but need to be mentioned here because the dorsal glands and vent glands of males of many taxa secrete onto the epidermis surrounding the posterior end of the cloaca rather than into the cloaca itself or in addition to sites within the cloaca (Sever, 1991a). In the Salamandridae, secretion onto the epidermis external to the cloaca is restricted to terrestrial breeders (Brizzi, Delfino, & Jantra, 1996). In the Plethodontidae, these glands are rather reduced compared to those in other families (Sever, 1994). Males of *Rhyacotriton olympicus* (Rhyacotritonidae) have greatly enlarged vent glands that secrete through pores lateral to the cloacal orifice (Sever, 1988). Specialized male dorsal or vent glands are secondarily lacking in some taxa, and this loss is often associated with neoteny or derived courtship patterns (Sever, 1991a; Brizzi, Calloni, Delfino, & Tanteri, 1995; Brizzi, Delfino, Rebelo, & Sever, 1999). As with other cloacal glands, these glands are controlled by gonadal steroid hormones.

3.10.4. Genial glands

Genial glands are an autapomorphy for the salamandrid genus *Notophthalmus* (Salamandridae). Genial glands are found at the base of three or four shallow invaginations ('pits') posterior to the eye (Hilton, 1902; Rogoff, 1927). The term 'genial' is from the Latin root *gena*, meaning the cheek or chin. At the base of the pits are numerous acinar glands consisting in the active condition of cuboidal epithelial cells that contain a colloidal eosinophilic substance.

Pool and Dent (1977) studied the hormonal control of the genial glands of *N. viridescens*. Both males and females have genial glands, which are similar in structure and secretory activity, but females only have about a third as many glands as males. In other species in the genus (*Notophthalmus meridionalis* and *Notophthalmus perstriatus*), genial glands have only been reported in males (Mecham, 1967; 1968). When newts are in breeding condition, the genial glands produce large quantities of a glycoproteinaceous product, and secretory cells become quiescent during the non-breeding season (summer).

In gonadectomized male newts with quiescent glands, the glands remain unchanged after treatment with saline, prolactin, or T, but are transformed to the breeding state by treatment with PRL and T in combination (Pool & Dent, 1977). Genial glands from ovariectomized females respond similarly to PRL in combination with T, but fail to respond to PRL with E_2. Thus, females also require a steroid plus PRL to induce hypertrophy of the genial glands, but that steroid is not E_2. Pool and Dent (1977) proposed that the T used in their experiments served as a substrate for formation of a steroid that is active in the female newt. Perhaps estrogens in a form other than E_2 could be as effective as T in stimulating the activity of female genial glands.

3.10.5. Mental glands

Sexually dimorphic chin glands have been reported in males of one species in the Salamandridae and occur widely in the Plethodontidae. During courtship in the salamandrid *T. torosa*, the male restrains the female by dorsal amplexus, during which he clasps the female with both his forelimbs and hindlimbs. The male slides forward so that his submandibular area can be rubbed across the female's snout (Davis & Twitty, 1964; Arnold, 1972). According to Smith (1941), the male is applying substances from specialized skin glands that can be distinguished from mucous and granular glands by the nature of their secretions. These specialized submandibular glands are absent in females of *T. torosa*. Smith (1941) speculated that males of *Taricha rivularis* would also have specialized chin glands because of similarities in amplectic behavior with *T. torosa*.

Outside of the breeding season, the specialized courtship glands are difficult to distinguish from mucous or granular glands (Smith, 1941). Although Smith's (1941) observations of unique submandibular glands in male *Taricha* are interesting, they have not subsequently been verified. If such glands exist, they certainly have been independently derived within the genus.

Specialized chin glands occur in males of many of the Plethodontidae but are lacking in some representatives of each clade (Houck & Sever, 1994). These glands are often referred to in the older literature as 'mental hedonic glands' (see Sever (1976a) for a review). Because the designation 'hedonic' refers to pleasure-giving, Arnold (1977) suggested avoidance of the judgmental term, and these chin glands are now referred to as 'mental courtship glands' or simply 'mental glands' (Houck & Sever, 1994), or sometimes as 'submandibular glands' (Rollmann, Houck, & Feldhoff, 1999). The term 'mental' is from the Latin *mentum*, meaning the chin. The first good anatomical study of these glands was by Noble (1927), and notable subsequent studies include those by Weichert (1945), Truffelli (1954), Lanza (1959), and Sever (1976a).

Generally, the glands are either in a pad of short, dorsoventrally oriented tubules that secrete over a small to large area of the skin of the lower jaw, or the glands form a fan-shaped cluster, antero-posteriorly oriented, of short (desmognathines) or long (many *Eurycea* and *Oedipina*) tubules that secrete at the apex of the lower jaw. In those species in which the nature of the secretions has been analyzed, the secretions have been shown to contain glycoproteins (Houck & Sever 1994; Feldhoff, Rollmann, & Houck, 1999). In *P. shermani*, a 22-kd protein called plethodontid receptive factor (PRF) has been isolated and shown to increase female receptivity during courtship (Rollman et al., 1999). Variation in PRF among populations of *P. shermani* has been demonstrated (Rollmann, Houck, & Feldhoff, 2000).

Sever (1976b) reported that cirri and mental glands can develop in female *E. quadridigitata* injected 2−4 times with 0.1 ml T enanthate over 28 days. The individual glands of the mental glands that formed on the females were larger in size but fewer in number than those found in males of similar size. The induced mental glands were derived from dermal mucous glands, the only glands otherwise found in the dermis of the lower jaw of females.

3.10.6. Nasolabial glands (cirri)

Members of the Plethodontidae possess clusters of glands in the lateral nasal region that are distinct from the more medial intermaxillary glands (Whipple 1906; Seifert 1937). These glands are called the nasolabial glands and they secrete alongside a groove that passes from the external naris to the edge of the upper lip. The nasolabial glands and the nasolabial groove are autapomorphies for the Plethodontidae. When a plethodontid taps its snout to the substrate, capillary action transfers aqueous odor solutions from the tip of the labia into the nasal cavity, where the solutions are delivered to the VNO (Brown 1968; Dawley 1998).

During the breeding season in males of many plethodontid species, hypertrophy of the nasolabial glands results in protrusions below the upper lip called cirri. A cirrus, therefore, is composed of hypertrophied nasolabial glands and incorporates the nasolabial groove (Sever, 1975; 1980). In some *Eurycea*, the growth into elongate cirri is especially prominent. The differential hypertrophy of nasolabial glands in males is thought to aid in the detection of pheromones used to locate potential mates (Sever 1980; Dawley 1992; Dawley & Crowder, 1995). For hormonal control, see Section 3.10.5: Sever (1976) induced growth of mental glands and cirri in females of *E. quadridigitata* by 2−4 injections of 0.1 ml T enanthate over 28 days. As in males, the nasolabial groove continued onto the naris. The only difference in external appearance of the cirri of males and the induced cirri of females was that the cirri of females were usually wider than those of males.

3.10.7. Skin glands of Taricha

In male *Taricha* (Salamandridae), an overall cutaneous hypertrophy occurs during the aquatic breeding stage (Halliday, 1998). Whether just one type of skin gland or all types are involved has not been determined. In *Hynobius nigrescens* (Hynobiidae), cutaneous hypertrophy associated with aquatic breeding occurs in both males and females and apparently involves only the serous glands (Hasumi & Iwasawa, 1990). The structural changes from the terrestrial type skin to the aquatic type skin are induced by PRL (Dent, 1975).

4. FUTURE WORK

As indicated in our preceding review, we know little or nothing about hormonal involvement in the development and function of various sex accessory structures (i.e., oviductal sperm storage tubules of *Ascaphus*, the uterus of viviparous amphibians, the penis of *Ascaphus*, etc.) and secondary sexual characteristics (i.e., tusks and spines, the VNO, various salamander courtship glands, etc.). Thus, much basic research is still needed, and, indeed, we cannot claim that hormonal control of a sex accessory structure or secondary characteristic of any amphibian has been completely elucidated.

Conducting studies in a phylogenetic context is important to fully understand the role of hormonal

mechanisms in the evolution of sex accessory structures and secondary characteristics. For example, a group of southeast Asian frogs (e.g., the *Rana blythi* species group) have evolutionarily lost many of the typical anuran secondary sexual characteristics; they have no vocal sacs, no advertisement call, no enlarged forelimbs, and no nuptial pads (Emerson, Rowsemitt, & Hess, 1993; Emerson, 1996). These changes are associated with low androgen levels that seem to have allowed the evolution of derived male parental care. As another example, Hayes (1997) argues that the difference in color pattern between two species of *Hyperolius* (one being sexually dimorphic and the other not) is a result of constraints due to the underlying hormonal basis of color change in these two species. We encourage the use of phylogenies for interpreting the evolutionary context of variation in hormone action among species.

Mechanistic studies examining the role of steroids on nervous tissue during sexual maturation will help to explain the development of dimorphism and monomorphism in secondary characteristics. The rapid (non-genomic) effects of steroids and their potential influence on secondary characteristics have not been studied. For example, E_2 affects ion transport in frog skin (Harvey, Alzamora, Healy, Renard, & Doolan, 2002) and this could potentially have dimorphic consequences.

Especially missing from this review are studies on the hormonal control of the sex accessory structures and secondary characteristics of caecilians. Head size dimorphism (Teodecki, Brodie, Formanowicz, & Nussbaum, 2008) and sexual differences in growth rates (Kupfer, Kramer, & Himstedt, 2004) have been reported, but the hormonal bases of these traits are not known.

Finally, more studies are needed that investigate the role of estrogens in males and androgens in females, and the synergistic effects of these hormones on secondary characteristics in both sexes. Exploring the hormonal basis of secondary sexual characteristics and more generally of secondary characteristics will prove to be a rich and rewarding enterprise.

ABBREVIATIONS

AVT Arginine vasotocin
B Corticosterone
CG Chorionic gonadotropin
DDE Dichlorodiphenyldichloroethylene
DDT Dichlorodiphenyltrichloroethane
DHEA Dehydroepiandrosterone
DHT 5α-Dihydrotestosterone
DOCA Deoxycorticosterone acetate
E_2 17β-estradiol
GnRH Gonadotropin-releasing hormone
GTH Gonadotropins
hCG Human chorionic gonadotropin
LH Luteinizing hormone
MIH Müllerian-inhibiting hormone
OXY Oxytocin
P_4 Progesterone
$PGF_{2\alpha}$ Prostaglandin $F_{2\alpha}$
PRF Plethodontid receptive factor
PRL Prolactin
T Testosterone
TSH Thyrotropin
VNO Vomeronasal organ

REFERENCES

Akbarsha, M. A., Jancy, G. M., Smita, M., & Oommen, O. V. (2006). Caecilian male Müllerian gland, with special reference *to Uraeotyphlus narayani*. In J.-M. Exbrayat (Ed.), *"Reproductive Biology and Phylogeny of Gymnophiona (Caecilians)"* (pp. 157–182). Enfield, NH: Science Publishers Inc.

Arnold, S. J. (1972). *The Evolution of Courtship Behavior in Salamanders*. Ann Arbor: University of Michigan. Ph.D. Dissertation.

Arnold, S. J. (1977). The evolution of courtship behavior in New World salamanders with some comments on Old World salamanders. In D. H. Taylor, & S. I. Guttman (Eds.), *"The Reproductive Biology of Amphibians"* (pp. 141–183). New York, NY: Plenum Press.

Benson, D. G. (1965). *Chemical and Morphological Changes in the Cloacal Glands of the Newt, Tritus viridescens, as induced by Harmonal Alterations*. Charlohesville: University of Virginia. Ph.D. Dissertation.

Boisseau, C. (1975). Étude ultrastructurale de l'oviducte du triton *Pleurodeles waltl* Michah. III. Actgion de l'estradiol 17β sur la différenciation des cellules sécrétrices de l'oviducte moyen de la femelle immature. *Gen. Comp. Endocrinol., 27*, 430–449.

Brizzi, R., Calloni, C., & Delfino, G. (1991). Tail base glands in European plethodontid salamanders with some comments on their biological and phylogenetic significance. *Amphibia-Reptilia, 12*, 357–372.

Brizzi, R., Delfino, G., & Jantra, S. (1996). Comparative anatomy and evolution of the male dorsal glands in the Salamandridae (Amphibia, Urodela). *Acta. Biologie. Benrodis., 8*, 61–77.

Brizzi, R., Delfino, G., & Jantra, S. (2003). An overview of breeding glands. In B. G. M. Jamieson (Ed.), *"Reproductive Biology and Phylogeny of Anura"* (pp. 253–317). Enfield, NH: Science Publishers, Inc.

Brizzi, R., Delfino, G., & Pellegrini, R. (2002). Specialized mucous glands and their possible adaptive role in the males of some species of *Rana* (Amphibia, Anura). *J. Morphol., 254*, 328–341.

Brizzi, R., Calloni, C., Delfino, G., & Tanteri, G. (1995). Notes on the male cloacal anatomy and reproductive biology of *Euproctus montanus* (Amphibia: Salamandridae). *Herpetologica, 51*, 98–118.

Brizzi, R., Delfino, G., Rebelo, R., & Sever, D. M. (1999). The male cloaca of *Chioglossa lusitanica* lacks dorsal glands: The possible correlation with the courtship mode. *J. Herpetol., 33*, 220–228.

Brodie, E. D., Jr., & Gibson, L. S. (1969). Defensive behavior and skin glands of the northwestern salamander, *Ambystoma gracile*. *Herpetologica, 25*, 187–194.

Brown, C. W. (1968). Additional observations on the function of the nasolabial groove of plethodontid salamanders. *Physiol. Zool., 39*, 357–367.

Chieffi-Baccari, G., D'Matteo, L., D'Istria, M., Minucci, S., Serino, I., & Varriale, B. (1993). The effects of gonadectomy and testosterone treatment on the Harderian gland of the green frog, *Rana esculenta*. *Cell. Tissue. Res., 273*, 201–208.

Clark, E. J., Norris, D. O., & Jones, R. E. (1995). Interactions of gonadal steroids and pesticides (DDT, DDE) on gonaduct growth in larval tiger salamanders, *Ambystoma tigrinum*. *Gen. Comp. Endocrinol., 109*, 94–105.

Conaway, C. H., & Metter, D. E. (1967). Skin glands associated with breeding in *Microhyla carolinensis*. *Copeia, 1967*, 672–673.

Darwin, C. (1871). *The Descent of Man and Selection in Relation to Sex*. New York, NY: Modern Library, Random House.

Davis, W. C., & Twitty, V. C. (1964). Courtship behavior and reproductive isolation in the species of *Taricha* (Amphibia, Caudata). *Copeia, 1964*, 601–610.

Dawley, E. M. (1992). Sexual dimorphism in a chemosensory system: the role of the vomeronasal organ in salamander reproductive behavior. *Copeia, 1992*, 113–120.

Dawley, E. M. (1998). Olfaction. In H. Heatwole, & E. M. Dawley (Eds.), *"Amphibian Biology", Vol. 3* (pp. 711–742). Australia: Chipping Norton: Surrey Beatty and Sons, Sensory Perception.

Dawley, E. M., & Crowder, J. (1995). Sexual and seasonal differences in the vomeronasal epithelium of the red-backed salamanders (*Plethodon cinereus*). *J. f. Comp. Neurol., 359*, 382–390.

Dent, J. N. (1975). Integumentary effects of prolactin in the lower vertebrates. *Am. Zool., 15*, 923–935.

Dent, J. N. (1982). On the interaction of thyroxine and of oxytocin with prolactin on the growth of the tail fin in the red-spotted newt. *Gen. Comp. Endocrinol., 47*, 269–275.

Deviche, P., Propper, C. R., & Moore, F. L. (1990). Neuroendocrine, behavioral, and morphological changes associated with the termination of the reproductive period in a natural population of male rough-skinned newts (*Taricha granulosa*). *Horm. Behav., 24*, 284–300.

D'Istria, M., Delrio, G., & Chieffi, G. (1975). Receptors for sex hormones in the skin of Amphibia. *Gen. Comp. Endocrinol., 26*, 281–283.

D'Istria, M., Picilli, A., Basile, C., Delrio, G., & Chieffi, G. (1982). Morphology and biochemical variations in the skin of *Rana esculenta* during the annual cycle. *Gen. Comp. Endocrinol., 48*, 20–24.

D'Istria, M., Chieffi-Baccari, G., D'Matteo, L., Minucci, S., Varriale, B., & Chieffi, G. (1991). Androgen receptor in the Harderian gland of *Rana esculenta*. *J. Endocrinol., 129*, 227–232.

Dorlöchter, M., Astrow, S. H., & Herreta, A. A. (1994). Effects of testosterone on a sexually dimorphic frog muscle: Repeated *in vivo* observations and androgen receptor distribution. *J. Neurobiol., 25*, 897–916.

Duellman, W. E., & Trueb, L. (1986). *Biology of Amphibians*. New York, NY: McGraw-Hill Book Co.

Ehmcke, J., Wistuba, J., Clemen, G., & Schlatt, S. (2003). Targeted expression of androgen receptors in tooth-forming tissues of a neotropical salamander (*Bolitoglossa schizodactyla*) enables region-specific formation of dimorphic types of teeth in the male. *Gen. Comp. Endocrinol., 134*, 26–35.

Emerson, S. B. (1996). Phylogenies and physiological processes—The evolution of sexual dimorphism in southeast Asian frogs. *Systematic Biology, 45*, 278–289.

Emerson, S. B., & Boyd, S. K. (1999). Mating vocalizations of female frogs: Control and evolutionary mechanisms. *Brain Behav. Evol., 53*, 187–197.

Emerson, S. B., Rowsemitt, C. N., & Hess, D. L. (1993). Androgen levels in a Bornean voiceless frog, *Rana blythi*. *Can. J. Zool., 71*, 196–203.

Exbrayat, J.-M. (2006). Endocrinology of reproduction in Gymnophiona. In J.-M. Exbrayat (Ed.), *"Reproductive Biology and Phylogeny of Gymnophiona (Caecilians)"* (pp. 183–230). Enfield, NH: Science Publishers, Inc.

Exbrayat, J.-M., & Delsol, M. (1988). Oviparité et développement inter-utérin chez les Gymnophiones. *Bull. Soc. Herp. Fr., 45*, 27–36.

Exbrayat, J.-M., & Estabel, J. (2006). Anatomy with particular reference to the reproductive system. In J.-M. Exbrayat (Ed.), *"Reproductive Biology and Phylogeny of Gymnophiona (Caecilians)"* (pp. 79–155). Enfield, NH: Science Publishers, Inc.

Exbrayat, J.-M., & Hraoui-Bloquet, S. (2006). Viviparity in *Typhlonectes compressicauda*. In J.-M. Exbrayat (Ed.), *"Reproductive Biology and Phylogeny of Gymnophiona (Caecilians)"* (pp. 325–357). Enfield, NH: Science Publishers, Inc.

Feldhoff, R. C., Rollmann, S. M., & Houck, L. D. (1999). Chemical analysis of courtship pheromones in a plethodontid salamander. In R. E. Johnson, D. Müller-Schwarze, & P. W. Sorensen (Eds.), *"Advances in Chemical Signals in Vertebrates"* (pp. 117–125). New York, NY: Kluwer Academic/Plenum Publishers.

Fernández, S. N., & Ramos, I. (2003). Endocrinology of reproduction. In B. G. M. Jamieson (Ed.), *"Reproductive Biology and Phylogeny of Anura"* (pp. 74–111). Enfield, NH: Science Publishers, Inc., 7.

Fernández, S. N., Mansilla, C., & Miceli, D. C. (1984). Hormonal regulation of an oviducal protein involved in *Bufo arenarum* fertilization. *Comp. Biochem. Physiol., 78A*, 147–152.

Fischer, L. M., & Kelley, D. B. (1991). Androgen receptor expression and sexual differentiation of effectors for courtship song in *Xenopus laevis*. *Neurosciences, 3*, 469–480.

Fitch, H. S. (1956). A field study of the Kansas ant-eating frog, *Gastrophryne olivacea*. *Univ. Kans. Publ. Mus. Nat. Hist., 8*, 275–306.

Frost, D. R., Grant, T., Faivovich, J., Bain, R. H., Hass, A., Haddad, C. F. B., et al. (2006). The amphibian tree of life. *Bull. Am. Mus. Nat. Hist., 297*, 1–370.

Girgenrath, M., & March, R. K. (2003). Season and testosterone affect contractile properties of fast calling muscles in the gray tree frog *Hyla chrysoscelis*. *Am. J. Physiol. Regul. Integr. Comp. Physiol., 284*, R1513–R1520.

Greenberg, B. (1942). Some effects of testosterone on the sexual pigmentation and other sex characters of the cricket frog (*Acris gryllus*). *J. Exp. Zool., 91*, 435–451.

Greven, H. (2003). Larviparity and pueriparity. In D. M. Sever (Ed.), *"Reproductive Biology and Phylogeny of Urodela"* (pp. 447–475). Enfield, NH: Science Publishers, Inc.

Greven, H., & Guex, G.-D. (1994). Structural and physiological aspects of viviparity in *Salamandra salamandra*. In H. Greven, & B. Thiesmeier (Eds.), *"Biology of Salamandra and Mertensiella"* (pp. 139–160). Bonn, Germany: Mertensiella Supplement 4, DGHT.

Halliday, T. (1998). Sperm competition in amphibians. In T. R. Birkhead, & A. P. Moeller (Eds.), *"Sperm Competition and Sexual Selection"* (pp. 465–502). London: Academic Press.

Hardy, M. P., & Dent, J. N. (1987). Hormonal facilitation in the release of sperm from the spermatheca of the red-spotted newt. *Experientia, 15*, 302–304.

Harvey, B. J., Alzamora, R., Healy, V., Renard, C., & Doolan, C. M. (2002). Rapid responses to steroid hormones: from frog skin to human colon. A homage to Hans Ussing. *Biochim. Biophys. Acta., 1556*, 116–128.

Hasumi, M., & Iwasawa, H. (1990). Seasonal changes in body shape and mass in the salamander, *Hynobius nigrescens*. *J. Herpetol., 24*, 113–118.

Hayes, T. B. (1997). Hormonal mechanisms as potential constraints on evolution: Examples from the Anura. *Am. Zool., 37*, 482−490.

Hayes, T. B., & Menendez, K. P. (1999). The effect of sex steroids on primary and secondary sex differentiation in the sexually dichromatic reedfrog (*Hyperolius argus*: Hyperolidae) from the Arabuko Sokoke Forest of Kenya. *Gen. Comp. Endocrinol., 115*, 188−199.

Heller, H., Ferreri, E., & Leathers, D. H. G. (1970). The effect of neurohypophysial hormones on the amphibian oviduct *in vitro*, with some remarks on the histology of this organ. *J. Endocrinol., 47*, 495−509.

Hilton, W. A. (1902). A structural feature connected with the mating of *Diemyctylus viridescens*. *Am. Nat., 34*, 643−651.

Himes, P. J., & Hadley, M. E. (1971). *In vitro* effects of steroid hormones on frog melanophores. *J. Invest. Dermatol., 57*, 337−342.

Hoffman, E. A., & Blouin, M. S. (2000). A review of colour and pattern polymorphisms in anurans. *Biol. J. Linn. Soc., 70*, 633−665.

Houck, L. D., & Arnold, S. J. (2003). Courtship and mating behaviour. In D. M. Sever (Ed.), *"Reproductive Biology and Phylogeny of Urodela"* (pp. 383−424). Enfield, NH: Science Publishers, Inc.

Houck, L. D., & Sever, D. M. (1994). Role of the skin in reproduction and behaviour. In H. Heatwole, & G. T. Barthalmus (Eds.), *"Amphibian Biology, Vol. 1: The Integument"* (pp. 351−381). Chipping Norton, Australia: Surrey Beatty and Sons.

Inger, R. I. (1954). Systematics and zoogeography of Phillippine Amphibia. *Fieldiana, Zool., 33*, 181−531.

Iwata, T., Toyoda, F., Yamamoto, K., & Kikuyama, S. (2000). Hormonal control of urodele reproductive behavior. *Comp. Biochem. Physiol. Part. B. Biochem. Mol. Biol., 26*, 221−229.

Iwasawa, H., & Kobayashi, M. (1974). Effects of testosterone and estradiol on the development of sexual characters in young *Rana nigromaculata*. *Biol. Reprod., 11*, 398−405.

Jaeger, R. G., & Gabor, C. R. (1993). Intraspecific chemical communication by a terrestrial salamander via the postcloacal gland. *Copeia, 1993*, 1171−1174.

Jego, P. (1977). Action différentielle de l'oestradiol sur les activités de la fucosyltransferase et de la galactosyltransférase de l'oviducte du triton Pleurodéle. *Gen. Comp. Endocrinol., 31*, 475−481.

Kelley, D. B. (1986). Neuroeffectors for vocalization in *Xenopus laevis*: hormonal regulation of sexual dimorphism. *J. Neurobiol., 17*, 231−248.

Kelley, D. B., & Dennison, J. (2004). The vocal motor neurons of *Xenopus laevis*. Development of sex differences in axon number. *J. Neurobiol., 21*, 869−882.

Klewen, R. (1988). *Die Landsalamander Europas, teil 1. Die Gattungen* Salamandra *und* Mertensiella*. Die Neue Brehm-Buecherei*. Germany: Wittensberg-Lutherstadt.

Kikuyama, S., Nakano, R., & Yasumasu, I. (1975). Synergistic action of prolactin and androgen on the cloacal glands of the newt. *Comp. Biochem. Physiol., 51*, 823−826.

Kikuyama, S., Seshimo, H., Shirama, K., Kato, T., & Noumura, T. (1986). Interaction of prolactin and sex steroids in the oviduct and tail of newts, *Cynops pyrrhogaster. Zool. Sci., 3*, 131−138.

Kikuyama, S., Tanaka, S., & Moore, F. (2003). Endocrinology of reproduction. In D. M. Sever (Ed.), *"Reproductive Biology and Phylogeny of Urodela"* (pp. 275−321). Enfield, NH: Science Publishers, Inc.

Kikuyama, S., Toyoda, F., Ohmiya, Y., Matsuda, K., Tanaka, S., & Hayashi, H. (1995). Sodefrin: A female-attracting peptide pheromone in newt cloacal glands. *Science, 267*, 1643−1645.

Kikuyama, S., Yazawa, T., Abe, S., Yamamoto, K., Iwata, T., Hoshi, K., et al. (2000). Newt prolactin and its involvement in reproduction. *Can. J. Pharmacol., 78*, 984−993.

Krenz, J. D., & Sever, D. M. (1995). Mating and oviposition in paedomorphic *Ambystoma talpoideum* precedes the arival of terrestrial males. *Herpetologica, 51*, 387−393.

Kupfer, A., Kramer, A., & Himstedt, W. (2004). Sex-related growth patterns in a caecilian amphibian (genus *Ichthyophis*): evidence from laboratory data. *J. Zool., 262*, 173−178.

Lamotte, M., & Xavier, F. (1972). Reserches sur le développement embryonnaire de *Nectophrynoides occidentalis* Angel, amphibian anoure vivipare. I. Les principaux traits morphologiques et biométriques du développement. *Ann. Emb. Morphol., 5*, 315−340.

Lanza, B. (1959). Il corpo ghiandolare mentoniero dei Plethodontidae (Amphibia, Caudata). *Monit. Zool. Ital., 67*, 15−53.

Larson, A., Weisrock, D. W., & Kozak, K. H. (2003). Phylogenetic systematics of salamanders (Amphibia: Urodela), a review. In D. M. Sever (Ed.), *"Reproductive Biology and Phylogeny of Urodela"* (pp. 31−108). Enfield, NH: Science Publishers, Inc.

Licht, L. E., & Sever, D. M. (1991). Cloacal anatomy of metamorphosed and neotenic salamanders. *Can. J. Zool., 69*, 2230−2233.

Lynch, L. C., & Blackburn, D. G. (1995). Effects of testosterone administration and gonadectomy on nuptial pad morphology in overwintering male leopard frogs, *Rana pipiens*. *Amphib.-Reptil., 16*, 113−121.

Mary, M. N., & Trauth, S. E. (2006). Histology and histochemistry of caudal courtship glands in three Arkansas plethodontid salamanders. *Herpetological Review, 37*, 280−284.

Mecham, J. S. (1967). *Notophthalmus perstriatus*. *Cat. Am. Amphib. Reptil., 38*, 1−2.

Mecham, J. S. (1968). *Notophthalmus meridionalis*. *Cat. Am. Amphib. Reptiles, 74*, 1−2.

Metter, D. E., & Conaway, C. H. (1969). The influence of hormones on the development of breeding glands in *Microhyla*. *Copeia, 1969*, 621−622.

Moore, F. L., & Deviche, P. (1988). Neuroendocrine processing of environmental information in amphibians. In M. H. Stetson (Ed.), *"Processing of Environmental Information in Vertebrates"* (pp. 19−45). New York, NY: Springer-Verlag.

Navas, C. A., & James, R. S. (2007). Sexual dimorphism of extensor carpi radialis muscle size, isometric force, relaxation rate and stamina during the breeding season of the frog Rana temporaria Liannaeus 1758. *J. Exper. Biol., 210*, 715−721.

Newman, W. R. (1954). A new plethodontid salamander from southwestern Virginia. *Herpetologica, 10*, 9−14.

Noble, G. K. (1925). An outline of the relation of ontogeny to phylogeny within the Amphibia I. *Am. Mus. Novit., 165*, 1−17.

Noble, G. K. (1927). The plethodontid salamanders, some aspects of their evolution. *Am. Mus. Novit., 360*, 1−5.

Noble, G. K. (1929). The relation of courtship to the secondary sexual characters of the two-lined salamander, *Eurycea bislineata* (Green). *Am. Mus. Novit., 362*, 1−5.

Noble, G. K. (1931). The hedonic glands of the plethodontid salamanders and their relation to the sex hormones. *Anat. Rec., 48*, 57−58, (abstract).

Noble, G. K., & Pope, S. H. (1929). The modification of the cloaca and teeth of the adult salamander, *Desmognathus*, by testicular transplants and by castration. *J. Exper. Biol., 6*, 399−411.

Noble, G. K., & Putnam, P. G. (1931). Observations on the life history of *Ascaphus truei* Stejneger. *Copeia, 1931*, 97−101.

Norris, D. O. (1987). Regulation of male gonaducts and sex accessory structures. In D. O. Norris, & R. E. Jones (Eds.), *"Hormones and Reproduction in Fishes, Amphibians, and Reptiles"* (pp. 327−354). New York, NY: Plenum Press.

Norris, D. O., & Moore, F. L. (1975). Antagonism of testosterone-induced cloacal development by estradiol-17B in immature larval tiger salamanders (*Ambystoma tigrinum*). *Herpetologica, 31*, 255−263.

Norris, D. O., Carr, J. A., Summers, C. H., & Featherston, R. (1997). Interactions of androgens and estradiol on sex accessory ducts of larval tiger salamanders, *Ambystoma tigrinum*. *Gen. Comp. Endocrinol., 106*, 348−355.

Norris, D. O., Norman, M. F., Pancak, M. K., & Duvall, D. (1985). Seasonal variations in spermatogenesis, testicular weights, vasa deferentia, and androgen levels in neotenic male tiger salamanders, *Ambystoma tigrinum*. *Gen. Comp. Endocrinol., 60*, 51−57.

Pool, T. B., & Dent, J. N. (1977). The ultrastructure and the hormonal control of product synthesis in the hedonic glands of the red-spotted newt *Notophthalmus viridescens*. *J. Exp. Zool., 201*, 177−202.

Polzonetti-Magni, A., Carnevali, O., Yamamoto, K., & Kikuyama, S. (1995). Growth hormone and prolactin in amphibian reproduction. *Zool. Sci., 12*, 683−694.

Potter, K. A., Bose, T. O., & Yamaguchi, A. (2005). Androgen-induced vocal transformation in adult female African calwed frogs. *J. Neurophysiol, 94*, 415−428.

Poynton, J. C. (1964). The Amphibia of southern Africa. *Ann Natal. Mus., 17*, 1−334.

Regnier, M., & Herrera, A. A. (1993). Differential sensitivity to androgens within a sexually dimorphic muscle of male frogs (*Xenopus laevis*). *J. Neurobiol., 24*, 1215−1228.

Rehberg, F. (1981). Nachtrag au "*Mertensiella luschani*, der Lykische salamander" in Herpetofauna 9, seite 16. *Herpetofauna, 10*, 23.

Rogoff, J. L. (1927). The hedonic glands of *Triturus viridescens*: a structural and morphological study. *Anat. Rec., 34*, 132−133, (abstract).

Rollmann, S. M., Houck, L. D., & Feldhoff, R. C. (1999). Proteinaceous pheromone affecting female receptivity in a terrestrial salamander. *Science, 285*, 1907−1909.

Rollmann, S. M., Houck, L. D., & Feldhoff, R. C. (2000). Population variation in salamander courtship pheromones. *J. Chem. Ecol., 26*, 2713−2724.

Sassoon, D. A., Gray, G. E., & Kelley, D. B. (1987). Androgen regulation of muscle fiber type in the sexually dimorphic larynx of *Xenopus laevis*. *J. Neurosci., 7*, 3198−3206.

Schultschik, G. (1994). Zur Fortpflanzungsbiologie von *Mertensiella caucasica* (Waga, 1876) (Urodela: Salamandridae). *Abhandlungen und Berichte für Naturkunde Magdeburg, 17*, 163−175.

Seifert, H. (1937). Untersuchungen űber die Műndhohlendrussen der urodelen Amphibien. *Gegenbaurs Morphologisches Jahrbuech, 70*, 173−216.

Sidor, C. A., & Blackburn, D. G. (1998). Effects of testosterone administration and castration on the forelimb musculature of male leopard frogs, *Rana pipiens*. *J. Exp. Zool. partA: Comp. Exp. Biol., 280*, 28−37.

Sever, D. M. (1975). Morphology and seasonal variation of the nasolabial glands of *Eurycea quadridigitata* (Holbrook). *J. Herpetol., 9*, 337−348.

Sever, D. M. (1976a). Morphology of the mental hedonic gland clusters of plethodontid salamanders (Amphibia: Caudata). *J. Herpetol., 10*, 222−239.

Sever, D. M. (1976b). Induction of secondary sexual characters in *Eurycea quadridigitata*. *Copeia, 1976*, 830−833.

Sever, D. M. (1980). Observations on the nasolabial groove of the plethodontid salamander *Eurycea quadridigitata*. *Proc. Indiana. Acad. Sci., 89*, 421−424.

Sever, D. M. (1985). Sexually dimorphic glands of *Eurycea nana, Eurycea neotenes* and *Typhlomolge rathbuni* (Amphibia: Plethodontidae). *Herpetologica, 41*, 71−84.

Sever, D. M. (1988). Male *Rhyacotriton olympicus* (Dicamptodontidae: Urodela) has a unique cloacal vent gland. *Herpetologica, 44*, 274−280.

Sever, D. M. (1989). Caudal hedonic glands in salamanders of the *Eurycea bislineata* complex (Amphibia: Plethodontidae). *Herpetologica, 45*, 322−329.

Sever, D. M. (1991a). Comparative anatomy and phylogeny of the cloacae of salamanders (Amphibia: Caudata). I. Evolution at the family level. *Herpetologica, 47*, 165−193.

Sever, D. M. (1991b). Comparative anatomy and phylogeny of the cloacae of salamanders (Amphibia: Caudata). II. Cryptobranchidae, Hynobiidae and Sirenidae. *J. Morphol., 207*, 283−301.

Sever, D. M. (1994). Comparative anatomy and phylogeny of the cloacae of salamanders (Amphibia: Caudata). VII. Plethodontidae. *Herpetological Monographs, 8*, 276−337.

Sever, D. M., & Houck, L. D. (1985). Spermatophore formation in *Desmognathus ochrophaeus*. *Copeia, 1985*, 394−402.

Sever, D. M., Rania, L. C., & Krenz, J. D. (1996). Reproduction of the salamander *Siren intermedia* Le Conte with especial reference to oviducal anatomy and evidence for sperm storage and internal fertilization. *J. Morphol., 227*, 335−348.

Sever, D. M., Sparreboom, M., & Schultschik, G. (1997). The dorsal tail tubercle of *Mertensiella caucasica* and *M. luschani* (Amphibia: Salamandridae). *J. Morphol., 232*, 93−105.

Sever, D. M., Moriarty, E. C., Rania, L. C., & Hamlett, W. C. (2001). Sperm storage in the oviduct of the internal fertilizing frog, *Ascaphus truei*. *J. Morphol., 248*, 1−21.

Sever, D. M., Hamlett, W. C., Slabach, R., Stephenson, B., & Verrell, P. A. (2003). Internal fertilization in the Anura with special reference to mating and female sperm storage in *Ascaphus*. In B. G. M. Jamieson (Ed.), *"Reproductive Biology and Phylogeny of Anura (Amphibia)"* (pp. 319−341). Enfield, NH: Science Publishers, Inc.

Shine, R. (1979). Sexual selection and sexual dimorphism in the Amphibia. *Copeia, 1979*, 297−306.

Sidor, C. A., & Blackburn, D. G. (1998). Effects of testosterone administration and castration on the forelimb musculature of male leopard frogs, *Rana pipiens*. *J. Exp. Zool. Part A: Comp. Exp. Biol.,* 280, 28−37.

Siegel, D. S., Sever, D. M., Schriever, T. A., & Chabarria, R. E. (2008). Ultrastructure and histochemistry of the adhesive breeding glands in male *Gastrophryne carolinensis* (Amphibia: Anura: Microhylidae). *Copeia, 2008*, 877−881.

Simons, R. R., & Felgenhauer, B. E. (1992). Identifying areas of chemical signal production in the red-backed salamander, *Plethodon cinereus*. *Copeia, 1992*, 776−781.

Singhas, C. A., & Dent, J. N. (1975). Hormonal control of the tail fin and of the nuptial pads in the male red-spotted newt. *Gen. Comp. Endocrinol, 26*, 382−393.

Slater, J. R. (1931). The mating of *Ascaphus truei* Stejneger. *Copeia, 1931*, 62−63.

Smith, R. E. (1941). Mating behavior in *Triturus torosus* and related newts. *Copeia, 1941*, 255−262.

Staub, N. L., & DeBeer, M. (1997). The role of androgens in female vertebrates: A review. Gen. Comp. *Endocrinol., 108*, 1−24.

Staub, N. L., & Paladin, J. (1997). The presence of modified granular glands in male and female *Aneides lugubris* (Amphibia: Plethodontidae). *Herpetologica, 53*, 339−344.

Staub, N. L., Palmer, C. A., Carnes, A., Quitiquit, C., & Susantio, M. (2005). A dorsal tail tubercle containing hypertrophied granular and mucous glands is present in female *Salamandra luschani* (Salamandridae). *Herpetologica, 61*, 415−421.

Taylor, E. H. (1920). Philippine Amphibia. *Philipp. J. Sci., 16*, 213−359.

Teodecki, E. E., Brodie, E. D., Jr., Formanowicz, D. R., Jr., & Nussbaum, R. A. (2008). Head dimorphism and burrowing speed in the African caecilian *Schistometopum thomense* (Amphibia: Gymnophiona). *Herpetologica, 54*, 154−160.

Thibert, P. (1986). Androgen sensitivity of skeletal muscle: Nondependence on the motor nerve in the frog forearm. *Exp. Neurol., 91*, 559−570.

Thomas, E. O., & Licht, P. (1993). Testicular and androgen dependence of skin gland morphology in the anurans, *Xenopus laevis* and *Rana pipiens*. *J. Morphol., 215*, 195−200.

Thomas, E. O., Tsang, L., & Licht, P. (1993). Comparative histochemistry of the sexually dimorphic skin glands of anuran amphibians. *Copeia, 1993*, 133−143.

Tobias, M. L., & Kelley, D. B. (1995). Sexual differentiation and hormonal regulation of the laryngeal synapse in *Xenopus laevis*. *J. Neurobiol., 28*, 515−526.

Tobias, M. L., Marin, M. L., & Kelley, D. B. (1993). The roles of sex, innervation, and androgen in laryngeal muscle of *Xenopus laevis*. *J. Neurosci., 13*, 324−333.

Tojio, Y., & Iwasawa, H. (1977). Effects of sex hormones on the development of gonads and sexual characters in young frogs of *Rana japonica*. *Zool. Mag., 86*, 20−28.

Tonutti, E. (1931). Beitrage zur Kenntnis der Gymnophionen. XV. Das Genital-system. *Morphologisches Jahrbuch, 68*, 151−292.

Toyoda, F., Hayashi, H., Ohmiya, Y., Tanaka, S., Mochida, H., Matsuda, K., et al. (1995). Une nouvelle phérome sexualle isolée á partir des glands abdominals du Triton male, *Cynops pyrrhogaster*. *Comptes Rendus des Séances de la Societé de Biologie, 189*, 1153−1148.

Toyoda, F., Tanaka, S., Matsuda, K., & Kikuyama, S. (1994). Hormonal control of response to and secretion of sex attractants in Japanese newts. *Physiol. Behav., 55*, 569−576.

Trauth, S. E., Sever, D. M., & Semlitsch, R. D. (1994). Female cloacal anatomy of paedomorphic *Ambystoma talpoideum* (Caudata: Ambystomatidae), with comments on intermorph mating and sperm storage. *Can. J. Zool., 72*, 2147−2157.

Trauth, S. E., Smith, R. D., Cheng, A., & Daniel, P. (1993). Caudal hedonic glands in the dark-sided salamander, *Eurycea longicauda melanopleura* (Urodela: Plethodontidae). *Proc. Arkansas. Acad. Sci., 47*, 151−153.

Truffelli, G. T. (1954). A macroscopic and microscopic study of the mental hedonic gland clusters of some plethodontid salamanders. *Univ. Kans. Sci. Bull., 36*, 1−39.

Uribe, M. C. A. (2001). Reproductive systems of caudate amphibians. In H. M. Dutta, & J. S. Data Munshi (Eds.), *"Vertebrate Functional Morphology. Horizon of Research in the 21st Century"* (pp. 267−293). Enfield, NH: Science Publishers, Inc.

Uribe Aranzábal, M. C. (2003). The testes, spermatogenesis, and male reproductive ducts. In D. M. Sever (Ed.), *"Reproductive Biology and Phylogeny of Urodela"* (pp. 183−202). Enfield, NH: Science Publishers, Inc.

Varriale, B., & Serino, I. (1994). The androgen receptor mRNA is up-regulated by testosterone in both the Harderian gland and thumb pad of the frog, *Rana esculenta*. *J. Steroid. Biochem. Mol. Biol., 51*, 259−265.

Vilter, V., & Lugand, A. (1959). Trophisme inter-utérin et croissance embryonnaire chez *Nectophrynoides occidentalis* Angel crapaud totalement vivipare du Mont Nimba (Haute-Guinée). *Comptes Rendus des Séances de la Societé de Biologie, 153*, 29−32.

Visser, J. J., Cei, J. M., & Gutierrez, L. S. (1982). The histology of dermal glands of mating *Breviceps* with comments on their possible functional value in microhylids (Amphibia:Anura). *South. Afr. J. Zool., 17*, 24−27.

Wager, V. A. (1965). *The Frogs of South Africa*. Capetown, South Africa: Purnell and Sons.

Wake, M. H. (1977). The reproductive biology of caecilians: an evolutionary perspective. In D. H. Taylor, & S. I. Guttman (Eds.), *"The Reproductive Biology of Amphibians"* (pp. 73−101). New York, NY: Plenum Press.

Wake, M. H. (1978). The reproductive biology of *Eleutherodactylus jasperi* (Amphibia, Anura, Leptodactylidae), with comments on the evolution of live-bearing systems. *J. Herpetol., 12*, 121−133.

Wake, M. H. (1980). The reproductive biology of *Nectophrynoides malcolmi* (Amphibia: Bufonidae), with comments on the evolution of reproductive modes in the genus Nectophrynoides. *Copeia, 1980*, 193−209.

Wake, M. H. (1981). Structure and function of the male Müllerian gland in caecilians (Amphibia: Gymnophiona), with comments on its evolutionary significance. *J. Herpetol., 15*, 17−22.

Wake, M. H. (1993). Evolution of oviductal gestation in amphibians. *J. Exp. Zool., 282,* 477−506.

Wake, M. H. (2006). A brief history of research on gymnophionan reproductive biology and development. In J.-M. Exbrayat (Ed.), *"Reproductive Biology and Phylogeny of Gymnophiona (Caecilians)"* (pp. 1−37). Enfield, NH: Science Publishers, Inc.

Wake, M. H., & Dickie, R. (1998). Oviduct structure and function and reproductive modes in amphibians. *J. Exp. Zool., 282*, 477−506.

Weichert, C. K. (1945). Seasonal variation in the mental gland and reproductive organs of the male *Eurycea bislineata*. *Copeia, 1945*, 78−84.

Whipple, I. L. (1906). The naso-labial groove of lungless salamanders. *Biol. Bull., 11*, 1−26.

Wiens, J. J., Bonett, R. M., & Chippindale, P. T. (2005). Ontogeny discombobulates phylogeny: paedomorphosis and higher-level salamander relationships. *Systematic Biology, 54*, 91−110.

Wilder, I. W. (1925). *The Morphology of Amphibian Metamorphosis*. Northampton, MA: Smith College.

Williams, T. A., & Larsen, J. H., Jr. (1986). New function for the granular skin glands of the eastern long-toed salamander, *Ambystoma macrodactylum columbianum*. *J. Exp. Zool., 239*, 329−333.

Williams, A. A., Martan, J., & Brandon, R. A. (1985). Male cloacal gland complexes of *Eurycea lucifuga* and *Eurycea longicauda* (Amphibia: Plethodontidae). *Herpetologica, 41*, 272−281.

Witschi, E. (1942). Hormonal regulation of development in lower vertebrates. Cold Spring Harbor Symp. *Quant. Biol., 10*, 35−39.

Woodley, S. K. (2007). Sex steroid hormones and sexual dimorphism of chemosensory structures in a terrestrial salamander (*Plethodon shermani*). *Brain Res., 1138*, 95−103.

Wu, K. H., Tobias, M. L., & Kelley, D. B. (2003). Estrogen receptor expression in laryngeal muscle in relation to estrogen-dependent increases in synapse strength. *Neuroendocrinol., 78*, 72−80.

Yamamoto, K., Kawai, Y., Hayashi, T., Ohe, Y., Hayashi, H., Toyoda, F., et al. (2000). Silefrin, a sodefrin-like pheromone in the abdominal gland of the sword-tailed newt, *Cynops ensicauda*. *Federation of European Biochemical Societies Letters, 472*, 267−270.

Yamamoto, K., Toyoda, F., Tanaka, S., Hayashi, H., & Kikuyama, S. (1996). Radioimmunoassay of a newt sex pheromone, sodefrin, and the influence of hormones on its level in the abdominal gland. *Gen. Comp. Endocrinol., 104*, 356−363.

Yang, C., Zhang, Y., & Cui, H. (2005). Correlation between the changes of microstructure and testosterone in nuptial pad of the frog, *Rana chensinensis*. *Zool. Res., 2005*, 638−644.

Chapter 6

Stress and Reproduction in Amphibians

James A. Carr
Texas Tech University, Lubbock, TX, USA

SUMMARY
The competing selective pressures of successfully reproducing and avoiding predation can have an impact on many aspects of an animal's behavior and physiology. At stake in this tradeoff is survival, as animals engaged in reproduction may deplete energy stores necessary for avoiding predation and may be more vulnerable to predation or pathogens while engaged in reproductive behavior. The impact of this tradeoff in shaping endocrine and neuronal pathways is seen clearly in amphibians. Exposure of amphibians to a wide variety of different stressors activates the hypothalamus–pituitary–adrenal (HPA) axis and elevates plasma levels of the glucocorticoid hormone corticosterone, which can act at multiple levels to influence gametogenesis, sex steroid synthesis, and reproductive behavior. Glucocorticoids are also required to supply nutrients for energetically costly reproductive behaviors such as calling and amplexus. This chapter will summarize recent developments in our understanding of the neural and endocrine pathways that mediate stressor effects on reproduction and the role of glucocorticoids in energy metabolism during reproduction in amphibians. Special attention is given to how multiple stressors related to changes in habitat quality may have an impact on reproduction and the health of amphibian populations.

1. INTRODUCTION

In the Panhandle and Southern Plains of West Texas there are more than 20 000 ephemeral ponds (playa lakes) that serve as breeding ponds for several species of amphibian. One commodity that is not in abundance in this part of the USA is rainfall, and, when heavy rains return each year for a brief period in late May and early June, the playas explode with breeding choruses of toads and frogs, including the New Mexico spadefoot toad (*Spea multiplicata*) and the Great Plains toad (*Bufo cognatus*). Standing in the middle of one of these choruses can be deafening–estimates of the number of individuals visiting playas during the breeding season range in the tens of thousands (Gray, Smith, Miller, & Bursey, 2007). For these explosively breeding animals, calling and amplexus are necessarily robust as they may have only one short opportunity to breed each year. Yet, within hours of capture and arrival at the laboratory, males will slowly stop calling and amplexing males will release their partners (Carr, unpublished). Similar effects of capture and captivity on reproductive behavior in amphibians have been reported by others (Licht, McGreery, Barnes, & Pang, 1983; Paolucci, Esposito, Difiore, & Botte, 1990; Gobbetti & Zerani, 1996). At the heart of this stress response are a suite of physiological changes that have evolved to inhibit reproduction in response to a threat. Animals are more vulnerable to predators when engaged in reproductive behavior or searching for a mate (Lima & Dill, 1990; Magnhagen, 1991), and it makes biological sense that mechanisms have evolved to rapidly inhibit reproduction when an animal is threatened. What is utterly unique and fascinating about stress as a physiological response is the variety of *different* sensory stimuli that evoke this response. The non-selectivity of stress as a physiological response may have bearing on the broader concern for how multiple and seemingly unrelated changes in global climate, pollution, and habitat quality affect reproduction and the health of amphibian populations worldwide. This chapter will review the evidence that exposure to stressors alters the activity of the hypothalamus–pituitary–adrenal (HPA) axis and influences reproduction in amphibians.

2. DEFINITIONS OF STRESS

The core concepts that lie at the heart of the stress response are well known to any freshman biology student: in response to a threat, an animal must increase blood flow and energy (in the form of nutrients) to organs critical for responding to the threat, while functions not critical for immediate survival (digestion, reproduction) are put on hold. Exposure of animals to many different foreign agents or unpredictable changes in their environment leads to a nearly immediate secretion of catecholamines from adrenal medulla cells and a slower (within minutes) increase in the activity of the HPA axis and elevation of the plasma levels of glucocorticoid hormones (Selye, 1956). According to Selye (1936),

animals respond to physical or psychological challenges in three distinct stages, which he termed the general adaptation syndrome (GAS): the alarm reaction, the stage of resistance, and, after prolonged exposure, the stage of exhaustion. Selye used the engineering term stress to describe the physiological state resulting in the GAS and described causative agents as stressors (Selye, 1956). A key concept underlying GAS theory is the relationship between stress and adaptation; in other words, that stress is a response used to adapt to the presence of a stressor. This concept is similar to Galen's belief that equilibrium of the four humors within the body reflects health (*eucrasia*) and that disease is associated with a 'disproportionate blend' or imbalance (*dyscrasia*) in the same (Brock, 1916). In more modern terms, we would recognize this as the concept that the physiological changes that occur during disease are an organism's way of returning the body to 'normal;' i.e., attempting to maintain homeostasis.

Is stress an attempt to return the body to 'normal'? The fact that stress and activation of the HPA axis are in many cases inextricably linked has led to confusion about the subjective nature and precise definitions of stress. For example, do alterations in plasma glucocorticoids in response to changing energy demands reflect a stress response? Clearly Selye believed that they did not (Selye, 1956), but none-the-less the role of glucocorticoids in adaptation to changing energy demands throughout the life cycle is difficult to separate from the concept of stress. These issues are critically important from a pragmatic point of view —if an endocrinologist or physiologist measures an increase in blood glucocorticoid levels, are the animals stressed?

Contributing to the difficulties in defining stress is the fact that most interpretations of Walter Cannon's concept of homeostasis (Cannon, 1932) revolve around a somewhat rigid adjustment of internal parameters to a fixed set-point that is required for the maintenance of life. This is not to say that Cannon's concept of homeostasis does not adequately capture the plasticity that must exist in adapting to perturbations in homeostasis. Getting a walnut stuck in your throat (respiratory acidosis) will always lead to the same mathematically predictable (Davenport, 1974) changes in renal bicarbonate transport, regardless of a person's age, socioeconomic status, or sex, because maintenance of pH is required for cell viability. More recent studies have elaborated on the concept of homeostasis to consider how set-points change throughout an animal's life cycle or in response to dramatic changes in the environment. The concept of 'allostasis' (Sterling & Eyer, 1988) adds some real-world perspective to traditional concepts of homeostasis in that it differentiates between maintenance of set-points required for cell viability (homeostasis) and processes that maintain physiological systems in balance in response to unpredictable changes in the environment or when changes in life history occur (McEwen & Wingfield, 2003). Within the larger context of allostasis, at least as defined by McEwen and Wingfield (2003), stress is an event (rather than a response) that engages endocrine and behavioral coping mechanisms, which are part of the process of allostasis but which occur in addition to those changes imposed during the life cycle. The concept of allostasis is immediately translatable to understanding how amphibians may adapt to a rapidly and constantly changing environment (global climate change, water-borne contaminants) in addition to the demands of the normal life cycle (requirement of increased HPA axis activity for metamorphosis; reproduction), and allows us to place stress and the mediators of allostasis (increased glucocorticoid and adrenomedullary catecholamine secretion) in a real-world context as an important mechanism for adapting to these changes.

Few would disagree with the statement that a response to stress in the short term is adaptive and helps animals to cope with immediate changes in their environment. However, prolonged activation of the HPA axis or adrenal catecholamine secretion can lead to serious deficits in immune function, energy balance, and health, and ultimately a negative impact on individual fitness. 'Allostatic load' is a concept that has been developed to describe the *cumulative* impact of physiological coping mechanisms, regardless of whether they occur in response to a stressful situation or as part of the adaptation to changes that is part of the normal life cycle (increased energy demands during reproduction, for example). In particular, unpredictable changes in the environment (changes in water quality; hydroperiod; water contamination) can increase allostatic load because these changes may place increased energy demands on individuals—demands that require additional secretion of glucocorticoid and adrenomedullary hormones. In theory, increased allostatic load can lead to a condition called 'allostatic overload' if unpredictable changes in the environment increase the cost of maintaining homeostasis beyond the ability of the organism to maintain a positive energy balance, or if energy demands are not exceeded but stress-induced food intake causes an animal to deposit and store more energy than it needs (McEwen & Wingfield, 2003). While data exist to support the concept of allostatic overload in homeotherms (Wingfield, Moore, & Farner, 1983; Smith et al., 1994; Herring & Gawlik, 2007), the immediate applicability of this concept to amphibians, at least to the extent that it relies on negative energy balance as an indicator of allostatic overload, is not clear. Walsberg (2003) points out that negative energy balance is a normal part of the life cycle (in particular on a seasonal basis) for many poikilothermic vertebrates. None-the-less, with respect to how stress may contribute to allostatic load and the potential ramifications for reproduction in amphibians, it is clear that negative energy balance or reduced body condition can potentially reduce reproductive success in

amphibians by limiting the amount of time during which males of some species can call (Ryan, 1985; Woolbright & Stewart, 1987) or by reducing fecundity (Prado & Haddad, 2005). So, at least from a theoretical standpoint, any unpredictable change in the environment that reduces resources or increases energy demands may contribute to allostatic load and may adversely impact reproductive effort if allostatic overload is reached.

3. THE HYPOTHALAMUS–PITUITARY–ADRENAL (HPA) AXIS IN AMPHIBIANS

In adult amphibians, corticosterone (CORT) and aldosterone are the principal adrenal steroids circulating in the blood (Carr & Norris, 2005), and both epinephrine and norepinephrine are present in measurable amounts in the amphibian circulation, although epinephrine predominates (Withers, Hillman, & Kimmel, 1988; Hillman, Withers, & Kimmel, 1998; Carr & Zozarro, 2004). Corticosterone and aldosterone are produced from cholesterol in a series of enzymatic reactions by cells that do not form a discrete adrenal cortex, as in mammals, but that are intermingled with kidney tissue and catecholamine-producing cells. A practical aspect of this anatomical arrangement is that it is difficult to isolate adrenocortical cells from kidney cells in amphibians, which is why the term 'interrenal gland' has been widely used to refer to this gland. Because of the number of enzymes involved in CORT biosynthesis, there are multiple targets available for pharmacological manipulation. Corticosterone synthesis can be inhibited by metyrapone, which inhibits the activity of 11β-hydroxylase (P450c11) or by cyanoketone, which inhibits 3β-hydroxysteroid dehydrogenase activity by occupying the enzyme's binding site for pregnenolone. Because cyanoketone works so far upstream in the steroid biosynthetic pathway, this drug blocks gonadal as well as interrenal steroid synthesis. Mitotane (o,p′-dichlorodiphenyldichloroethane (DDD)) has been used successfully to inhibit CORT synthesis in mammals and birds but for reasons unknown at present has no effect on plasma CORT levels in amphibians (Breuner, Jennings, Moore, & Orchinik, 2000).

It is generally believed that corticosterone is carried in blood plasma by a binding protein that can affect the transport and delivery of this steroid to target tissues. All vertebrates produce a protein that is secreted into the blood and binds glucocorticoids with high affinity: a so-called corticosteroid-binding globulin (CBG). Corticosteroid-binding globulins are small proteins that belong to a larger class of serine protease inhibitors (Serpin, gene id: SERPIN, clade A, member 6; i.e., SERPINA6), one the best-known being alpha 1 anti-trypsin inhibitor, which inhibits elastase activity. (Note: while CBGs belong to the serpin family, based on similarities in protein structure, they do not have any intrinsic ability to inhibit proteases.) These proteins are poorly characterized in amphibians and CBG binding has been thoroughly characterized in only two species, the tiger salamander *Ambystoma tigrinum* (Orchinik, Matthews, & Gasser, 2000) and the Southern toad *Bufo terrestris* (Ward, Fontes, Breuner, & Mendonça, 2007). Changes in CBG levels can alter the availability of CORT, as a steroid bound to CBG is unavailable to interact with receptors in target tissues. The amount of 'free' CORT available to interact with target tissues can be calculated by measuring the amount of total circulating hormone, the maximal binding capacity of CBG, and the affinity of the hormone for CBG (Barsano & Baumann, 1989). The rank order affinity of biologically active steroids for the amphibian CBG shows some interesting patterns (Table 6.1). First, there are large species differences in the affinity of the synthetic glucocorticoid dexamethasone to bind to CBG. Dexamethasone binds with high affinity to the *Ambystoma* CBG (Orchinik et al., 2000) but shows no appreciable affinity for CBG in *Bufo* (Ward, Fontes, Breuner, Mendonça, 2007). Secondly, the androgen dihydrotestosterone binds with higher affinity to CBGs from both species than CORT, the putative endogenous ligand for CBG. High affinity of androgens for plasma CBG has been reported for other amphibian species (Martin & Ozon, 1975) suggesting that CBG may be something of a misnomer for the CBG that is present in amphibian blood.

Corticosterone is not stored to any appreciable extent within interrenal gland cells but is synthesized upon demand in response to corticotropin (ACTH) acting on melanocortin 2 receptors (MC2R) in the interrenal gland. The MC2R has a higher affinity for ACTH than melanotropin (MSH) (Mountjoy, Robbins, Mortrud, & Cone, 1992) and, based on studies in mammals, is localized to adrenocortical cells,

TABLE 6.1 Constants for the inhibition of [3H] corticosterone binding to corticosteroid-binding globulin in blood plasma

	Ki	
Competitor	**Ambystoma**	**Bufo**
Dexamethasone	0.87 ± 0.07	< 400
Dihydrotestosterone	0.75 ± 0.15	1.08 ± 0.71
Corticosterone	1.85 ± 0.23	16.3 ± 2.88
Testosterone	2.12 ± 0.28	104.6 ± 14.5
Progesterone	6.11 ± 1.69	75.1 ± 18.9
Estradiol	27.3 ± 2.65	312 ± 63.6

Mean ± SEM (standard error of the mean) inhibitory constants (Ki) from Orchinik, Matthews & Gasser (2000) for *Ambystoma* and from Ward, Fontes, Breuner & Mendonça (2007) for *Bufo*.

although the precise location of these receptors has not been examined in any amphibian species to date.

Once released into the blood, CORT that is not bound to CBG (i.e., free CORT) can interact with high-affinity receptors on target tissues to alter protein gene transcription. All vertebrates possess intracellular cytosolic glucocorticoid receptors (cGR) that belong to a large family of nuclear hormone receptors that includes androgen, estrogen, thyroid hormone, and retinoic acid receptors (Carr & Norris, 2005). The unbound cGR belongs to a protein complex that also includes at least two different heat shock proteins (hsp90 and hsp70) as well as a number of less-well-characterized proteins. Binding of glucocorticoids to the cGR induces a conformational change in the cGR complex that causes dissociation of the cGR/hsp complex and translocation of the bound cGR to the nucleus. Once inside the nucleus there are two modes of action: the bound cGR can bind to the glucocorticoid response elements in the promoter region of glucocorticoid-sensitive genes (transactivation) or the bound cGR can interact directly with other transcription factors (transrepression) (see Stahn & Buttgereit (2008) for a more thorough treatment of transactivation and transrepression mechanisms). Mifepristone (RU486) is an antagonist of the amphibian cGR and has been used to block cGR action in some studies (Crespi & Denver, 2004). Very little information is available on the tissue distribution of the cGR in amphibians. Yao et al. (2008b) examined the anatomical localization of cGR in the brain of the African clawed frog *Xenopus laevis*. High densities of immunoreactive cGR were found in the pituitary gland and hypothalamus, and in forebrain areas such as the medial pallium (Yao et al., 2008b), an area thought to be homologous to the hippocampus in mammals. Cytosolic glucocorticoid receptors occur in several peripheral tissues including the testes (Denari & Ceballos, 2006), liver, kidney, and skin (Orchinik et al., 2000).

The ability of CORT administration to rapidly inhibit motor behaviors involved in reproduction (see Section 6.4 for more details) led to a search for a transmembrane receptor that might mediate rapid actions of glucocorticoids by linking to intracellular signal transduction pathways. The rationale underlying this hypothesis is that actions of CORT mediated by the traditional cGR occur too slowly (requiring receptor translocation, changes in gene transcription, and protein synthesis) to account for the rapid actions of corticosterone on behavior. Evidence of a cell membrane (mGR) was first reported by Orchinik, Murray, and Moore (1991), who identified a high-affinity mGR in synaptic membranes isolated from rough-skinned newt (*Taricha granulosa*) brain. An interesting feature of this mGR that is quite different from the cGR is its lack of affinity for dexamethasone (Orchinik et al., 1991), a synthetic glucocorticoid that has a high affinity for the cGR. The mGR in newts appears to be a G-protein coupled receptor (Orchinik, Murray, Franklin, & Moore, 1992) with an apparent molecular weight of 63 kD (Evans, Murray, & Moore 2000). Interestingly, the mGR in salamander neuronal membranes does not appear to be linked to a G-protein signaling pathway (Orchinik et al., 2000). Other labs have reported the existence in neuronal (Towle & Sze, 1983) and mononuclear cell membranes (Sackey, Watson, & Gametchu, 1997; Chen, Watson, & Gametchu, 1999; Gametchu, Chen, Sackey, Powell, & Watson, 1999; see also Song and Buttgereit (2006) for a review) of an mGR. The mononuclear cell mGR may be a splice variant of the cGR, as both membrane and cytosolic GRs share common antibody epitopes (Buttgereit, Straub, Wehling, & Burmester, 2004). The primary amino acid structure of mGRs remains unresolved.

3.1. Regulation of the Hypothalamus–pituitary–adrenal (HPA) Axis

The primary regulation of ACTH secretion is via the 41-amino-acid corticotropin-releasing factor (CRF). The gene encoding CRF belongs to a family of four paralogous genes encoding CRF and the urocortin-1, -2, and -3 (UCN-1, -2, and -3) peptides. Interestingly, the UCN-2 gene has not yet been identified in the amphibian genome (Boorse, Crespi, Dautzenberg, & Denver, 2005). Sauvagine (SVG), a 40-amino-acid peptide initially purified from the frog *Phyllomedusa sauvagei* (Montecucchi & Henschen, 1981) appears to be a member of the CRF family, based upon sequence homology, physiological actions, and receptor binding affinity, although, at present, mRNA encoding this peptide has been reported only in *P. sauvagei* (GENBANK accession number AY943910). Corticotropin-releasing factor peptides act on two types of G-protein coupled receptors, the CRF-R1 and CRF-R2 receptors, each encoded by different genes and each with different ligand binding preferences (Dautzenberg, Dietrich, Palchaudhuri, & Spiess, 1997; Dautzenberg et al., 2001). In amphibians, as in mammals, CRF binds preferentially to the CRF-R1, whereas UCN-1 binds to the CRF-R2 with slightly greater affinity (Dautzenberg et al., 2001). Urocortin-3 is highly selective for the CRF-R2 (Boorse et al., 2005). The potency of UCN-3 at the CRF-R2 is consistent with receptor binding data, as the EC50s for cyclic AMP stimulation in HEK293 cells expressing the xCRF-R2 or xCRF-R1 are 0.29 nM and 584 nM, respectively (Boorse et al., 2005). Relative to UCN-3, SVG is at least an order of magnitude more potent at stimulating cAMP accumulation in cells expressing xCRF-R2 (Boorse et al., 2005). Thus, both UCN-3 and SVG appear to be selective agonists for the CRF-R2, with UCN-3 exhibiting more selectivity but SVG exhibiting more potency.

It is generally believed that CRF is the principal endogenous hormone regulating ACTH secretion from

pituitary corticotropes in amphibians, based on the following lines of evidence: CRF neurons are located in key hypothalamic and limbic brain areas known to be involved in coping with stress and hypothalamic CRF neurons project to the median eminence (Tonon et al., 1985; Carr & Norris, 1990; Yao, Westphal, & Denver, 2004; Calle et al., 2005); CRF concentrations in the hypothalamus are altered following stressor exposure (Boorse & Denver, 2004); and administration of CRF stimulates ACTH (Tonon et al., 1986) and CORT secretion (Boorse & Denver, 2004). Although pituitary CRF receptors have not been extensively characterized in amphibians, initial work in *X. laevis* suggests that the anterior pituitary, which houses the cells that produce ACTH, expresses CRF-R1 receptors (Calle et al., 2006; Ito, Okada, Noriyuki, & Kikuyama, 2006). There is evidence that the anterior pituitary possesses CRF-R2 receptors (Okada et al., 2007), although whether these receptors reside on corticotropes in the amphibian pituitary gland is not yet known. There are some data indicating that arginine vasotocin (AVT) also is a potent stimulator of pituitary ACTH *in vitro* (Tonon et al., 1986), although the role of endogenous AVT in regulating CRF secretion during stress in amphibians is not known.

4. NEURONAL CIRCUITS MEDIATING ENDOCRINE RESPONSE TO STRESSORS

The release of CRF is the primary stimulus for increased HPA activity during stress in all vertebrates, and there has been significant progress made in elucidating the stimulatory and inhibitory neuronal pathways regulating CRF secretion during stress. In mammals, there is evidence for at least three major stimulatory pathways regulating CRF neurons during stress (Ziegler & Herman, 2002): an ascending brainstem pathway conveying visceral sensory and pain information (Sawchenko et al., 1996); a pathway conveying information from circumventricular organs (subfornical organ, area postrema) receiving blood-borne chemical signals; and a basal forebrain pathway involved in integrating more complex sensory information (social status, novel environment, predator) (Ziegler & Herman, 2002). Evidence suggests that information regarding stressors that activate visceral sensory (chemoreceptors, baroreceptors, etc.), and pain pathways is carried to the hypothalamus via ascending noradrenergic neurons in the A2 noradrenergic cell group (in the nucleus of the solitary tract), the A1 cell group in the rostral ventral medulla, and in the locus coeruleus (Sawchenko et al., 1996; Herman, McCreary, Bettenhausen, & Ziegler, 2002). Finally, perception of so-called 'psychological stressors' involving the integration of complex sensory modalities (social stress) modulates CRF secretion through direct, projecting limbic system pathways and local hypothalamic circuits that may also provide input on homeostatic set-points such as blood glucose and body temperature to CRF neurons (Ziegler & Herman, 2002). It is plausible that all of these pathways operate in amphibians (Figure 6.1), although the afferent innervation of CRF neurons has not been studied in detail in any amphibian species. Amphibians possess noradrenergic cell groups in the vicinity of the nucleus of the solitary tract (NTS), the rostral ventral medulla, and locus coeruleus (González & Smeets, 1993; 1995), although there is at present no direct evidence that noradrenergic neurons directly innervate CRF neurons in amphibians. Likewise, while there is evidence of direct innervation of the hypothalamus by neurons in subpallial homologs of the medial and central amygdala (Moreno & González, 2003; 2007a; 2007b), it is unknown whether CRF neurons are directly innervated by these pathways.

A critical factor in limiting the response to stress is the inhibition of CRF-producing neurons by glucocorticoids. In mammals, inhibition of CRF and ACTH secretion involves both direct and indirect pathways, through activation of cGR in the pituitary gland, hypothalamic CRF neurons, and hippocampal neurons that regulate paraventricular nucleus (PVN) CRF neurons. Recent data indicate that similar regulatory pathways exist in amphibians (Yao & Denver, 2007; Yao, Stenzel-Poore, & Denver, 2007; Yao, Schulkin, & Denver 2008a). Orchinik et al. (1991) demonstrated high-affinity [3H]CORT binding sites in the preoptic nucleus, a major location of CRF neurons in the amphibian brain (Tonon et al., 1985; Olivereau, Vandesande, Boucique, Ollevier, & Olivereau, 1987; Bhargava & Rao, 1993; Yao et al., 2004; Calle et al., 2005). Immunocytochemical studies using antisera developed against *Xenopus* cGR have shown the cGR protein expressed in the pituitary gland and brain areas heavily populated with CRF neurons (the bed nucleus of the stria terminalis; the preoptic area), as well as the dorsal medial pallium (Yao, Hui, & Denver, 2008b). Amphibian CRF genes contain glucocorticoid response elements (Yao et al., 2007) and glucocorticoids inhibit forskolin-stimulated CRF gene promotor activation *in vitro* (Yao et al., 2008a). Manipulation of plasma glucocorticoid levels leads to expected changes in CRF content in the preoptic area and BnST, suggesting that glucocorticoid regulation of CRF gene expression is evolutionarily conserved (Yao et al., 2008a).

5. RESPONSE OF THE AMPHIBIAN ENDOCRINE SYSTEM TO STRESSORS

Exposure to a wide variety of physiological and psychological stressors activates the HPA axis and adrenomedullary catecholamine secretion in amphibians, including causing increased conspecific density/crowding (Moore & Miller, 1984; Moore & Zoeller, 1985; Glennemeier & Denver,

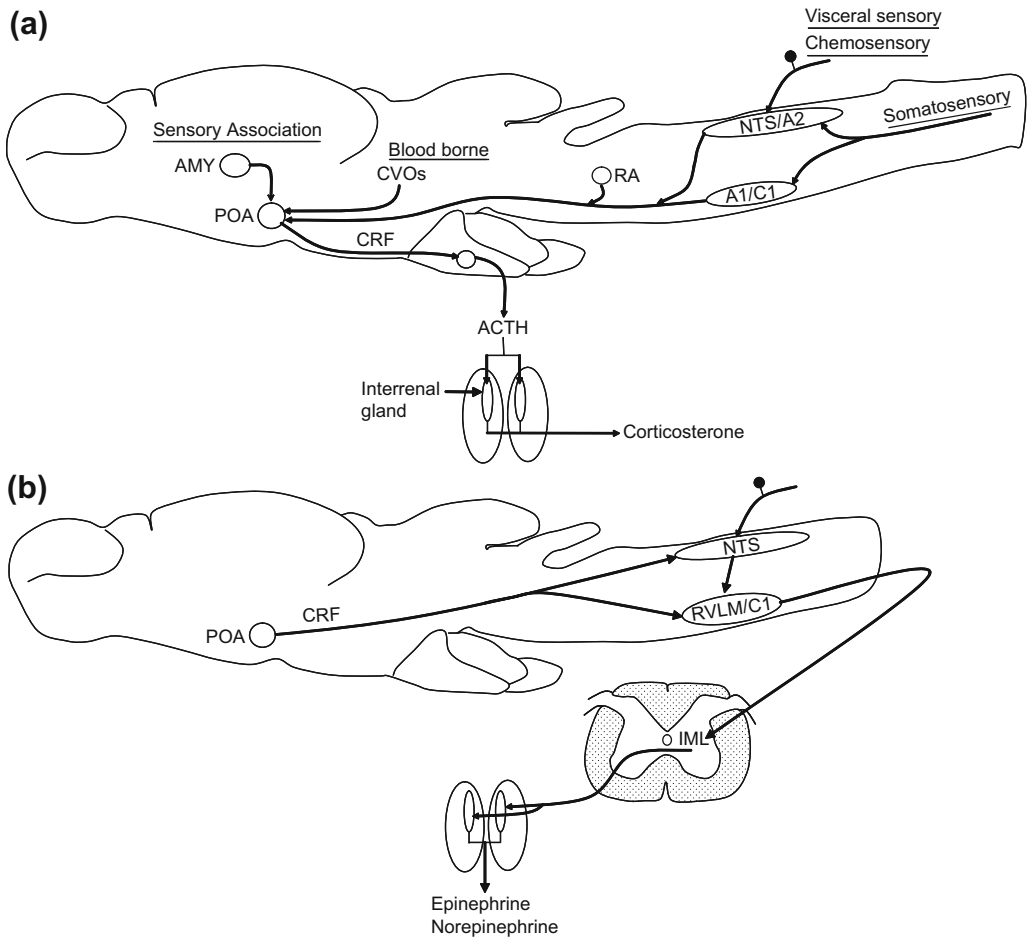

FIGURE 6.1 Neuronal pathways for activating the HPA axis and interrenal catecholamine secretion during stress in amphibians. (a) Three putative stimulatory pathways regulating corticotropin-releasing factor (CRF) neurons in the amphibian preoptic area (POA) (*adapted from Sawchenko, 1987; Herman, McCreavy, Bettenhausen & Ziegler 2002; Ziegler & Herman, 2002*). Somatic sensory, visceral sensory, and chemosensory information associated with physiological or interoceptive stressors (ether exposure, low blood pressure, low blood sugar, pain) converge in ascending pathways originating in the brainstem, whereas chemical mediators in the blood (such as cytokines) may gain access through circumventricular organs (CVO) such as the pineal organ, choroids plexus, or paraphysis (Kemnitz, Fox, & McNulty, 1990). Information regarding more complex stressors (restraint, social stress, crowding) is processed in sub-pallial limbic areas such as the amygdala (AMG) that project to the POA and hypothalamus. Catecholaminergic cell groups homologous to the A2 and A1 noradrenergic cells groups have been identified in amphibians (Gonzalez & Smeets, 1993; 1995), although direct projections between these cells and CRF neurons in the POA have not been confirmed and the pathways shown are based on well-described connections in the mammalian brain (Sawchenko & Swanson, 1982) and the fact that physiological stressors (ether, hemorrhage) known to act via this pathway in mammals elicit a stress response in amphibians. Likewise, although tracing studies have confirmed AMG projections to the POA and hypothalamus, and there are several lines of evidence supporting a role for the AMG in multimodal sensory association and autonomic regulation in amphibians (Laberge, Muhlenbrock-Lenter, Grunwald & Roth 2006), a precise role for AMG in afferent regulation of CRF neurons remains speculative, although such pathways are well-established in mammals (see Ziegler and Herman (2002) for a review). C1, C1 adrenergic nucleus; RA, raphe nucleus; NTS, nucleus of the solitary tract. (b) Theoretical pathways for regulation of interrenal catecholamine secretion during stress. Descending projections from CRF neurons in the POA may directly or indirectly modulate premotor sympathetic nervous system cells via direct projections to the C1 adrenergic area of the rostral ventrolateral medulla (RVLM) or via innervation of the NTS, respectively. Rostral ventrolateral medulla neurons in turn may innervate cholinergic preganglionic sympathetic neurons (Accordi, 1991) in the intermediolateral cell column (IML) of the spinal cord (Funakoshi & Nakano, 2007) that project to the catecholamine-producing cells in the interrenal gland. Direct projections from hypothalamic CRF neurons to the RVLM (Milner, Reis, Pickel, Aicher, & Giuliano, 1993) and NTS (Sawchenko, 1987) exist in mammals and immunohistochemical studies have confirmed the presence of a ventral medullary catecholamine cell group in amphibians (Gonzalez & Smeets, 1993; 1995).

2002), shaking (Yao et al., 2005; Bonett, Hu, Bagamasbad, & Denver, 2008), restraint (Juráni, Murgas, Mikulaj, & Babusíková, 1973; Tureková & Juráni, 1978), confinement (Licht et al., 1983; Belden, Moore, Wingfield, & Blaustein, 2005), desiccation (Denver, 1998; Denver, Mirhadi, & Phillips, 1998), and reduced caloric intake (Hu, Crespi, & Denver, 2008). Exposure to ether vapors, a well characterized interoceptive stressor in mammals that activates ascending brainstem pathways regulating CRF neurons (Emmert & Herman, 1999), is a potent stimulator of

plasma CORT (Leboulenger, Dupont, Vaudry, & Vaillant, 1976; Olsen, Lovering, & Carr, 1999) and plasma catecholamines (Carr, unpublished) in amphibians. Thus, while precise information on the neurochemical anatomy of the afferent circuitry stimulating CRF secretion during stress may be sketchy, afferent pathways conveying information about systemic or interoceptive stressors may be wired in a fashion similar to that observed in mammals (Figure 6.1). Since the processing of complex sensory information in the amphibian brain occurs by definition through sub-cortical pathways, it is difficult to directly compare the pathways involved in processing psychological stressors by the amphibian brain to what is believed to occur in mammals. In rats, it is thought that the sensory and association cortex are involved in the initial integration of stressful stimuli before sending output to the amygdala (Ziegler & Herman, 2002), which filters this information before activating CRF neurons in the PVN via direct and indirect neuronal pathways. The simple fact that new sensory-associated parts of the limbic system have evolved in amniotes (basolateral complex in mammals (e.g., Laberge, Mühlenbrock-Lenter, Grunwald, & Roth, 2006)) may mean that amphibians are more limited in terms of the complexity of sensory information processing. However, without neuroanatomical studies on the neuronal processing of psychological stressors in amphibians, even this remains speculative. It is certainly plausible to speculate that the exposure of amphibians to complex stressors that involve multiple sensory modalities (such as increased conspecific density or predator cues) might trigger amygdalar cell groups (Laberge et al., 2006), which in turn activate the HPA axis. Amphibians exhibit pronounced aversive and avoidance behaviors when exposed to a variety of predatory cues, and, while there is abundant evidence in the mammalian literature indicating that exposure to predator cues activates the HPA axis (see Roseboom et al., 2007), there are no data yet indicating a similar response in amphibians. Interestingly, there is some evidence that exposure to tadpole alarm pheromone induced by the presence of a predator (dragonfly larvae) actually *suppresses* the HPA axis (Fraker et al., 2009). Further, reduced HPA activity appears to be required for quiescent anti-predator behavior in tadpoles (Fraker et al., 2009).

Although most studies in amphibians have examined the effects of an acute stressor on the HPA axis, there are data indicating that early life stress can have long-lasting effects on growth, development, and HPA activity in amphibians, as has been well-documented in mammals. Exposure to increased conspecific density during the larval period can negatively affect post-metamorphic and juvenile growth (Goater, 1994) and elevate plasma CORT levels (Glennemeier & Denver, 2002). Likewise, food restriction during the larval period elevates whole-body CORT and reduces size at metamorphosis (Hu et al., 2008). Interestingly, treatment of tadpoles with exogenous CORT at doses designed to mimic the elevation in corticosterone that occurs during caloric restriction caused a decreased number of immunoreactive GR in animals examined two months after metamorphosis (Hu et al., 2008). These data suggest that exposure to stressors during the larval period can affect the HPA axis and also growth and metabolism long after the stressor is terminated.

5.1. Effects of Water Quality and Water-borne Contaminants on the Hypothalamus–pituitary–adrenal (HPA) axis

Global declines in amphibian populations have raised concerns that alterations in water quality might have sub-lethal impacts on growth, development, and reproduction (see Chapter 11, this volume). Because of their thin skin and aquatic or semi-aquatic life cycles, amphibians represent a worst case scenario for exposure to aquatic contaminants. There are some data examining the impact of water quality and water-borne contaminants on the HPA axis in amphibians. Exposure of amphibians to polychlorinated biphenyls (Glennemeier & Denver, 2001) alters HPA activity. The exposure of male adult *X. laevis* to a mixture of nine pesticides elevated plasma corticosterone (Hayes et al., 2006).

5.2. Multiple Stressor Effects on the Hypothalamus–pituitary–adrenal (HPA) Axis as a Result of Decreased Habitat Quality

Decreased habitat quality could act as a stressor to amphibians, although pinpointing exactly which environmental components may be adversely affecting amphibian health can be difficult. In theory, many factors (changes in hydroperiod, land use practices, reduced water quality, increased pathogen numbers) may lead independently to decreased habitat quality. A few studies have examined the effects of reduced habitat quality on the HPA axis. Mudpuppies (*Necturus maculosus*) collected from sites contaminated with PCBs and organochlorine pesticides showed a reduced CORT response to stressor exposure compared to animals collected at reference sites (Gendron, Bishop, Fortin, & Hontela, 1997). Male *B. terrestris* inhabiting a coal ash-polluted site had higher levels of plasma CORT than males at a reference site (Hopkins, Mendonça, & Congdon, 1997). Moreover, when males from the reference site were transferred to the polluted site, they exhibited higher plasma CORT within 10 days of transfer (Hopkins et al., 1997). Interestingly, males from the polluted site continued to have elevated plasma CORT after being transferred to the laboratory, and

were refractory to an ACTH challenge (Hopkins, Mendonça, & Congdon, 1999). Toads exposed to coal ash waste also exhibit differences in the ratio of plasma CBG levels to total CORT (Ward et al., 2007). Toads transferred from a reference site to a coal ash site showed the expected elevation in plasma CORT but exhibited no change in circulating CBG levels. As a consequence, plasma levels of free (unbound) CORT were significantly elevated in these animals (Ward et al., 2007). This suggests that exposure to the poor-quality habitat may lead to increases in plasma CORT that are unbuffered by plasma CBG (Ward et al., 2007). The precise cause for the elevation in plasma CORT is not clear, although the coal ash waste sites are characterized by a variety of trace metals that might impact the HPA axis either directly, by altering the synthesis or turnover of HPA axis hormones, or indirectly, by increasing energy demands in order to metabolize and eliminate the pollutant. Analysis of trace elements in individual *B. terrestris* inhabiting coal waste sites indicates that these animals have greater tissue burdens of arsenic, selenium, and vanadium than animals from reference sites (Hopkins, Mendonça, Rowe, & Congdon, 1998). Altering the landscape through which amphibians migrate to breeding ponds may also introduce a stressor. Homan et al. (2003) found that male spotted salamanders (*Ambystoma maculatum*) migrating over pavement had higher plasma corticosterone levels than males migrating through undisturbed forest.

5.3. Contribution of Multiple Stressors to Allostatic Load and Overload

As outlined above, reductions in habitat quality can lead to increased activity of the HPA axis and therefore could contribute to allostatic load in amphibian populations. Increased pollution, pathogen abundance, and changes in food availability in theory could all act independently to contribute to cumulative allostatic load and overload (Figure 6.2) by increasing HPA axis activity. In the case of exposure to multiple pollutants, the cellular mechanism of action of the toxicant is not necessarily as important in determining allostatic load as whether each toxicant increases energy expenditure in the exposed animal (Figure 6.2). Any type of unpredictable event that requires additional energy expenditure may negatively impact resource allocation for development or reproduction if resources are limited. Given worldwide declines in amphibian populations and the fact that multiple factors may contribute to some declines, it is important to consider the concept of allostatic load when examining how multiple stressors may adversely affect amphibian populations and reproductive effort.

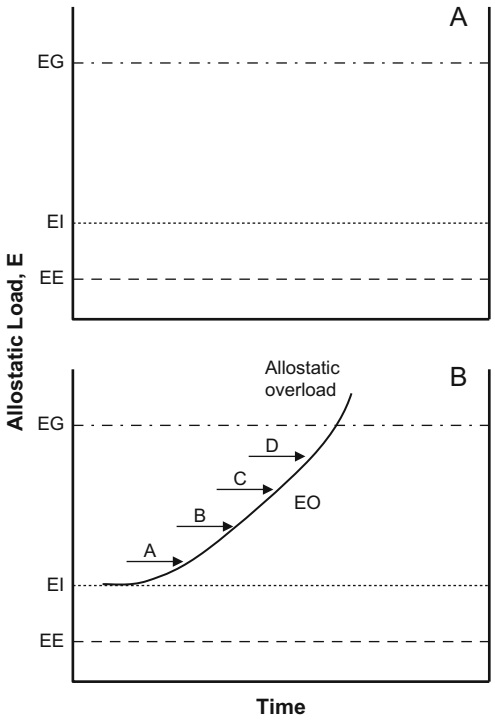

FIGURE 6.2 Theoretical depiction of how multiple environmental stressors might contribute to allostatic load and allostatic overload in amphibians, based upon the energetic requirements (E) of an amphibian during its life cycle (*adapted from McEwen & Wingfield, 2003*). EE represents the basic energy requirements needed to maintain cellular processes vital to homeostasis (ion transport, maintenance of body fluid pH, etc.), EI represents the energy required to forage and sequester food, and EG represents the energy resources available to the organism in the environment. Although all of these parameters would be expected to fluctuate during the course of the year, they are drawn as straight lines for the sake of simplicity. EO represents the additional energy required to metabolize and excrete pollutants or acquire resources in lower quality habitats. Multiple stressors (A, B, C, D) over time may contribute individually to the cumulative effect of EO and allostatic overload if EO surpasses EG, leading to increased glucocorticoid secretion. In a case where A,B,C, and D are different contaminants, the individual mode of action for each contaminant is not as important in determining its contribution to allostatic load as is the energy requirement placed on the organism to metabolize and excrete each contaminant.

6. INHIBITORY EFFECTS OF STRESS AND THE HPA AXIS ON REPRODUCTION

Exposure to noxious stimuli or direct administration of HPA axis hormones can inhibit reproduction at several levels, by reducing sex steroid synthesis, by interfering with gametogenesis, and/or by inhibiting reproductive behavior.

6.1. Effects of Stressors and Hypothalamus–pituitary–adrenal (HPA) Axis Hormones on Plasma Sex Steroid Levels

Several studies indicate that exposure to mild stressors such as restraint or crowding can very quickly increase plasma

levels of corticosterone and reduce plasma sex steroid levels within hours of stressor onset. In the process of studying seasonal changes in plasma gonadotropins and sex steroid levels, Licht et al. (1983) observed that transport of American bullfrogs (*Rana catesbeiana*) to the laboratory or maintenance of the animals in capture bags led to an elevation in plasma CORT and reductions in sex steroid levels to baseline levels within 24 hours of capture, and that the inhibitory effects of captivity on plasma sex steroid levels seemed most pronounced in male frogs. A similar negative correlation between plasma CORT and plasma sex steroids levels within hours of captivity has been observed in other amphibian species including the edible frog *Rana esculenta* (Paolucci et al., 1990; Zerani, Amabili, Mosconi, & Gobbetti, 1991). The fact that plasma CORT levels increase shortly after the onset of captivity (Licht et al., 1983; Paolucci et al., 1990; Zerani et al., 1991; Coddington & Cree, 1995; Cooperman, Reed, & Romero, 2004; Mosconi et al., 2006) and are generally, though not always (Coddington & Cree, 1995), negatively correlated with plasma sex steroid levels, suggests that CORT may act to lower plasma sex steroids during captivity. A role for elevated plasma CORT levels in lowering plasma sex steroid secretion after captivity is supported by data showing that administration of exogenous corticosterone lowers plasma sex steroids levels (Moore & Zoeller, 1985; Paolucci et al., 1990; Burmeister, Somes, & Wilczynski, 2001). There are a few exceptions to the negative correlation between CORT and plasma sex steroids. Moore and Zoeller (1985) found that exposing newts to crowded conditions, while significantly reducing testosterone, had no significant effect on plasma CORT. Similarly, Houck, Mendonça, Lynch, and Scott (1996) found that captive male marbled salamanders (*Ambystoma opacum*) had lower plasma androgen levels but identical plasma CORT titers compared to field-caught male salamanders. Both of these studies were performed in urodeles and, aside from a lack of data on CBG levels and free vs. bound CORT titers, there are no *a priori* reasons as to why stress sensitivity would be lower in urodele amphibians than in anuran amphibians. However, these data may suggest that other factors aside from CORT may be involved in stress-induced reductions in plasma sex steroid levels. Houck et al. (1996) argued that plasma CORT and plasma testosterone may be dissociated in *A. opacum*. This idea was based in part on data from Orchink, Licht, and Crews (1988) showing that plasma CORT is elevated in amplexing male amphibians. As discussed below, elevated plasma corticosterone during amplexus or calling is not unusual given that both behaviors are energetically costly and CORT is an important glucocorticoid in amphibians. In fact, exogenous administration of CORT is only effective at inhibiting androgen-dependent calling at relatively high concentrations (Burmeister et al., 2001). Alternative explanations for the lack of negative correlation between plasma CORT and plasma testosterone may be that the interrenal response to captivity was reduced once the males were acclimated to captive conditions. Houck et al. (1996) collected plasma samples for CORT analysis 10 days after establishment of captive conditions. In addition, CORT responsiveness to stressors may vary seasonally. There are well-described annual (Leboulenger et al., 1982) and seasonal (Pancak & Taylor, 1983) patterns in plasma CORT that may underlie stress responsiveness.

In a study examining captivity effects on plasma steroid levels in American bullfrogs, Licht et al. (1983) noticed that plasma androgens appeared to be more sensitive to captivity stress in male frogs. In female bullfrogs, there were no significant changes in plasma estradiol or testosterone concentrations within the first 20 hours of capture (Licht et al., 1983). Interestingly, others have reported a lack of sensitivity in plasma sex steroids levels to stress. There were no changes in plasma sex steroid levels in vitellogenic whistling frogs (*Litoria ewingii*) within 24 hours of capture, even though plasma CORT levels rose steadily after capture (Coddington & Cree, 1995).

6.2. Effects of Stress and Hypothalamus–pituitary–adrenal (HPA) Axis Hormones on Gonadotropin–releasing hormone (GnRH), Gonadotropins, and Sex Steroid Synthesis

Corticosterone may act at any one of numerous levels to reduce plasma sex steroid levels by interfering with gonadotropin-releasing hormone (GnRH) or gonadotropin secretion, by acting directly within gonadal tissue to inhibit steroid synthesis or by altering the metabolism and transport of sex steroid hormones. Gonadotropin levels are significantly reduced within hours of capture in bullfrogs (Licht et al., 1983), possibly due to altered levels of GnRH release. Moore and Zoeller (1985) showed that exposure to an acute stressor (crowding) or exogenous administration of CORT altered brain GnRH content in rough-skinned newts. A direct action of CORT on gonadal steroid synthesis also is possible. Studies in mammals have shown that glucocorticoids can act directly within the testis to inhibit testosterone production via interaction with cGR (Monder, Hardy, Blanchard, & Blanchard, 1994; Monder, Miro, Marandici, & Hardy, 1994). Amphibian testicular tissue also possesses a cGR that is principally located in Sertoli and Leydig cells (Denari & Ceballos, 2006). However, testicular tissue in both mammals and amphibians contains 11β-hydroxylase, an enzyme that can act to protect against glucocorticoid action via degradation of CORT (Denali & Ceballos, 2005). While this enzyme might help to adjust the seasonal availability of CORT within testicular tissue, it is possible that elevated concentrations reached during an acute stress could swamp the protective actions of this enzyme.

6.3. Effects of Stress and the Hypothalamus–pituitary–adrenal (HPA) Axis on Gametogenesis

Stress may affect gametogenesis in amphibians. Stress associated with captivity reduced oviductal weight and the number of estrogen-synthesizing ovarian follicle cells in female Indian skipper frogs (*Rana cyanophlyctis*) (Pancharatna & Saidapur, 1992). Lowered plasma testosterone elicited by CORT administration was associated with reduced spermatogenesis in *Bufo melanostictus* (Biswas, Chaudhuri, Sarkar, & Sengupta, 2000). Further, Tsai, Lunden, & Jones (2003) reported that subchronic (20 day) CORT treatment selectively reduced the number of spermatids in male leopard frogs (*Rana pipiens*).

6.4. Effects of Stress and Hypothalamus–pituitary–adrenal (HPA) Axis Hormones on Reproductive Behavior

Exposing amphibians to noxious stimuli or the administration of exogenous corticosterone inhibits reproductive behavior. Courtship behavior in rough-skinned newts was inhibited in a dose-dependent and rapid fashion by exogenous administration of CORT, mimicking the inhibitory effect of stressors on reproductive behavior in these animals (Moore & Miller, 1984). The effects of CORT are not pharmacological, as manipulation of endogenous corticosterone secretion has the expected effects on behavior. Administration of a dose of CRF sufficient to elevate plasma CORT reduced courtship and these effects were blocked with the CORT synthesis inhibitor metyrapone (Moore & Miller, 1984). An interesting feature of the CORT effects is that they occur much more quickly (less than eight minutes (Orchinik et al., 1991)) than one would expect based on a genomic mechanism of action. Moreover, dexamethasone, a synthetic glucocorticoid that does not have high affinity for the membrane amphibian mGR, failed to inhibit courtship after peripheral administration. These observations were used in part to formulate the hypothesis that CORT acts through a non-genomic mechanism involving membrane receptor-coupled signal transduction pathways to inhibit courtship behavior. As discussed previously, there are data supporting the existence of mGR in the amphibian central nervous system (CNS) (Orchinik et al., 1991; 1992; Moore & Evans, 1999; Orchinik et al., 2000). While the precise molecular structure of the amphibian membrane mGR has not been elucidated, there is abundant evidence supporting the existence of mGRs in the mammalian literature (see Song & Buttgereit, 2006; Stahn & Buttgereit, 2008). It is important to note that, while rapid CORT action may indicate the involvement of non-genomic signaling pathways, rapid action of glucocorticoids does not necessarily require the presence of an mGR. Corticosterone is known to elicit rapid (within seconds) effects in the CNS via cytoslic receptors that directly interact with intracellular signal transduction pathways (Liu et al., 2008). Other examples of rapid effects mediated by the cGR receptor include physicochemical interactions with plasma and mitochondrial membranes (Song & Buttgereit, 2006). Overall, however, the congruity between the lack of dexamethasone binding to the newt mGR and the inability of this steroid to inhibit courtship argues against a role for cGR in mediating the inhibitory action of CORT on courtship behavior.

In theory, there are two ways in which CORT might act to inhibit reproductive behavior in amphibians. To the extent that reproductive behavior is associated with plasma sex steroid levels, CORT might interfere with behavior indirectly by impacting gonadal steroid synthesis. Although CORT does affect GnRH content (Moore & Zoeller, 1985), this possibility seems unlikely given the very rapid nature of the CORT effects. Alternatively, CORT may act directly within the CNS to influence motor pathways regulating courtship behavior. There is substantial data indicating a direct effect of CORT on neuronal firing in pre-motor areas of the brainstem as well as in the spinal cord (Figure 6.3). Corticosterone reduces the firing of medullary neurons within minutes and reduces the sensory responsiveness of medullary neurons to increased cloacal pressure, a normal sensory stimulus in newt courtship (Rose, Moore, & Orchinik, 1993; Rose, Kinnaird, & Moore, 1995; Rose, Marrs, & Moore, 1998). Dexamethasone, which does not bind to the newt mGR (Orchinik et al., 1991), failed to elicit the same effects on neuronal firing (Rose et al., 1993). Interestingly, CORT may also act on intraspinal pathways regulating clasping behavior. Male rough-skinned newts with a spinal cord transection, which eliminates descending input from medullary pre-motor areas, exhibit a strong clasping response that continues even in the absence of behavioral cues for release, suggesting that medullary neurons projecting to the spinal cord contribute to the inhibitory regulation of clasping (Rose, 2000). Corticosterone reduced the maintenance of the clasp after termination of cloacal stimulation in spinally transected newts without affecting the initiation of the clasp or the strength of the clasp during cloacal stimulation (Lewis & Rose, 2003), suggesting that CORT does not directly affect sensory afferents carrying input from the cloaca or output from somatic motor neurons innervating clasping muscles. Instead, CORT appears to affect maintenance of clasping after cloacal stimulation, possibly by interfering with a so-called intraspinal 'clasp generator' (Rose, 2000). Consistently with other studies in this model, dexamethasone failed to elicit the response observed with CORT

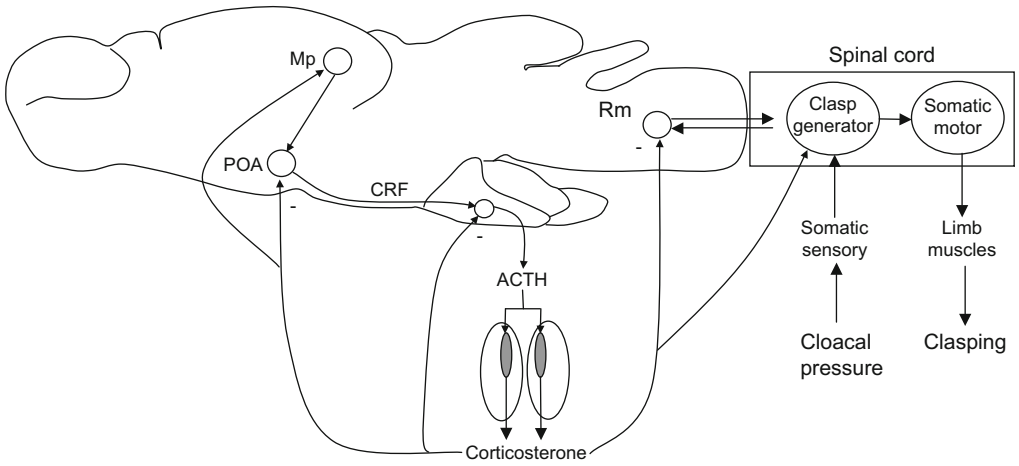

FIGURE 6.3 Targets for corticosterone in the amphibian CNS during stress. Corticosterone acts on the cytosolic glucocorticoid receptor (cGR) in the pituitary gland, preoptic area (POA), and dorsal medial pallium (Mp) to inhibit CRF production (Yao, Schulkin & Denver, 2008a; Yao, Hu & Denver 2008b). Corticosterone acts on cell membrane glucocorticoid receptor (mGR) in neurons of the medial reticular nucleus (Rm) (Rose, Moore & Orchinik, 1993; Rose, Marrs & Moore 1998) and spinal cord (Lewis & Rose, 2003) to inhibit clasping behavior.

(Lewis & Rose, 2003; Rose et al., 1993), arguing against a role for the traditional cGR in this response.

The mechanisms mediating the effects of CORT on neuronal firing have recently been elucidated by Coddington, Lewis, Rose, and Moore (2007) (Figure 6.4). Administration of endocannabinoid receptor agonists to male newts inhibits clasping behavior (Soderstrom, Leid, Moore, & Murray, 2000; Coddington & Moore, 2003) and *in situ* hybridization studies indicate that cells in the rostromedial medulla of the brainstem express mRNA encoding cannabinoid type 1 (CB_1) receptors (Hollis, Coddington, & Moore, 2006). Pretreatment of male newts with the cannabinoid receptor antagonist AM281 prevents stress-induced inhibition of clasping and ameliorates the inhibition of clasping observed after peripheral administration of CORT (Coddington et al., 2007). Further, pretreatment with AM281 prevented CORT-induced changes in spontaneous or stimulated (via cloacal pressure) neuronal firing in the rostral medial medulla (Coddington et al., 2007). These data are consistent with the hypothesis that CORT released during stress causes the release of endocannabinoid signaling molecules in the brainstem that act to modulate the responsiveness to sensory stimulation from the cloaca.

6.5. Multiple Stressor and Habitat Quality Effects on Reproduction

Reduced habitat quality can indirectly affect reproductive success and population health by reducing the number of young of the year recruited into the population. Rowe, Hopkins, and Coffman (2001) demonstrated that 100% of *B. terrestris* larvae developing in surface waters contaminated with coal ash waste containing several trace elements (As, Cd, Cr, Cu, Se, and others) failed to metamorphose due to reduced resources and direct toxicity of the trace elements.

A few studies have examined how multiple changes in habitat quality and agrochemical exposure associated with agricultural land use may affect reproduction and population size. Knutson et al. (2004) found no differences in amphibian species richness or reproductive success in ponds surrounded by row crops compared to natural wetlands or ponds surrounded by non-grazed pasture. Sex ratio, size, and age profiles were found to be similar for populations of African clawed frogs (*X. laevis*) inhabiting ponds in maize- and non-maize-growing areas of South Africa (DuPreez et al., 2005). Gray and Smith (2005) reported that body mass and length were 10−148% greater for post-metamorphic amphibians captured at natural grassland playas than at cropland playas in the US Southern High Plains. Since body size and condition are related to reproductive success in amphibians, these data suggest that land use practices may impact local amphibian populations in this area.

7. ROLE OF GLUCOCORTICOIDS IN MEETING ENERGY DEMANDS DURING REPRODUCTION

Reproduction in amphibians is energetically costly. Amphibians may remain in amplexus for more than a day and males may call for several days in a row. It has been estimated that calling is the most energetically demanding behavior that an amphibian (or any ectotherm) can undertake (Taigen & Wells, 1985) and increases metabolic rate more than 20 times over basal levels (Wells, 2001). As discussed previously, transition between different reproductive behaviors may represent several different allostatic

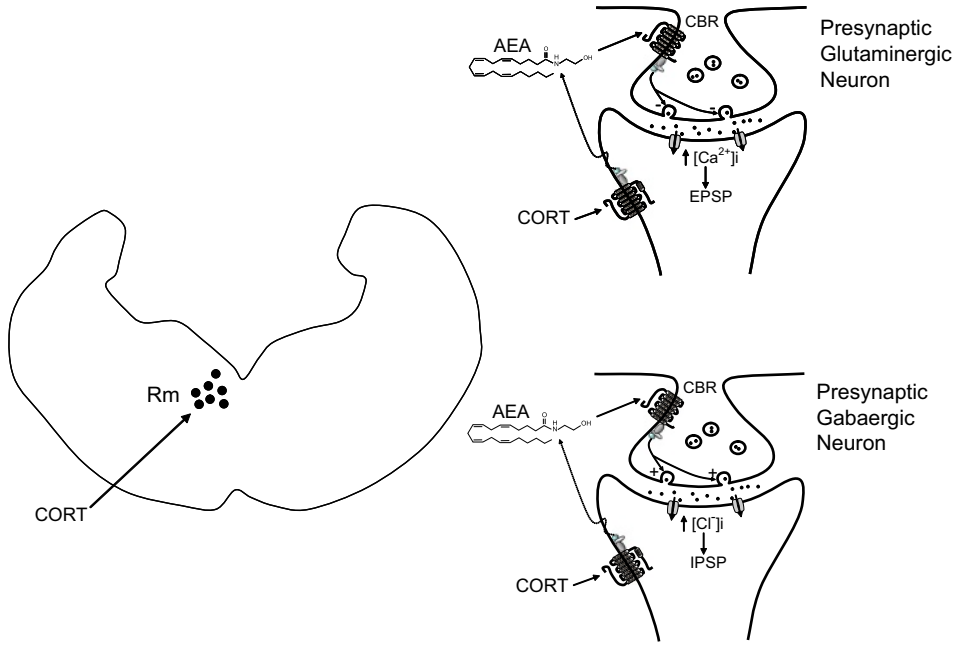

FIGURE 6.4 Neurochemical mechanisms mediating the effects of corticosterone on neuronal activity via the mGR. Corticosterone acts on cell membrane glucocorticoid receptors (mGR) in the medial reticular nucleus (Rm) to stimulate the release of endocannabinoids (Coddington, Lewis, Rose & Moore, 2007) such as anandamide (AEA). Endocannabinoids in turn act upon a G-protein coupled receptor (CBR) on reticulospinal neurons (Hollis, Coddington & Moore, 2006) to reduce glutaminergic and enhance gabaergic neurotransmission (Di, Malcher-Lopes, Halmos, & Tasker, 2003; Di, Malcher-Lopes, Marcheselli, Bazan, & Tasker, 2005), thereby reducing excitatory postsynaptic potentials (epsps) and increasing inhibitory postsynaptic potentials (ipsps) in postsynaptic cells and ultimately reducing the firing rate of reticulospinal neurons involved in clasping. *Adapted from Di, Malcher-Lopes, Halmos, & Tasker, (2003); Di, Malcher-Lopez, Marchelli, Bazan & Tasker (2005).*

states depending upon energy demands, and plasma glucocorticoids may play an important role in supplying energy for calling behavior and amplexus. Plasma levels of CORT are greater in calling than non-calling male anurans (Mendonça, Licht, Ryan, & Barnes, 1985; Leary, Jessop, Garcia, & Knapp, 2004) and are positively correlated with condition factor (Leary et al., 2004), a ratio of body weight to length that is correlated with fitness. Plasma CORT is positively correlated with the rank order of calling effort for a range of anuran species (Emerson & Hess, 2001). Plasma glucocorticoids also are elevated in males engaged in amplexus (Orchinik et al., 1988). The fact that calling behavior can be induced by exogenous administration of androgens or AVT, and that calling males have higher testosterone levels than non-calling males (Marler & Ryan, 1996), raises an interesting problem, as a number of studies indicate that administration of CORT decreases plasma sex steroid levels in amphibians (see Section 6.1) and plasma CORT levels tend to correlate negatively with plasma androgen levels (Licht et al., 1983; Orchinik et al., 1988; Marler & Ryan, 1996; Burmeister et al., 2001). This inverse relationship between plasma CORT and plasma androgens led Emerson (2001) to propose the 'energetics—hormone—vocalization model,' which hypothesizes that plasma CORT is elevated during calling to help with energy demands until levels are high enough to inhibit testosterone synthesis (Figure 6.5). At this stage, the individual stops calling and energy demands return to basal levels. Once plasma glucocorticoids levels are below the threshold for inhibiting androgen synthesis, calling resumes. However, Leary et al. (2004) failed to find an inverse relationship between plasma CORT and androgen levels in two species of *Bufo*. They proposed a modified model based upon expenditure of energy reserves, plasma CORT, plasma androgens, and AVT. In this model, plasma androgen levels exhibit minor cyclical fluctuations that are not associated with time of day or plasma CORT but are sufficient to periodically stimulate AVT production in the hypothalamus, which is required for calling (Boyd 1994a; 1994b).

8. ADAPTIVE SIGNIFICANCE OF STRESS-INDUCED INHIBITION OF REPRODUCTION

What are the selective pressures that have favored inhibition of reproduction by high plasma CORT levels? Reproductive behavior in amphibians is very energetically costly and expending stored energy during reproduction may limit energy stores that could be called upon in the future to avoid a predator or seek refuge. Alternatively,

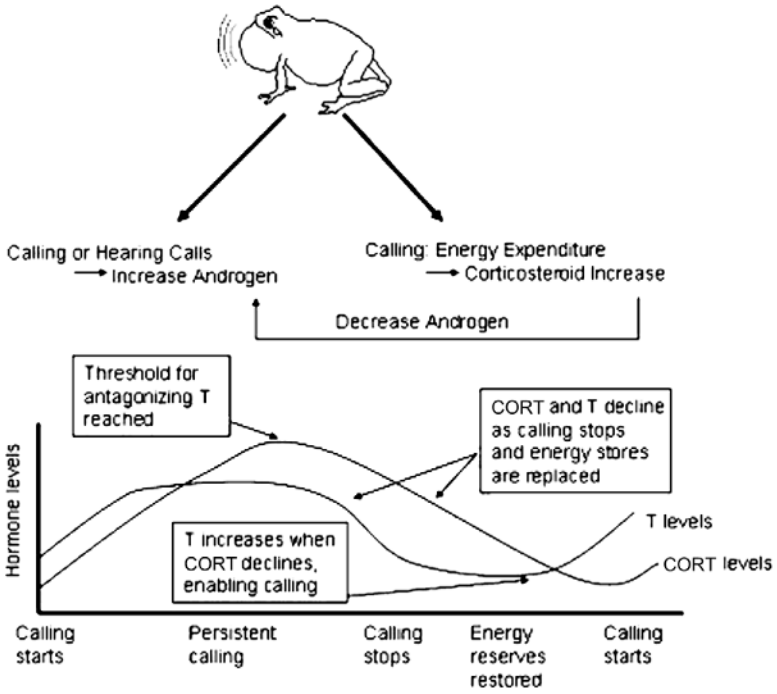

FIGURE 6.5 The reciprocal relationship between plasma glucocorticoids and plasma androgens is important for maintaining a balance between reproductive effort and energy supply according to the energetics–hormone–vocalization model proposed by Emerson (2001). At the beginning of vocalization, both corticosterone and testosterone plasma levels begin to rise in order to facilitate calling and to supply energy for calling, respectively. Once a threshold level of plasma corticosterone is reached, plasma testosterone begins to decline, calling stops, and energy supplies are restored. *Reproduced with permission from Wilczynski, Lynch, and O'Bryant (2005).*

engaging in reproductive behavior and large breeding choruses can make amphibians more vulnerable to predation (Tuttle & Ryan, 1981; Ryan, 1985) or more susceptible to pathogens (such as the chytrid fungus) and parasites (such as blood-sucking flies (Bernal, Rand, & Ryan, 2006; Bernal, Page, Rand, & Ryan, 2007)). Perhaps the HPA axis serves in part to balance the tradeoffs between predation risk and reproduction and in a sense reduce the 'survival cost' (Magnhagen, 1991) of reproduction. Frog-eating bats and blood-sucking flies are attracted to the calling of the male túngara frog (*Physalaemus pustulosus*) (Tuttle & Ryan, 1981; Ryan, 1985; Bernal et al., 2006; Bernal, Rand, & Ryan, 2007). Males of this species exhibit a number of adaptations to reduce predation risk during mating (Bernal et al., 2007a), including stopping their calls (Ryan, 1985). Although there are no data at present showing that exposure to predators leads to increased HPA activity in amphibians, predator-induced activation of the HPA axis has been demonstrated in mammals (see Park, Zoladz, Conrad, Fleshner, & Diamond, 2008).

ABBREVIATIONS

ACTH	Corticotropin
AEA	Anandamide
AMG	Amygdala
AVT	Arginine vasotocin
cAMP	Cyclic adenosine monophosphate
CB_1	Cannabinoid type 1
CBG	Corticosteroid-binding protein
cGR	Cytosolic glucocorticoid receptor
CNS	Central nervous system
CORT	Corticosterone
CRF-R1	Corticotropin-releasing factor receptor 1
CRF-R2	Corticotropin-releasing factor receptor 2
DDD	Dichlorodiphenyldichloroethane
E	Energetic requirements
GAS	General adaptation syndrome
GnRH	Gonadotropin-releasing hormone
HPA	Hypothalamus–pituitary–adrenal
IML	Intermediolateral cell column
MC2R	Melanocortin 2 receptor
mGR	Membrane-bound glucocorticoid receptor
Mp	Medial pallium
MSH	Melanotropin
NTS	Nucleus of the solitary tract
P450c11	11β-hydroxylase
PVN	Paraventricular nucleus
Rm	Medial reticular nucleus
RU-486	Mifepristone
RVLM	Rostral ventrolateral medulla
serpin	Serine protease inhibitors
SVG	Sauvagine
UCN	Urocortin (UCN-1, UCN-2, UCN-3)

REFERENCES

Accordi, F. (1991). The chromaffin cells of urodele amphibians. *J. Anat., 179*, 1−8.

Barsano, C. P., & Baumann, G. (1989). Editorial: simple algebraic and graphic methods for the apportionment of hormone (and receptor) into bound and free fractions in binding equilibria; or how to calculate bound and free hormone? *Endocrinology, 124*, 1101−1106.

Belden, L. K., Moore, I. T., Wingfield, J. C., & Blaustein, A. R. (2005). Corticosterone and growth in Pacific Treefrog (*Hyla regilla*) tadpoles. *Copeia, 2005*, 424−430.

Bernal, X. E., Rand, A. S., & Ryan, M. J. (2006). Acoustic preferences and localization performance of blood-sucking flies (*Corethrella coquillett*) to tungara frog calls. *Behav. Ecol., 17*, 709−715.

Bernal, X. E., Rand, A. S., & Ryan, M. J. (2007b). Sexual differences in the behavioral response of tungara frogs, *Physalaemus pustulosus*, to cues associated with increased predation risk. *Ethology, 113*, 755−763.

Bernal, X. E., Page, R. A., Rand, A. S., & Ryan, M. J. (2007a). Natural history miscellany—Cues for eavesdroppers: Do frog calls indicate prey density and quality? *Amer. Nat., 169*, 409−415.

Bhargava, S., & Rao, P. D. (1993). Distribution of corticotropin-releasing factor immunoreactive neurons in the brain of the tiger frog, *Rana tigrina*. *Neurosci. Lett., 154*, 27−30.

Biswas, N. M., Chaudhuri, G. R., Sarkar, M., & Sengupta, R. (2000). Influence of adrenal cortex on testicular activity in the toad during the breeding season. *Life. Sci., 66*, 1253−1260.

Bonett, R. M., Hu, F., Bagamasbad, P., & Denver, R. J. (2008). Stressor and glucocorticoid-dependent induction of the immediate early gene Kruppel-like factor 9 (KLF9): implications for neural development and plasticity. *Endocrinology, 150*, 1757−1765.

Boorse, G. C., & Denver, R. J. (2004). Expression and hypophysiotropic actions of corticotropin-releasing factor in *Xenopus laevis*. *Gen. Comp. Endocrinol., 137*, 272−282.

Boorse, G. C., Crespi, E. J., Dautzenberg, F. M., & Denver, R. J. (2005). Urocortins of the South African clawed frog, *Xenopus laevis*: conservation of structure and function in tetrapod evolution. *Endocrinology, 146*, 4851−4860.

Boyd, S. K. (1994a). Arginine vasotocin facilitation of advertisement calling and call phonotaxis in bullfrogs. *Horm. Behav., 28*, 232−240.

Boyd, S. K. (1994b). Gonadal steroid modulation of vasotocin concentrations in the bullfrog brain. *Neuroendocrinology, 60*, 150−156.

Breuner, C. W., Jennings, D. H., Moore, M. C., & Orchinik, M. (2000). Pharmacological adrenalectomy with mitotane. *Gen. Comp. Endocrinol., 120*, 27−34.

Brock, A.J., 1916. Galen, *On the Natural Faculties, 170 AD*. English Translation, New York: G.P. Putnam's Sons.

Burmeister, S., Somes, C., & Wilczynski, W. (2001). Behavioral and hormonal effects of exogenous vasotocin and corticosterone in the green treefrog. *Gen. Comp. Endocrinol., 122*, 189−197.

Buttgereit, F., Straub, R. H., Wehling, M., & Burmester, G. R. (2004). Glucocorticoids in the treatment of rheumatic diseases: an update on the mechanisms of action. *Arthritis. Rheum., 50*, 3408−3417.

Calle, M., Corstens, G. J., Wang, L., Kozicz, T., Denver, R. J., Barendregt, H. P., et al. (2005). Evidence that urocortin I acts as a neurohormone to stimulate alpha MSH release in the toad *Xenopus laevis*. *Brain. Res., 1040*, 14−28.

Calle, M., Jenks, B. G., Corstens, G. J., Veening, J. G., Barendregt, H. P., & Roubos, E. W. (2006). Localisation and physiological regulation of corticotrophin-releasing factor receptor 1 mRNA in the *Xenopus laevis* brain and pituitary gland. *J. Neuroendocrinol., 18*, 797−805.

Carr, J. A., & Norris, D. O. (1990). Immunohistochemical localization of corticotrophin-releasing factor and arginine vastocin-like immnnoreactivities in the train and pitutiary of the American bullforg (*Rana catesbeiana*) during development and metamorphosis. *Gen. Comp. Endocrinol., 78*, 180−188.

Carr, J. A., & Norris, D. O. (2005). The hypothalamus−pituitary Axis. In D. O. Norris, & J. A. Carr (Eds.), *"Endocrine Disruption: Biological Bases for Health Effects in Wildlife and Humans"* (pp. 58−86). N.Y: Oxford University Press.

Carr, J. A., & Zozzaro, P. E. (2004). The toad iris assay: a simple method for evaluating CRH action on the sympathetic nervous system. *Gen. Comp. Endocrinol., 135*, 134−141.

Cannon, W. B. (1932). *The Wisdom of the Body.* New York: Norton.

Chen, F., Watson, C. S., & Gametchu, B. (1999). Association of the glucocorticoid receptor alternatively-spliced transcript 1A with the presence of the high molecular weight membrane glucocorticoid receptor in mouse lymphoma cells. *J. Cell. Biochem., 74*, 430−446.

Coddington, E. J., & Cree, A. (1995). Effect of acute captivity stress on plasma concentrations of corticosterone and sex steroids in female whistling frogs, *Litoria ewingi*. *Gen. Comp. Endocrinol., 100*, 33−38.

Coddington, E., & Moore, F. L. (2003). Neuroendocrinology of context-dependent stress responses: vasotocin alters the effect of corticosterone on amphibian behaviors. *Horm. Behav., 43*, 222−228.

Coddington, E., Lewis, C., Rose, J. D., & Moore, F. L. (2007). Endocannabinoids mediate the effects of acute stress and corticosterone on sex behavior. *Endocrinology., 148*, 493−500.

Cooperman, M. D., Reed, J. M., & Romero, L. M. (2004). The effects of terrestrial and breeding densities on corticosterone and testosterone levels in spotted salamanders, *Ambystoma maculatum*. *Can. J. Zool., 11*, 1795−1803.

Crespi, E. J., & Denver, R. J. (2004). Ontogeny of corticotropin-releasing factor effects on locomotion and foraging in the Western spadefoot toad (*Spea hammondii*). *Horm. Behav., 46*, 399−410.

Dautzenberg, F. M., Dietrich, K., Palchaudhuri, M. R., & Spiess, J. (1997). Identification of two corticotropin-releasing factor receptors from *Xenopus laevis* with high ligand selectivity: unusual pharmacology of the type 1 receptor. *J. Neurochem., 69*, 1640−1649.

Dautzenberg, F. M., Py-Lang, G., Higelin, J., Fischer, C., Wright, M. B., & Huber, G. (2001). Different binding modes of amphibian and human corticotropin-releasing factor type 1 and type 2 receptors: evidence for evolutionary differences. *J. Pharmacol. Exp. Ther., 296*, 113−120.

Davenport, H. W. (1974). *The ABCs of Acid Base Chemistry* (Sixth Edition). Chicago: University of Chicago Press.

Denari, D., & Ceballos, N. R. (2006). Cytosolic glucocorticoid receptor in the testis of *Bufo arenarum*: seasonal changes in its binding parameters. *Gen. Comp. Endocrinol., 147*, 247−254.

Denver, R. J. (1998). Hormonal correlates of environmentally induced metamorphosis in the Western spadefoot toad, *Scaphiopus hammondii*. *Gen. Comp. Endocrinol., 110*, 326−336.

Denver, R. J., Mirhadi, N., & Phillips, M. (1998). Adaptive plasticity in amphibian metamorphosis: response of *Scaphiopus hammondii* tadpoles to habitat desiccation. *Ecology, 79*, 1859−1872.

Di, S., Malcher-Lopes, R., Halmos, K. C., & Tasker, J. G. (2003). Nongenomic glucocorticoid inhibition via endocannabinoid release in the hypothalamus: a fast feedback mechanism. *J. Neurosci., 23*, 4850−4857.

Di, S., Malcher-Lopes, R., Marcheselli, V. L., Bazan, N. G., & Tasker, J. G. (2005). Rapid glucocorticoid-mediated endocannabinoid release and opposing regulation of glutamate and -aminobutyric acid inputs to hypothalamic magnocellular neurons. *Endocrinology, 146*, 4292−4301.

Du Preez, L. H., Solomon, K. R., Carr, J. A., Giesy, J. P., Gross, T. S., Kendall, R. J., Smith, E. E., Van Der Kraak, G., & Weldon, C. (2005). Population structure characterization of the clawed frog (*Xenopus laevis*) in corn-growing versus non-corn-growing areas in South Africa. *African. J. Herpetol., 54*, 61−68.

Emerson, S. B. (2001). Male advertisement calls: behavioral variation and physiological processes. In M. J. Ryan (Ed.), *"Anuran Communication"* (pp. 36−44). Washington: Smithsonian Institution Press.

Emerson, S., & Hess, D. L. (2001). Glucocorticoids, androgens, testis mass, and the energetics of vocalization in breeding male frogs. *Horm. Behav., 39*, 59−69.

Emmert, M. H., & Herman, J. P. (1999). Differential forebrain c-fos mRNA induction by ether inhalation and novelty: evidence for distinctive stress pathways. *Brain. Res., 845*, 60−67.

Evans, S. J., Murray, T. F., & Moore, F. L. (2000). Partial purification and biochemical characterization of a membrane glucocorticoid receptor from an amphibian brain. *J. Steroid. Biochem. Mol. Biol., 72*, 209−221.

Funakoshi, K., & Nakano, M. (2007). The sympathetic nervous system of anamniotes. *Brain. Behav. Evol., 69*, 105−113.

Fraker, M. E., Cuddapah, V., McCollum, S. A., Hu, F., Relyea, R., Hempel, J., & Denver, R. J. (2009). Characterization of an alarm pheromone secreted by amphibian tadpoles that induces behavioral inhibition and suppression of the neuroendocrine stress axis. *Horm. Behav.,* in press.

Gametchu, B., Chen, F., Sackey, F., Powell, C., & Watson, C. S. (1999). Plasma membrane-resident glucocorticoid receptors in rodent lymphoma and human leukemia models. *Steroids, 64*, 107−119.

Gendron, A. D., Bishop, C. A., Fortin, R., & Hontela, A. (1997). *In vivo* testing of the functional integrity of the corticosterone-producing axis in mudpuppy (amphibia) exposed to chlorinated hydrocarbons in the wild. *Environ. Toxicol. Chem., 16*, 1694−1706.

Glennemeier, K. A., & Denver, R. J. (2001). Sublethal effects of chronic exposure to an organochlorine compound on northern leopard frog (*Rana pipiens*) tadpoles. *Environ. Toxicol., 16*, 287−297.

Glennemeier, K. A., & Denver, R. J. (2002). Role for corticoids in mediating the response of *Rana pipiens* tadpoles to intraspecific competition. *J. Exp. Zool., 292*, 32−40.

Goater, C. P. (1994). Growth and survival of postmetamorphic toads—interactions among larval history, density, and parasitism. *Ecology, 75*, 2264−2274.

Gobbetti, A., & Zerani, M. (1996). Possible mechanism for the first response to short captivity stress in the water frog, *Rana esculenta. J. Endocrinol., 148*, 233−239.

González, A., & Smeets, W. J. (1993). Noradrenaline in the brain of the South African clawed frog *Xenopus laevis*: a study with antibodies against noradrenaline and dopamine-beta-hydroxylase. *J. Comp. Neurol., 331*, 363−374.

González, A., & Smeets, W. J. (1995). Noradrenergic and adrenergic systems in the brain of the urodele amphibian, *Pleurodeles waltlii*, as revealed by immunohistochemical methods. *Cell. Tissue. Res., 279*, 619−627.

Gray, M. J., & Smith, L. M. (2005). Influence of land use on postmetamorphic body size of playa lake amphibians. *J. Wildlife. Management., 69*, 515−524.

Gray, M. J., Smith, L. M., Miller, D. L., & Bursey, C. R. (2007). Influences of agricultural land use on *Clinostomum attenuatum* metacercariae prevalence in Southern Great Plains amphibians, U.S.A. Herp. *Conservation. Biol., 2*, 23−28.

Hayes, T. B., Case, P., Chui, S., Chung, D., Haeffele, C., Haston, K., et al. (2006). Pesticide mixtures, endocrine disruption, and amphibian declines: are we underestimating the impact? *Environ. Health. Perspect., 114*(Suppl 1), 40−50.

Herman, J. P., McCreary, B. J., Bettenhausen, K., & Ziegler, D. R. (2002). Neurocircuit activation of the hypothalamo−pituitary−adrenocortical axis: Roles for ascending noradrenergic systems. In R. McCarty, G. Aguilera, E. Sabban, & R. Kvetnansky (Eds.), *"Stress: Neural, endocrine and molecular studies."* (pp. 19−24). London, UK: CRC Press.

Herring, G., & Gawlik, D. E. (2007). The role of stress proteins in the study of allostatic overload in birds: use and applicability to current studies in avian ecology. *Scientific World Journal., 7*, 1596−1602.

Hillman, S., Withers, P., & Kimmel, P. (1998). Plasma catecholamines with hemorrhage in the bullfrog, *Rana catesbeiana. J. Exp. Zool., 280*, 174−181.

Hollis, D. M., Coddington, E., & Moore, F. L. (2006). Neuroanatomical distribution of cannabinoid receptor gene expression in the brain of the rough-skinned newt, *Taricha granulosa. Brain. Behav. Evol., 67*, 135−149.

Homan, R. N., Regosin, J. V., Rodrigues, D. M., Reed, J. M., Windmiller, B. S., & Romero, L. M. (2003). Impacts of varying habitat quality on the physiological stress of spotted salamanders (*Ambystoma maculatum*). *Anim. Conservation, 6*, 11−18.

Hopkins, W. A., Mendonça, M. T., & Congdon, J. D. (1997). Increased circulating levels of testosterone and corticosterone in southern toads, *Bufo terrestris*, exposed to coal combustion waste. *Gen. Comp. Endocrinol., 108*, 237−246.

Hopkins, W. A., Mendonça, M. T., & Congdon, J. D. (1999). Responsiveness of the hypothalamo—pituitary—interrenal axis in an amphibian (*Bufo terrestris*) exposed to coal combustion wastes. *Comp. Biochem. Physiol. C. Pharmacol. Toxicol. Endocrinol., 122*, 191−196.

Hopkins, W. A., Mendonça, M. T., Rowe, C. L., & Congdon, J. D. (1998). Elevated trace element concentrations in southern toads, *Bufo terrestris*, exposed to coal combustion waste. *Arch. Environ. Contam. Toxicol., 35*, 325−329.

Houck, L. D., Mendonça, M. T., Lynch, T. K., & Scott, D. E. (1996). Courtship behavior and plasma levels of androgens and corticosterone in male marbled salamanders, *Ambystoma opacum* (ambystomatidae). *Gen. Comp. Endocrinol., 104*, 243−252.

Hu, F., Crespi, E. J., & Denver, R. J. (2008). Programming neuroendocrine stress axis activity by exposure to glucocorticoids during postembryonic development of the frog, *Xenopus laevis. Endocrinology., 149*, 5470−5481.

Ito, Y., Okada, R., Noriyuki, T., & Kikuyama, S. (2006). Cloning and distribution of the bullfrog type 1 and type 2 corticotropin-releasing factor receptors. *Gen. Comp. Endocrinol., 146*, 291−295.

Juráni, M., Murgas, K., Mikulaj, L., & Babusíková, F. (1973). Effect of stress and environmental temperature on adrenal function in *Rana esculenta*. *J. Endocrinol., 57*, 385−391.

Kemnitz, C. P., Fox, L. M., & McNulty, J. A. (1990). Ultrastructure and organization of circumventricular organs and endolymphatic tubules in the treefrog, *Hyla versicolor. J. Morphol., 204*, 197−208.

Knutson, M. G., Richardson, W. B., Reineke, D. M., Gray, B. R., Parmelee, J. R., & Weick, S. E. (2004). Agricultural ponds support amphibian populations. *Ecol. Appl., 14*, 669−684.

Kupwade, V. A., & Saidapur, S. K. (1987). Effect of dexamethasone and ACTH on oocyte growth and recruitment in the frog *Rana cyanophlyctis* during the prebreeding vitellogenic phase. *Gen. Comp. Endocrinol., 65*, 394−398.

Laberge, F., Mühlenbrock-Lenter, S., Grunwald, W., & Roth, G. (2006). Evolution of the amygdala: new insights from studies in amphibians. *Brain. Behav. Evol., 67*, 177−187.

Leary, C. J., Jessop, T. S., Garcia, A. M., & Knapp, R. (2004). Steroid hormone profiles and relative body condition of calling and satellite toads: implications for proximate regulation of behavior in anurans. *Behav. Ecol., 15*, 313−320.

Leboulenger, F., Delarue, C., Belanger, A., Perroteau, I., Netchitailo, P., Leroux, P., et al. (1982). Direct radioimmunoassay for plasma corticosterone and aldosterone in frog. I. Validation of the methods and evidence for daily rhythms in a natural environment. *Gen. Comp. Endocrinol., 46*, 521−532.

Leboulenger, F., Dupont, W., Vaudry, H., & Vaillant, R. (1976). Role of various regulation factors of interrenal gland activity in response to stress in the green frog *Rana esculenta*. *C.R. Seances. Soc. Biol. Fil., 170*, 434−441.

Lewis, C. M., & Rose, J. D. (2003). Rapid corticosterone-induced impairment of amplectic clasping occurs in the spinal cord of roughskin newts (*Taricha granulosa*). *Horm. Behav., 43*, 93−98.

Licht, P., McGreery, B. R., Barnes, R., & Pang, R. (1983). Seasonal and stress related changes in plasma gonadotropins, sex steroids and corticosterone in the bullfrog, *Rana catesbeiana*. *Gen. Comp. Endocrinol., 50*, 124−145.

Lima, S. L., & Dill, L. M. (1990). Behavioral decisions made under the risk of predation: a review and prospectus. *Can. J. Zool., 68*, 619−640.

Liu, X. H., Zeng, J. W., Zhao, Y. D., Chen, P. H., Xiao, Z., & Ruan, H. Z. (2008). Rapid inhibition of ATP-induced currents by corticosterone in rat dorsal root ganglion neurons. *Pharmacology, 82*, 164−170.

Magnhagen, C. (1991). Predation risk as a cost of reproduction. *Trends. Ecol. Evol., 6*, 183−186.

Marler, C. A., & Ryan, M. J. (1996). Energetic constraints and steroid hormone correlates of male calling behavior in the túngara frog. *J. Zool. Lond., 240*, 397−409.

Martin, B., & Ozon, R. (1975). Steroid−protein interactions in non-mammalian vertebrates. II. Steroids binding proteins in the serum of amphibians; a physiological approach. *Biol. Reprod., 13*, 371−380.

McEwen, B. S., & Wingfield, J. C. (2003). The concept of allostasis in biology and biomedicine. *Horm. Behav., 43*, 2−15.

Mendonça, M. T., Licht, P., Ryan, M. J., & Barnes, R. (1985). Changes in hormone levels in relation to breeding behavior in male bullfrogs (*Rana catesbeiana*) at the individual and population levels. *Gen. Comp. Endocrinol., 58*, 270−279.

Milner, T. A., Reis, D. J., Pickel, V. M., Aicher, S. A., & Giuliano, R. (1993). Ultrastructural localization and afferent sources of corticotropin-releasing factor in the rat rostral ventrolateral medulla: implications for central cardiovascular regulation. *J. Comp. Neurol., 333*, 151−167.

Monder, C., Hardy, M. P., Blanchard, R. J., & Blanchard, D. C. (1994b). Comparative aspects of 11β-hydroxysteroid dehydrogenase. Testicular 11β- hydroxysteroid dehydrogenase: development of a model for the mediation of Leydig cell function by corticosteroids. *Steroids., 59*, 69−73.

Monder, C., Miro, V. Y., Marandici, A., and Hardy, M.P. (1994a). 11β-Hydroxysteroid dehydrogenase alleviates glucocorticoid-mediated inhibition of steroidogenesis in rat Leydig cells. *Endocrinology,* 134, 1199−1204.

Montecucchi, P. C., & Henschen, A. (1981). Amino acid composition and sequence analysis of sauvagine, a new active peptide from the skin of *Phyllomedusa sauvagei*. *Int. J. Pept. Protein. Res., 18*, 113−120.

Moore, F. L., & Evans, S. J. (1999). Steroid hormones use non-genomic mechanisms to control brain functions and behaviors: a review of evidence. *Brain. Behav. Evol., 54*, 41−50.

Moore, F. L., & Miller, L. J. (1984). Stress-induced inhibition of sexual behavior: corticosterone inhibits courtship behaviors of a male amphibian (*Taricha granulosa*). *Horm. Behav., 18*, 400−410.

Moore, F. L., & Zoeller, R. T. (1985). Stress-induced inhibition of reproduction: evidence of suppressed secretion of LH-RH in an amphibian. *Gen. Comp. Endocrinol., 60*, 252−258.

Moreno, N., & González, A. (2003). Hodological characterization of the medial amygdala in anuran amphibians. *J. Comp. Neurol., 466*, 389−408.

Moreno, N., & González, A. (2007a). Regionalization of the telencephalon in urodele amphibians and its bearing on the identification of the amygdaloid complex. *Front. Neuroanat., 1*, 1−12.

Moreno, N., & González, A. (2007b). Development of the vomeronasal amygdala in anuran amphibians: hodological, neurochemical, and gene expression characterization. *J. Comp. Neurol., 503*, 815−831.

Mosconi, G., Palermo, F., Carotti, M., Kikuyama, S., Yamamoto, K., & Polzonetti-Magni, A. M. (2006). Neuroendocrine modulation of stress response in the anuran, *Rana esculenta*. *Amphibia-Reptilia., 27*, 401−408.

Mountjoy, K. G., Robbins, L. S., Mortrud, M. T., & Cone, R. D. (1992). The cloning of a family of genes that encode the melanocortin receptors. *Science, 257*, 1248−1251.

Okada, R., Miller, M. F., Yamamoto, K., De Groef, B., Denver, R. J., & Kikuyama, S. (2007). Involvement of the corticotropin-releasing factor (CRF) type 2 receptor in CRF-induced thyrotropin release by the amphibian pituitary gland. *Gen. Comp. Endocrinol., 150*, 437−444.

Olivereau, M., Vandesande, F., Boucique, E., Ollevier, F., & Olivereau, J. M. (1987). Immunocytochemical localization and spatial relation to the adenohypophysis of a somatostatin-like and a corticotropin-releasing factor-like peptide in the brain of four amphibian species. *Cell. Tissue. Res., 247*, 317−324.

Olsen, C. M., Lovering, A. T., & Carr, J. A. (1999). Alpha-melanocyte stimulating hormone and habituation of prey-catching behavior in the Texas toad, *Bufo speciosus*. *Horm. Behav., 36*, 62−69.

Orchinik, M., Licht, P., & Crews, D. (1988). Plasma steroid concentrations change in response to sexual behavior in *Bufo marinus*. *Horm. Behav., 22*, 338−350.

Orchinik, M., Matthews, L., & Gasser, P. J. (2000). Distinct specificity for corticosteroid binding sites in amphibian cytosol, neuronal membranes, and plasma. *Gen. Comp. Endocrinol., 118*, 284−301.

Orchinik, M., Murray, T. F., & Moore, F. L. (1991). A corticosteroid receptor in neuronal membranes. *Science., 252*, 1848−1851.

Orchinik, M., Murray, T. F., Franklin, P. H., & Moore, F. L. (1992). Guanyl nucleotides modulate binding to steroid receptors in neuronal membranes. *Proc. Natl. Acad. Sci. U.S.A., 89*, 3830−3834.

Pancak, M. K., & Taylor, D. H. (1983). Seasonal and daily plasma corticosterone rhythms in American toads, *Bufo americanus*. *Gen. Comp. Endocrinol., 50*, 490−497.

Pancharatna, K., & Saidapur, S. K. (1992). A study of ovarian follicular kinetics, oviduct, fat body, and liver mass cycles in laboratory-maintained *Rana cyanophlyctis* in comparison with wild-caught frogs. *J. Morphol., 214*, 123−129.

Paolucci, M., Esposito, V., Difiore, M., & Botte, V. (1990). Effects of short postcapture confinement on plasma reproductive hormone and corticosterone profiles in *Rana esculenta* during the sexual cycle. *Bollettino. di. zoologia., 57*, 253−259.

Park, C. R., Zoladz, P. R., Conrad, C. D., Fleshner, M., & Diamond, D. M. (2008). Acute predator stress impairs the consolidation and retrieval of hippocampus-dependent memory in male and female rats. *Learn. Mem., 15*, 271−280.

Prado, C. P. A., & Haddad, C. F. B. (2005). Size-fecundity relationships and reproductive investment in female frogs in the Pantanal, southwestern Brazil. *Herp. J., 15*, 181−189.

Rose, J. D. (2000). Corticosteroid actions from neuronal membrane to behavior: neurophysiological mechanisms underlying rapid behavioral effects of corticosterone. *Biochem. Cell. Biol., 78*, 1−9.

Rose, J. D., Marrs, G. S., & Moore, F. L. (1998). Rapid, corticosterone-induced disruption of medullary sensorimotor integration related to suppression of amplectic clasping in behaving roughskin newts (*Taricha granulosa*). *Horm. Behav., 34*, 268−282.

Rose, J. D., Moore, F. L., & Orchinik, M. (1993). Rapid neurophysiological effects of corticosterone on medullary neurons: relationship to stress induced suppression of courtship clasping in an amphibian. *Neuroendocrinology, 57*, 815−824.

Rose, J. D., Kinnaird, J. R., & Moore, F. L. (1995). Neurophysiological effects of vasotocin and corticosterone on medullary neurons: implications for hormonal control of amphibian courtship behavior. *Neuroendocrinology, 62*, 406−417.

Roseboom, P. H., Nanda, S. A., Bakshi, V. P., Trentani, A., Newman, S. M., & Kalin, N. H. (2007). Predator threat induces behavioral inhibition, pituitary−adrenal activation and changes in amygdala CRF-binding protein gene expression. *Psychoneuroendocrinology, 32*, 44−55.

Rowe, C. L., Hopkins, W. A., & Coffman, V. R. (2001). Failed recruitment of southern toads (*Bufo terrestris*) in a trace element-contaminated breeding habitat: direct and indirect effects that may lead to a local population sink. *Arch. Environ. Contam. Toxicol., 40*, 399−405.

Ryan, M. J. (1985). *The Tungara frog: A study in sexual selection and communication*. Chicago: The University of Chicago Press.

Sackey, F. N., Watson, C. S., & Gametchu, B. (1997). Cell cycle regulation of membrane glucocorticoid receptor in CCRF-CEM human ALL cells: correlation to apoptosis. *Am. J. Physiol., 273*, E571−E583.

Sawchenko, P. E. (1987). Evidence for differential regulation of corticotropin-releasing factor and vasopressin immunoreactivities in parvocellular neurosecretory and autonomic-related projections of the paraventricular nucleus. *Brain. Res, 437*, 253−263.

Sawchenko, P. E., & Swanson, L. W. (1982). The organization of noradrenergic pathways from the brainstem to the paraventricular and supraoptic nuclei in the rat. *Brain Res., 257*, 275−325.

Sawchenko, P. E., Brown, E. R., Chan, R. K., Ericsson, A., Li, H. Y., Roland, B. L., et al. (1996). The Paraventricular nucleus of the hypothalamus and the functional neuroanatomy of visceromotor responses to stren. *Preg. Brain Res., 107*, 201−222.

Selye, H. (1936). A syndrome produced by diverse nocuous agents. *Nature, 138*, 32.

Selye, H. (1956). *The Stress of Life*. New York: McGraw Hill.

Soderstrom, K., Leid, M., Moore, F. L., & Murray, T. F. (2000). Behavioral, pharmacological, and molecular characterization of an amphibian cannabinoid receptor. *J. Neurochem., 75*, 413−423.

Song, I. H., & Buttgereit, F. (2006). Non-genomic glucocorticoid effects to provide the basis for new drug developments. *Mol. Cell. Endocrinol., 246*, 142−146.

Stahn, C., & Buttgereit, F. (2008). Genomic and nongenomic effects of glucocorticoids. *Nat. Clin. Pract. Rheumatol., 4*, 525−533.

Sterling, P., & Eyer, J. (1988). Allostasis: a new paradigm to explain arousal pathology. In S. Fisher, & J. Reason (Eds.), *"Handbook of Life Stress, Cognition and Health"* (pp. 629−649). New York: Wiley.

Taigen, T. L., & Wells, K. D. (1985). Energetics of vocalization by an anuran amphibian (*Hyla versicolor*). *J. Comp. Physiol. B., 155*, 163−170.

Tonon, M. C., Burlet, A., Lauber, M., Cuet, P., Jégou, S., Gouteux, L., Ling, N., & Vaudry, H. (1985). Immunohistochemical localization and radioimmunoassay of corticotropin-releasing factor in the forebrain and hypophysis of the frog *Rana ridibunda*. *Neuroendocrinology, 40*, 109−119.

Tonon, M. C., Cuet, P., Lamacz, M., Jegou, S., Cote, J., Gouteaux, L., Ling, N., Pelletier, G., & Vaudry, H. (1986). Comparative effects of corticotropin-releasing factor, arginine vasopressin, and related neuropeptides on the secretion of ACTH and alpha-MSH by frog anterior pituitary cells and neurointermediate lobes *in vitro*. *Gen. Comp. Endocrinol., 61*, 438−445.

Towle, A. C., & Sze, P. Y. (1983). Steroid binding to synaptic plasma membrane: Differential binding of glucocorticoids and gonadal steroids. *J. Steroid. Biochem., 18*, 135−143.

Tsai, P. S., Lunden, J. B., & Jones, J. T. (2003). Effects of steroid hormones on spermatogenesis and GnRH release in male Leopard frogs, *Rana pipiens*. *Gen. Comp. Endocrinol., 134*, 330−338.

Tureková, L., & Juráni, M. (1978). Participation of hypothalamus in the response of interrenal tissue to stress of frog *Rana ridibunda*. *Endocrinol. Exp., 12*, 171−178.

Tuttle, M. D., & Ryan, M. J. (1981). Bat predation and the evolution of frog vocalizations in the Neotropics. *Science, 214*, 677−678.

Walsberg, G. E. (2003). How useful is energy balance as a overall index of stress in animals? *Horm. Behav., 43*, 16−17.

Ward, C. K., Fontes, C., Breuner, C. W., & Mendonça, M. T. (2007). Characterization and quantification of corticosteroid-binding globulin in a southern toad, *Bufo terrestris*, exposed to coal-combustion-waste. *Gen. Comp. Endocrinol., 152*, 82−88.

Wells, K. D. (2001). The energetics of calling in frogs. In M. J. Ryan (Ed.), *"Anuran communication"* (pp. 45−60). Washington: Smithsonian Institution.

Wilczynski, W., Lynch, K. S., & O'Bryant, E. L. (2005). Current research in amphibians: studies integrating endocrinology, behavior, and neurobiology. *Horm. Behav., 48*, 440−450.

Wingfield, J. C., Moore, M. C., & Farner, D. S. (1983). Endocrine responses to inclement weather in naturally breeding populations of white-crowned sparrows. *Auk., 100*, 56−62.

Withers, P. C., Hillman, S. S., & Kimmel, P. B. (1988). Effects of activity, hemorrhage, and dehydration on plasma catecholamine levels in the marine toad (*Bufo marinus*). *Gen. Comp. Endocrinol., 72*, 63−71.

Woolbright, L. L., & Stewart, M. M. (1987). Foraging success of the tropical frog, *Eleutherodactylus coqui*—the cost of calling. *Copeia, 1987*, 69−75.

Yao, M., & Denver, R. J. (2007). Regulation of vertebrate corticotropin-releasing factor genes. *Gen. Comp. Endocrinol., 153*, 200−216.

Yao, M., Hu, F., & Denver, R. J. (2008b). Distribution and corticosteroid regulation of glucocorticoid receptor in the brain of *Xenopus laevis*. *J. Comp. Neurol., 508*, 967−982.

Yao, M., Stenzel-Poore, M., & Denver, R. J. (2007). Structural and functional conservation of vertebrate corticotropin-releasing factor genes: evidence for a critical role for a conserved cyclic AMP response element. *Endocrinology, 148*, 2518−2531.

Yao, M., Schulkin, J., & Denver, R. J. (2008a). Evolutionarily conserved glucocorticoid regulation of corticotropin-releasing factor expression. *Endocrinology, 149*, 2352−2360.

Yao, M., Westphal, N. J., & Denver, R. J. (2004). Distribution and acute stressor-induced activation of corticotrophin-releasing hormone neurones in the central nervous system of *Xenopus laevis*. *J. Neuroendocrinol., 16*, 880−893.

Zerani, M., Amabili, F., Mosconi, G., & Gobbetti, A. (1991). Effects of captivity stress on plasma steroid-levels in the green frog, *Rana esculenta*, during the annual reproductive-cycle. *Comp. Biochem. Physiol. A-Physiol., 98*, 491−496.

Ziegler, D. R., & Herman, J. P. (2002). Neurocircuitry of stress integration: Anatomical pathways regulating the hypothalamo−pituitary−adrenocortical axis of the rat. *Integ. Comp. Biol., 42*, 541−551.

Chapter 7

Maternal Adaptations to Reproductive Modes in Amphibians

Hartmut Greven
Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany

SUMMARY
This chapter surveys structural, physiological, and endocrinological adaptations as well as parental–offspring interactions in amphibians practicing oviductal, skin, vocal sac, and stomach incubation. In spite of this diversity of incubation sites, many parallels exist regarding basic requirements: the preparation of the incubation site; maintenance of a suitable milieu in the case of 'closed' brood chambers, e.g., by secretions and membrane-bound pumps; provision of the young with oxygen and removal of metabolic waste material, e.g., by thinning of the epithelia involved and increased vascularization; and in some cases allocation of food beyond the yolk reserves by uterine secretions, oophagy, epitheliophagy, adelphophagy, and/or a placenta. Hormones mediate the cyclical changes of most incubation sites, but knowledge of specific endocrinological control mechanisms is very sparse. In most oviductal brooders and possibly in a skin brooder, postovulatory follicles persist and secrete progesterone. There is evidence in some cases that eggs, embryos, and hatched larvae manipulate the parental tissue by secreting prostaglandins (and possibly other substances not yet identified) and by ingesting maternal tissue.

1. TOPICS AND TERMINOLOGY

Amphibians typically lay eggs in water and from these eggs larvae hatch and undergo metamorphosis to finally live (semi-) terrestrially. However, various types of parental investment in offspring after fertilization, collectively referred to as parental care (e.g., Lehtinen & Nussbaum, 2003), have evolved that may affect the structure, physiology, endocrinology, and behavior of both parent (mostly the mother) and offspring. Elaborate regulatory mechanisms and mutual influences can be expected when young develop completely or predominantly in or on the body of one of the parents.

The most common organ used for brooding or incubation inside the vertebrate body is the lower portion of the oviduct, usually called a uterus. Oviductal incubation has evolved repeatedly in the Amphibia and apparently several times in each of the three extant orders. This mode of incubation and release, or birth, of the young in a more or less advanced state is traditionally termed viviparity (this term is occasionally limited to metamorphosed juveniles). (For some objections against the use of the term viviparity see Packard (1989), Greven (2002; 2003b).) If the degree of maternal nutrition is known, lecithotrophic viviparity (or ovoviviparity; embryos are exclusively yolk-dependent) or matrotrophic viviparity (young are supplied by the parent with essential nutrition beyond the yolk during gestation) might be distinguished (for terminology see also Blackburn, 2000). In some cases, it may be more useful to employ terms that refer to the stage of the released offspring (Greven, 2002; 2003b) to qualify viviparity (a term that generally refers to 'any reproductive mode in which eggs are retained in the oviduct and offspring have live birth' (Lehtinen & Nussbaum, 2003, p. 360)) and to characterize more precisely the developmental stage of released young, which may reveal an oviparity–viviparity continuum. Thus, this chapter distinguishes oviparity as the release of non-inseminated eggs (occasionally termed ovuliparity) or inseminated eggs prior to karyogamy. Viviparity may be zygoparity (release of a zygote; not yet shown in any amphibian species to the author's knowledge), embryoparity (release of an embryo prior to hatching), larviparity (release of free-living larvae), or pueriparity (release of metamorphosed offspring).

Anura exhibit the most diverse incubation sites among amphibians. Besides the 'conventional' oviductal incubation, fertilized eggs or hatchlings may be carried in or on the dorsal skin, in the stomach of the female, or even in the vocal sac of the male for a substantial period of development, and may be released as larvae ('larviparity') or froglets ('pueriparity'). Further, skin brooding has evolved repeatedly in unrelated lineages. Among these various modes, Lehtinen and Nussbaum (2003) distinguish 'viviparity' (see Section 2.3); 'egg transport' and 'tadpole

transport' (physical relocation of eggs or tadpoles); and 'egg brooding' and 'tadpole brooding' (eggs or tadpoles are carried in or on the body of the parent until metamorphosis) in the brooding forms. However, this classification characterizes larviparity and pueriparity definitely present also in skin brooders as largely passive processes.

The selective pressures resulting in these reproductive modes differ among amphibians and within a given genus and are not yet fully understood in most cases. Generally, it can be assumed that the various modes increase survival of the offspring. However, one of the main selective factors in the evolution of reproductive modes appears to be parent–offspring conflict (e.g., Trivers, 1974; Crespi & Semeniuk, 2004). These aspects, however, are beyond the scope of this survey.

Considering basic adaptations and their regulation, many parallels exist between oviductal, skin-, vocal sac-, and stomach-brooders, either due to constraints caused by the 'substrate' (e.g., oviduct, skin) used and/or to the largely identical demands made on the parent and the offspring. To stress these parallels and for the sake of convenience, this chapter describes analogous phenomena with the same term; i.e., gestation period instead of incubation time; parturition or birth instead of release of the young; larviparity instead of release of larvae; etc.

The general control of reproduction appears to be largely similar in extant Amphibia, including the taxa treated herein. It involves external environmental cues responsible for the production of primary hormones in the hypothalamus, which in turn stimulate the pituitary to produce gonadotropins (GTHs); i.e., follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Both GTHs are involved in the control of ovarian steroid secretion; i.e., progesterone (P_4) and estradiol (E_2). The changes of plasma GTHs and gonadal steroids—closely related to follicular growth throughout the sexual cycle and their effect on the oviduct, spawning, and behavior—have been treated elsewhere (e.g., Kikuyama, Tanaka, & Moore, 2003; Fernández & Ramos, 2003; Norris & Lopez, 2005; Rastogi et al., 2005; Exbrayat, 2006a).

In the following, focus is given to some main problems shared by all brooding taxa; i.e., (1) provisioning an appropriate incubation site, (2) maintaining gestation, and (3) establishing maternal–embryonic relationships that include respiration and gas exchange, osmoregulation, elimination of waste products, immunological relationships (most species are likely to cope with specific immune responses as the embryo can be regarded as an allograft in its parental host), and allocation of food. One also must consider how the offspring may manipulate the mother (or father)—a process often closely linked to the transfer of nutrition—and parturition or birth. So far as it is available, information on regulative mechanisms is added.

None-the-less, the present survey suffers from the fact that knowledge of these topics is extremely fragmentary, that functional adaptations and hormonal regulation mostly have been derived from structural studies, and that in many cases only the basic phenomenon is known. Further, information is widely scattered in the literature and the limited space available has necessitated more selectivity and the citation of more summarizing literature than would be ideal. In spite of some objections (e.g., Smith & Chiszar, 2006), nomenclature and systematics follow Frost (2008).

2. OVIDUCTAL INCUBATION

The paired tubular oviducts of Amphibia are a secondary sex characteristic that develops under the influence of sex steroids (e.g., Norris & Lopez, 2005). When mature, oviducts open anteriorly with a funnel (ostium) into the body cavity and posteriorly into the cloaca. Their basic organization and structure appears to be similar in all Amphibia. Two or three structurally and functionally different main portions can be distinguished: (1) the short, straight, and simply organized pars recta (pr), which opens to the coelomic cavity with a dilated ostium and takes up the ovulated eggs; (2) the longer pars convoluta (pc); and (3) the short ovisac in oviparous species or the longer pars uterina (pu) in viviparous species. The ovisac or uterus is often considered as a part of the pc.

The oviduct consists, from outermost to innermost layers, of the thin epithelium of the visceral pleuroperitoneum, layers of loosely arranged smooth muscles increasing in number towards the uterus, vascularized connective tissue, and the mono-layered epithelium with various ciliated and non-ciliated secretory or glandular cells. The structure and histochemistry of the latter allow further subdivision of the pc. Changes of the oviduct associated with season and the reproductive cycles are dependent directly or indirectly upon estrogenic steroids. In the preovulatory period, the oviduct is preparing to accept oocytes. In the ovulatory period, the oviduct takes up the mature oocytes, encasing them with jelly layers secreted by the glandular tissue of the pc during their transport toward the cloaca. Pars recta secretions and pc-secreted jelly components are essential for fertilization in oviparous species, but this has not been studied in viviparous urodeles, anurans, or in any caecilian species (e.g., Urodela (e.g., Greven, 1998; 2002; 2003a); Gymnophiona or caecilians (e.g., Massood-Parveez & Nadkarni, 1991; Wake & Dickie, 1998; Exbrayat, 2006a); Anura (e.g., Fernández & Ramos, 2003)).

Oviductal incubation requires the transfer of sperm into and fertilization of eggs within the oviduct. In viviparous as well as in the majority of oviparous Urodela, females pick up spermatophores, which have been deposited by

the male during courtship. Spermatozoa are stored in the spermatheca, a system of tubules situated in the roof of the cloaca, and eggs either are inseminated when they pass the spermatheca in oviparous taxa or sperm migrate into the oviduct in viviparous taxa. Caecilians appear to lack a special spermatheca (for a review see Sever, 2002). In all caecilians, sperm are transferred by a specific copulation organ, the phallodeum, which is inserted into the female cloaca (e.g., Billo, Straub, & Senn, 1985). In anurans practicing oviductal incubation, mates presumably attach their cloacae to transfer sperm, but this has been verified only in a single species (for a review see Sever, Hamlett, Slabach, Stephenson, & Verell, 2003).

Fertilized eggs are retained in the uterus, where they develop until parturition. During the gestation period folliculogenesis and vitellogenesis should be inhibited to a certain extent and, depending on the intensity of mother−offspring relations, moderate to distinct changes of the uterus are observed. In all oviductal brooders, postovulatory corpora lutea (CL) have been found persisting longer than the ephemeral CL of oviparous amphibians. The activity span of the CL, however, differs greatly, with CL-degeneration (luteolysis) ranging from after mid-pregnancy to near term. Authors agree that in amphibians the CL play an important role in controlling gestation length (e.g., Xavier, 1987).

2.1. Urodela

Despite the fact that in most urodeles sperm are stored in the roof of the cloaca, which lies relatively near the mouth of the oviduct, oviductal incubation has evolved only in some Salamandridae, i.e., the species and subspecies of the genera *Salamandra* and *Lyciasalamandra* (formerly the viviparous species and subspecies of the genus *Mertensiella*). *Salamandra* spp. are distributed in Europe, North Africa, and the Near East; *Lyciasalamandra* spp. are found in southwestern Turkey and the adjacent Aegean islands. All are fully terrestrial after metamorphosis.

Reproductive strategies within *Salamandra salamandra* range from larviparity to pueriparity (ovoviviparity and viviparity according to Buckley, Alcobendas, García-Paris, and Wake (2007)) (Figure 7.1(c)). For example, *Salamandra salamandra salamandra* and *S. s. terrestris* give birth to larvae, *Salamandra salamandra fastuosa* delivers a smaller number of young near metamorphosis and occasionally transformed young, and *Salamandra salamandra. bernadezi* bears predominantly fully metamorphosed young (e.g., Joly, 1986). This diversity is achieved by heterochronic shifts (for further reading see Greven & Thiesmeier, 1994; Buckley et al., 2007). *Salamandra atra*, *Salamandra lanzai*, and *Lyciasalamandra* spp. are entirely pueriparous, bearing usually two metamorphosed young. Due to this variation and the various modes of nutrition of the intrauterine offspring, oviductal incubation is assumed to have evolved independently several times among these salamandrids (e.g., Veith, Steinfartz, Zardoya, Seitz, & Meyer, 1998; Buckley et al., 2007).

Most data regarding maternal adaptations are known from larviparous subspecies of *S. salamandra* and the pueriparous *S. atra*. Fertilization takes place in the upper part of the oviduct (*S. salamandra* (Joly & Boisseau, 1973; Zakrzewski, 1976)) or is secondarily shifted back to the cloaca (*S. atra* (reviewed by Greven, 1998; Guex & Greven, 1994; Greven, 2002)).

2.1.1. The oviduct and its changes during gestation

Compared to oviparous urodeles, the oviduct of larvi- and pueriparous salamandrids shows some modifications. The pc is less convoluted, and has only three or four (*S. atra*) glandular subdivisions, resulting probably in a reduced number of egg jelly layers. Especially the most posterior subdivision appears to be reduced in length, allowing for a longer pu. Histologically, tubular glands are abundant in the pc of S. *salamandra* and *Lyciasalamandra luschani*, whereas columnar gland cells are predominant in *S. atra*. Further, unlike oviparous species, gland cells of the pc secrete more acidic than neutral mucopolysaccharides probably resulting in the production of mechanically less resistant egg jelly layers (Vilter, 1986; Greven, 1998; 2002; 2003b).

Changes of the oviduct in the preovulatory period are poorly documented. Generally, oocyte maturation coincides with highly active glands in the oviduct (Joly & Picheral, 1972; Zakrzewski, 1976). Administration of E_2-benzoate during vitellogenesis strongly stimulates the production of jelly in *S. atra* (Guex & Greven, 1994). Glands and gland cells of the pc are refilled with secretory products relatively shortly after ovulation. This synthesis depends on pituitary activity (*S. atra*: Niederl, 1981; Vilter, 1986).

In spite of the small size of the eggs, there is not enough jelly produced to encase all ovulated oocytes following the sudden ovulation of numerous oocytes in *S. atra*. Usually, a single egg per uterus, the 'embryonic egg,' is coated with a complete set of jelly layers, which is tough and remarkably thick. The embryonic egg becomes fertilized when the jelly protrudes into the cloacal chamber and brings the uterine mouth close to the spermatheca. The eggs that are incompletely or not encased are not fertilized (counts of these unfertilized eggs vary in different studies and range from 20 to 104 (e.g., Häfeli, 1971; Vilter, 1986; Guex & Greven, 1994; Greven, 1998)). In *L. luschani* two eggs develop, but further details are unknown (Guex, 1994).

The uterus of all urodele species is aglandular. The simple cuboidal or flattened epithelium is thin, lacks ciliated cells, is secretory to a certain extent, and contributes to the uterine fluid (*S. salamandra*:Lostanlen, Boisseau, &

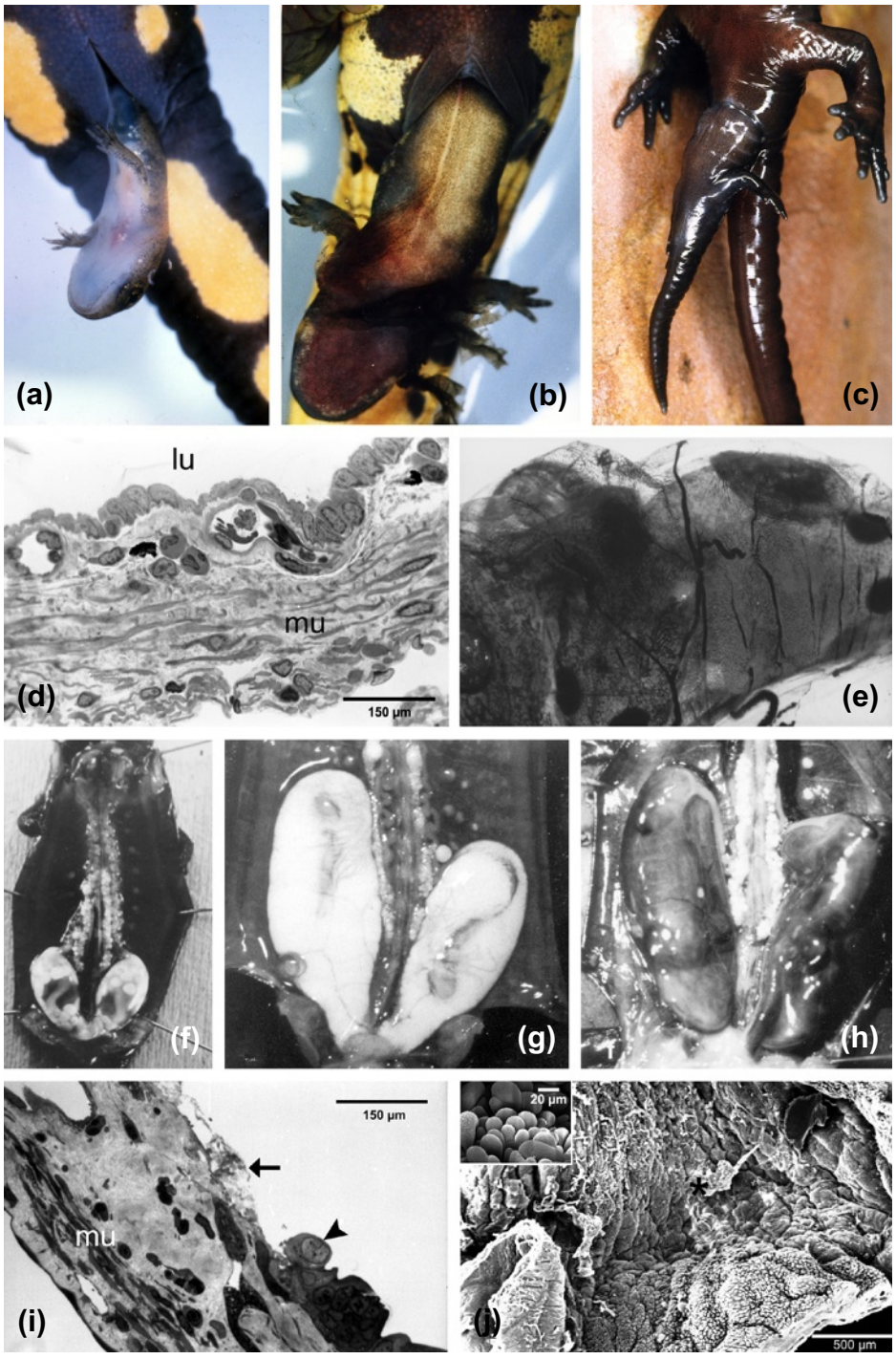

FIGURE 7.1 Oviductal incubation and various 'parities' in *Salamandra* spp. (a) Larviparity in *Salamandra salamandra* (b) Birth of an advanced larva in *Salamandra salamandra fastuosa*. (c) Pueriparity in *Salamandra atra*. *Copyright of (a–c) retained by W. Sauer, Marburg, Germany.* (d) Uterine epithelium of *S. salamandra* (semithin epoxy section stained with toluidine blue borax). (e) Vascularization of the uterine wall in *S. salamandra* (ink-injected and cleared preparation). (f) Embryos of *S. atra* shortly before hatching, stage 1. (g) Larvae at early stage 2 within the embryotrophic egg mass. (h) Transition from stage 2 to stage 3. Arrowheads indicate the location of the *zona trophica*. *(f–h) from Guex and Greven (1994).* (i) *Zona trophica* with large cells (arrowhead) and areas devoid of epithelial cells (arrow) in *S. atra* (semithin section stained with toluidine blue borax). (j) *Zona trophica*; note the grazed areas (asterisk). Inset: Cells bulging in the uterine lumen (SEM). l, lumen of the uterus; mu, muscle cells. See color plate section at the end of the book.

Joly, 1976)). The underlying muscles can be roughly differentiated in longitudinal and circular layers. The connective tissue layer is thick in non-pregnant females but thinner and attenuated in pregnant females. Vascularization is rich and most capillaries are situated immediately under the epithelium (Figure 7.1(d–e)). Most structural changes in the uterus of the larviparous *S. salamandra* can be attributed to mechanical stress exerted by the growing offspring rather than to hormonal effects. Such changes are the thinning of the uterine wall, including its epithelium, and stretching of tight junctions (zonulae occludentes) that seal the intercellular space between epithelial cells (see Greven, 1998; 2003b).

Transport capabilities of the uterine epithelium of pregnant females have been indirectly demonstrated by the basolateral distribution of Na^+-K^+-ATPase activity, by the accumulation of chloride and cations in the intercellular spaces, and by an ouabain-sensitive transport of ions towards the vascularized uterine connective tissue. Sodium content of the uterine fluid during pregnancy corresponds to that of the blood (approx. 80 mmol/l), but is considerably higher (ca. 290 mmol/l) in non-pregnant animals (e.g., Greven, 1998). This is indicative of a maternally controlled uterine environment. The membrane-bound Na^+-K^+-ATPase, characteristic of vertebrate epithelia, is very probably present in all epithelia of 'brood chambers.' In closed chambers this pump might be involved in creating a suitable environment for the developing offspring. Changes of ATPase activity during the reproductive cycle can be expected, but have not been studied in any 'brood chamber.'

Rough estimations of the density of the capillary network of the uterus did not account for an increase in vascularization in pregnant females of *S. salamandra* and *S. atra*, but dilated blood vessels are indicative of a higher blood pressure during gestation. Further, the appearance of the muscle layer does not change significantly during pregnancy (e.g., Lostanlen et al., 1976; Guex & Chen, 1986; Greven, 1998).

In *S. atra*, secretions of the uterine lining increase during pregnancy, namely when housing stage 3 larvae (see Section 2.3.4), and form the 'uterine milk.' Formation of a proliferating trophic zone at the anterior end of the uterus is seen at the end of stage 2 (e.g., Niederl, 1981; Guex & Chen, 1986; Guex & Greven, 1994).

Cytokines are known to be involved in immune and inflammatory reactions and in reproductive processes (Paulesu, 1997). The cytokine interleukin IL1B and its functional membrane receptor type 1 (IL1R1) have been demonstrated in the caudal oviduct of the oviparous (or ovuliparous) toad *Bufo bufo* with external fertilization, the oviparous newt *Triturus carnifex* with internal fertilization, and the pueriparous *S. lanzai*. Variation in cytokine expression in these disparate species may reflect different roles of IL1 in oviducts serving various reproductive roles. Authors have hypothesized that the expression of IL1 in *B. bufo* may be an immune response to environmental antigens mediated by IL1; in the two salamander species, however, expression of IL1 may be a response to paternal-derived antigens; e.g., the spermatozoa stored in the spermatheca or fertilized eggs retained in the uterus (Jantra et al., 2007).

2.1.2. Gestation time and endocrinological aspects

The length of gestation in urodeles depends on the species (and subspecies) and climatic conditions and varies from a few months (three to fourteen in larviparous *S.* s. *salamandra* and *S. s. terrestris*; five to twelve months in *L. luschani*) to some years (up to three years in *S. atra aurorae*; up to four or five years in *S. atra atra* and *S. lanzai*) (e.g., Häfeli, 1971; Vilter, 1986; Guex 1994; Guex & Greven, 1994).

Corpora lutea in *S. s. terrestris* are not vascularized and exhibit a cavity. Three to four months after ovulation they begin to degenerate. Granulosa cells show 3β-hydroxysteroid dehydrogenase (3β-HSD) activity and possess abundant smooth endoplasmic reticulum and mitochondria with tubular cristae. Preliminary *in-vitro* studies revealed the presence of enzymes necessary for converting pregnenolone to P_4 and P_4 to 17α-, 20α-, and 20α-hydroxyprogesterone in isolated CL. Ovariectomy of pregnant females either 15 days or several months after ovulation solely affected coherence of uterine capillaries and caused weakening of the epithelium (Lostanlen et al., 1976), but embryos remained alive (Joly & Picheral, 1972).

In *S. atra*, large numbers of CL persist in the ovary of gravid females for the first two years of gestation. Later, their numbers gradually decrease until parturition. This decrease is correlated with the growth of oocytes starting during stage 3 (Vilter V. & Vilter A., 1964; Niederl, 1981; Vilter, 1986). Corpora lutea also may control the activity of oviductal glands and stage 1 and stage 2 features of the uterus (Guex & Greven, 1994).

The few data available for plasma sex hormones during gestation in *S. s. terrestris* show high testosterone (T) levels increasing from the time after ovulation until the next ovulation and low E_2 levels during this period, perhaps triggering hepatic production of the yolk precursor protein, vitellogenin, related to postovulatory follicular growth. Levels of P_4 are very low during this same period. The plasma levels of P_4 and E_2 were low even at the beginning of gestation, when active CL were present and the amount of P_4 was high in the ovary (Joly, Chesnel, & Boujard, 1994).

Corpora lutea regress relatively earlier in *S. s. terrestris* than in the biennially reproducing *S. s. fastuosa*, in which growth of follicles and vitellogenesis starts not until

parturition and continues in the second year until the next ovulation. In this latter subspecies, high plasma levels of vitellogenin, E_2, and T were seen, and again low P_4 levels in the plasma but high levels in the ovary were measured. The reasons for the low plasma P_4 levels are unknown and authors have not found potential metabolites of P_4 (Joly et al., 1994; see also Xavier, 1987). Data from the annually reproducing *Salamandra infraimmaculata* only revealed that ovarian levels of E_2, T, and P_4, as well as 17α-hydroxy progesterone were higher in pregnant than in non-pregnant females (Degani, Sharon, & Warburg, 1997).

In the biennial cycle of *S. s. fastuosa*, the gonadotropic cells of the pituitary (identified by paraldehyde fuchsin staining) remained inactive during gestation (the first year of the cycle) but were large during vitellogenesis (the second year of the cycle). As females in the first and second year of the cycle can be found in the same geographic area, authors have suggested regulation of pituitary function by hormonal feedback rather than by external factors (e.g., Joly et al., 1994). The volume of the pituitary in *S. atra* decreases after ovulation (Vilter, 1986).

2.1.3. Mother–offspring interactions

2.1.3.1. Respiration and gas exchange

In larviparous species, exchange of gases takes place across the egg envelope encasing the offspring until birth, via the well-vascularized larval gills and the skin. In *S. atra*, gills reach their maximal relative length at stage 2, when larvae are hatched (e.g., Guex & Greven, 1994). A higher affinity of larval hemoglobin to oxygen relative to adult hemoglobin is expected but has not been studied.

2.1.3.2. Osmoregulation and excretion

Larval gills possess mitochondria-rich cells, which show carboanhydrase activity (studied after birth) and probably are involved in gas exchange and acid-base regulation (Lewinson, Rosenberg, & Warburg, 1984). The intrauterine offspring of *S. salamandra* are ureotelic. Urea is very likely removed via the uterine blood vessels, which is indicated by the higher content of urea nitrogen in the uterine fluid and blood plasma of pregnant females. Also, in *S. atra*, urea increases in the uterine fluid during pregnancy (Guex & Greven, 1994). The thyroid glands of intrauterine larvae of *S. salamandra* are relatively active just before and at the beginning of maternal hibernation. A relative high thyroxine concentration could keep the level of the somatotropic prolactin (PRL) low and may increase the activity of the urea cycle necessary for larval ureotelism (Schindelmeiser, 1985). After birth, larvae return partly to ammonotelism (Schindelmeiser & Greven, 1981; Schindelmeiser J., Schindelmeiser I., & Greven, 1983).

2.1.3.3. Immunology

In *S. salamandra*, the serum of pregnant females but not of non-pregnant females inhibits a cytotoxic reaction of maternal spleen cells against larval cells. One fraction of the serum specifically protects embryonic epithelial cells; a second fraction, however, was unspecific. An IgM and a α_2-macroglobulin, the latter linked with the immunosuppressive properties only during pregnancy, were involved in this process. The protective effect appeared to be enhanced with an increasing number of embryos in the uterus, and cytotoxicity and protection was largely specific for the female's own embryos (Badet, Chateuareynaud-Duprat, Mouches, Bove, & Metivaud, 1980; Badet, 1984).

2.1.3.4. Allocation of food

Eggs in larviparous species are richly yolked and measure approximately 5 mm in diameter: those of the pueriparous *S. atra* are smaller (2.0 to 2.2 mm (Vilter, 1986)). In larviparous species, the uterus appears not to be specialized for nutrient transfer. Nutrients are taken exclusively from yolk reserves (lecithotrophy), although a sparse uptake of amino acids by intrauterine embryos has been demonstrated (Lostanlen et al., 1976). However, this appears to be a common feature of non-keratinized epithelia. Consequently, the dry weight of developing *S. salamandra* decreases during the gestation period (Greven & Guex, 1994). Larvae can be removed from the uterus very early in development and reared in water (e.g., Joly & Picheral, 1972).

In pueriparous salamanders and those giving birth to young immediately before or after metamorphosis (e.g., *S. s. bernadezi*), the offspring initially feed upon yolk stores and later on degraded, unfertilized eggs (oophagy) and upon less developed siblings (adelphophagy) (e.g., Guex, 1994; Buckley et al., 2007). In *S. s. fastuosa*, a feeding behavior during gestation is more plastic. The number of fertilized and unfertilized eggs is variable, as are the size and weight of the developing offspring. Degrading unfertilized eggs and smaller siblings are cannibalized and the same female can bear facultatively numerous larvae and/or fully metamorphosed young (for further reading see Greven & Thiesmeier, 1994; Greven, 2003b). In *L. luschani*, one fertilized egg develops in each oviduct. After a lecithotrophic phase, intrauterine larvae feed on disintegrated eggs and the female gives birth to two fully metamorphosed young (Guex, 1994).

Epitheliophagy occurs in *S. atra* and is correlated with a long period of gestation. First, embryos feed on the yolk when still surrounded by the egg jelly (lecithotrophic phase; stage 1). After hatching, stage 2 larvae ingest the disintegrated undeveloped eggs (embryotrophic eggs) (Figure 7.1(f–g)). At the beginning of stage 3, the larvae have consumed these eggs and metamorphosis begins. Now they feed upon epithelial cells produced in the zona

trophica at the transition between the pc and pu (Figure 7.2(h)). The large cells of this zone bulge into the uterine lumen, become detached from the underlying connective tissue (probably by necrosis and/or apoptotic processes), float in the uterine lumen, and are ingested by the larvae. When the young are positioned with their heads toward the nutritive zone, they appear to scrape off these cells with a special dentition of the upper and lower jaw. Often more than half of the area of the zona trophica is free of epithelial cells and even blood vessels are opened (Figure 7.1(i–j)). The epithelium regenerates continuously from the remaining epithelium and probably from cells submerged in the connective tissue during the period in which the offspring feeds. The zona trophica develops only in the presence of embryos of the Schwalbe stage 3 and cannot be induced by progesterone and/or estrogenic steroids, indicating independence of maternal hormones. The nature of the stimulus is unknown, but prostaglandins produced from the larvae are suggested. (Guex & Chen, 1986; Guex & Greven, 1994; Greven, 1998; 2002; 2003b).

2.1.3.5. Parturition

Birth is triggered by hormonal and environmental factors, but details are unknown and nothing is known about factors signaling the pregnancy and/or readiness for birth except that perhaps secretions from the offspring's skin are involved (Guex & Greven, 1994). Premature birth can be induced with several pituitary hormones and external stress (changes in light, temperature, etc.) especially in the spring (Gasche, 1942), the most common time for parturition in temperate regions (e.g., Zakrzewski, 1976; Joly, 1986). The neurohypophysial hormone arginine vasotocin (AVT) evokes strong contractions of isolated oviducts of *S. salamandra* and *S. atra*. Pregnant females of *S. salamandra* injected intramuscularly with high doses of AVT gave birth to their larvae (Heller, Ferreri, & Leathers, 1970), but not *S. atra* females with stage 3 young. Perhaps a signal is needed from the intrauterine offspring about its developmental status, which permits the uterus to react (Greven & Guex, 1994). Generally, contractions of the uterine muscularis may help to expel the young, but in pueriparous species a more active role of the young has been reported (Häfeli, 1971).

Birth in larviparous species may take more than one day, with the largest bulk of larvae in the first day. In most cases, larvae are born quickly with the head or the tail presenting and with or without the egg capsule. In *S. atra* several weeks may separate the two parturitions (Figure 7.1(a–c)) (Guex & Greven, 1994; Greven, 2003b).

2.2. Gymnophiona

In Gymnophiona, oviductal incubation is more common. Previously it was assumed that approximately 50% of extant species are pueriparous (e.g., Wake, 1993). A more recent estimation narrows the presence of pueriparity to 24–30% of the genera and to 15–17% of the species (Wilkinson & Nussbaum, 1998). Oviductal incubation (viviparity) is suggested to have evolved independently at least four times (Gower, Giri, Dharne, & Shouche, 2008). Of the worldwide distributed Caeciliidae, the secondarily aquatic Typhlonectinae (e.g., *Typhlonectes*, *Chtonerpeton*) of South America and at least one terrestrial genus of the Scolecomorphinae from Africa as well as some other Caeciliidae (e.g., *Dermophis*, *Geotrypetes* (Figure 7.2(a)) are viviparous. Larviparity is unknown in Gymnophiona (Wilkinson & Nussbaum, 1998), but embryoparity (gastrula-to-neurula stages) has been reported from *Ichthyophis* cf. *kohtaoensis* (Ichthyophiidae) (Kupfer, Kramer, Himstedt, & Greven, 2006a).

Most details of maternal adaptations to oviductal incubation are known from two biennially reproducing species, the terrestrial *Dermophis mexicanus* and the aquatic *Typhlonectes compressicauda*. Oocytes are fertilized in the anterior part of the oviduct of both oviparous and viviparous species (e.g., Wake, 1968; Exbrayat, 2006a; 2006b; Exbrayat & Hraoui-Bloquet, 2006).

2.2.1. The oviduct and its changes during reproduction

Available studies on the oviduct of viviparous caecilians are either incomplete (e.g., Wake 1970) or suffer from an inadequate documentation (e.g., Exbrayat, 1984; 1988). Wake (1970) examined several oviparous and viviparous species, but did not distinguish oviductal divisions. Exbrayat (1984; 1988) described a short anterior moderately convoluted portion, which produces the egg envelope, and a posterior uterine portion in the oviduct of the viviparous *T. compressicauda*. The egg envelope, composed of neutral and acid mucosubstances in this species, is extremely reduced compared to the tough, multilayered egg jelly in oviparous species (Breckenridge & Jayasinghe, 1979).

The oviduct of the oviparous *Ichthyophis geddomei* reveals the pr, pc, and pu mentioned above (Masood-Parveez & Nadkarni, 1991), and this organization very probably holds for viviparous species (Figure 7.2(b)) in which, however, the pc appears comparatively short, exhibiting more glandular cells with acidic secretions.

A further characteristic probably specific to viviparous species has been described by Exbrayat (1989). In the oviparous *I. kohtaoensis*, the long oviducts open with a funnel-like ostium into the coelomic cavity at the level of the heart; i.e., far from the ovary. In contrast, in the viviparous *T. compressicauda*, a gutter equipped with ciliated cells and glandular cells parallels the ovary and the

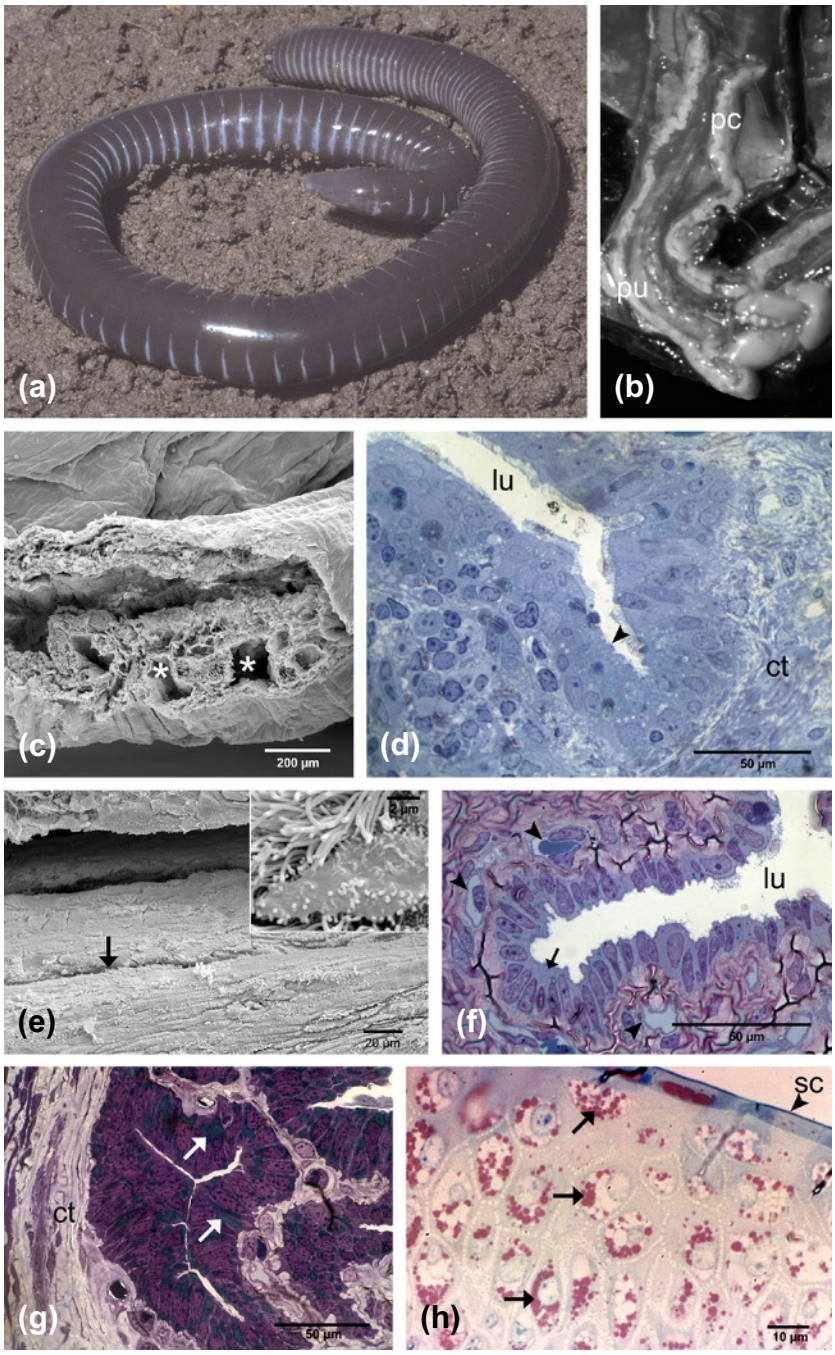

FIGURE 7.2 Oviductal incubation in Gymnophiona. (a) Female of the viviparous *Geotrypetes seraphini*. *Copyright retained by A. Kupfer, Jena, Germany.* (b–f) Oviduct of a non-reproductive female of *Typhlonectes natans*. (b) Pars convoluta (pc) and relatively straight pars uterine (PU). (c) Pars convoluta with large blood vessels (asterisks) (SEM). (d) Pars convoluta epithelium with ciliated cells and non-ciliated moderately secretory cells (arrowhead) on the bottom of a fold. (e) Uterus with flattened folds (arrow) covered with ciliated cells and cells with short microvilli (inset) (SEM). (f) Fold of the uterine epithelium fold; note capillaries (arrowheads) and very few lipid droplets (arrow) (semithin epoxy section, toluidine blue borax). (g) Uterine fold of a pregnant female of *Chtonerpeton indistinctum* with prismatic cells and accumulations of lipids (arrows) (semithin epoxy section, toluidine borax). (h) Lipid- (arrows) rich epidermis cells of the skin-feeding *Boulengerula taitanus* (semithin section, Nile blue). ct, connective tissue; lu, lumen of the oviduct; sc, stratum corneum. See color plate section at the end of the book.

relatively short oviduct, probably facilitating the transport of eggs to the ovary.

Judging from the available histological studies, cyclical changes during the reproductive cycle include folding of the oviductal wall and differentiation of glandular cells from largely undifferentiated cells in the pr and the pc (Figure 7.2(c–d)) before and during ovulation, secretion of the egg jelly in the pc, and regression of the pc epithelium

afterwards. The pu is lined by ciliated and rather undifferentiated cells in non-reproductive females (Figure 7.2 (e–f)). The uterine wall becomes strongly folded at ovulation; its glandular cells and small secretory cells produce predominantly acid mucous secretions. At sites where embryos rest, the uterus is highly vascularized. In interembryonic regions, ramifying projections of the uterine wall develop. Later in gestation, glandular areas are reduced and uterine areas are devoid of epithelial cells (e.g., Exbrayat, 1984; 1988; 2006a; Wake, 1970; 1977; 1993; Wake & Dickie, 1998).

Only two TEM studies of the pu exist. On the basis of exquisitely preserved tissue, Welsch, Müller, and Schubert (1977) described the uterus of a non-pregnant and a pregnant female of *Chtonerpeton indistinctum* and stated that in pregnant females (1) the circularly arranged muscle cells are smaller and are more distant from each other; (2) the loose vascularized connective tissue forms numerous variously shaped villi covered by the monolayered uterine epithelium; (3) epithelial cells, a single cell type only (ciliated cells were sporadically seen in non-pregnant females), are prismatic, bear microvilli, and contain secretory granules rich in carbohydrates and considerable amounts of lipid droplets (Figure 7.2(g)); and (4) the activity of several hydrolases is stronger than in non-pregnant females. Areas free of epithelial cells were not found in this species. The study of the uterus of female *T. compressicauda* in different stages of pregnancy (Hraoui-Bloquet, Scudie, & Exbrayat, 1994) suffers from poor fixation. The uterus in early stages of gestation with embryos still surrounded by egg jelly (stages 23–25 of 34 developmental stages; see Sammouri, Renous, Exbrayat, and Lescure (1990)) is lined by ciliated cells and secretory cells with a few lipid droplets. During stages 26–31, the number of lipid-rich cells increases and some cells seem to degenerate. Later, lipid-containing cells seem to be detached and large areas of the uterine wall appear to be denuded from the epithelium. The young are born at stage 34. The epithelium seems not to be restored until after birth.

2.2.2. Gestation time and endocrinological aspects

Gestation time among viviparous Gymnophiona varies from two months (*Gymnopis multiplicata*), to four to six months (*C. indistinctum*), to six to seven months or somewhat longer, depending on the temperature in species with a biennial cycle (*T. compressicauda* (Billo et al., 1975)) to approximately one year (*D. mexicanus* (e.g., Wake, 1980a; Exbrayat, 2006b)).

Corpora lutea have been demonstrated by histology in all species so far examined; they obviously persist throughout pregnancy (Wake, 1968; 1980a). Ovaries of *T. compressicauda* contain poorly vascularized CL with a central cavity in the first stages of pregnancy. They are filled with granulosa cells and capillaries during the middle and end of pregnancy ('compact' CL). Both show 3β-HSD activity. The number of CL remains rather constant through a great part of the gestation period but decreases before term. The remaining CL degenerate rapidly after parturition (Exbrayat & Collenot, 1983). *In-vitro* studies have revealed that ovaries of pregnant females convert labeled pregnenolone into P_4 (Exbrayat, 2006a).

In the pituitary of *T. compressicauda*, gonadotropic and lactotropic cells change their size (expressed as mean surface area of sections) during the reproductive cycle. Gonadotropic cells are well developed just before breeding (pre- and postovulatory period), decrease in size towards the end of pregnancy, and regress during the year of sexual inactivity. Prolactin cells are sparse but large in vitellogenic females from the beginning to the middle of pregnancy. Afterwards, they progressively decrease in size when CL regress (Exbrayat & Morel, 1990/1991). A study using immunohistochemistry and *in-situ* hybridization showed that the number of PRL cells remained relatively constant during the first stages of gestation, increased in females carrying free larvae with well-developed gills, and reached a maximum at parturition. Prolactin-coding mRNA expression remained constant during the first stages of gestation, increased during the final stages, and decreased markedly after parturition (Exbrayat & Morel, 1995). These results were supported by the strong expression of the long form of PRL receptor (PRL-R) mRNAs in ovaries with compact CL; i.e., during the middle and the end of pregnancy (Exbrayat & Morel, 2003). Authors have suggested that (1) PRL is needed to maintain persistence of CL and, thus, inhibition of uterine contractions; (2) the differentiation of the uterine wall is largely independent of CL or PRL in the first stages of pregnancy when CL still have a central cavity and sparse vascularization; and (3) the modifications of the uterus later in gestation are linked with the CL, which then might be under the control of PRL (see also Exbrayat, 2006a).

2.2.3. Mother–offspring interactions

2.2.3.1. Respiration and gas exchange

While still within the egg jelly, embryos develop a pair of external tri-ramous, well-vascularized gills. Gills degenerate before or disappear a short time after birth. In the Typhlonectinae, gills form a pair of large, highly vascularized sac-like structures (e.g., Wake, 1977; Exbrayat, 2006b). Oxygen affinity of the fetal blood is greater than in the adult, improving gaseous exchange between the oviduct and the fetal gills and skin (*T. compressicauda* (Toews & MacIntyre, 1977; Garlick et al., 1979)).

2.2.3.2. Osmoregulation and excretion

Although not explored, ureotelism of the young is expected, considering the limited fluid present in the uterus. Adults of *T. compressicauda* excrete both ammonia and urea (Stiffler, DeRuyter, & Talbot, 1994).

2.2.3.3. Immunology

Immune reactions toward the intrauterine offspring have been shown in *T. compressicauda* using cultivated fragments of larvae and larval gills. The serum of pregnant females inhibits a cytotoxic reaction of adult spleen cells against larval cells (Exbrayat, Pujol, & Hraoui-Bloquet, 1995).

2.2.3.4. Allocation of food

Eggs are small in viviparous caecilians (1−3 mm in diameter). Further, the number of newborns is small (e.g., 4−6 in *T. compressicauda*; 6−7 in *D. mexicanus*), but they are of considerable size, weighing 5−6 g (*T. compressicauda*) (e.g., Wake, 1977; 1980a; Exbrayat, 2003b), indicating intense matrotrophy.

The intrauterine offspring may gain nourishment via (1) a placenta, (2) lipid- and glycoprotein-rich secretions, (3) epitheliophagy, (4) oophagy, and/or (5) adelphophagy. All these options are realized in *Typhlonectes* spp.

In *T. compressicauda* and *T. natans*, the 'ectotrophoblast' and gills together with the vascularized uterus meet the definition of a placenta, which is 'an intimate apposition or fusion of the fetal organs to the maternal (or paternal) tissues for physiological exchange' (Mossman, 1937, p. 130). The 'ectotrophoblast' is a ventral coelomic pouch, which is covered with absorptive cells. It develops in stage 22 (Sammouri et al., 1990) (length of the embryo is 8 mm) and reaches its maximum in stage 23, when the yolk becomes reduced, and disappears by stage 28 when the gills enlarge. At stage 26, embryos of approximately 11 mm hatch (newborns measure 116 mm!). Parts of the enlarged gills closely appose the uterine epithelium and, when fully developed, their epithelial cells bear apical projections at the larval face and microvilli at the uterine face. Especially at the uterine face, gills are heavily vascularized. Ectotrophoblast and gills may take up nutritive substances from the uterine lumen, the former at the time when the yolk stores are rapidly depleting and gills have not reached their full form. Experimental evidence for the uptake of (macro)molecules, however, is missing, and the published TEM pictures to show this uptake are not convincing (e.g., Delsol, Exbrayat, Flatin, & Gueydan-Baconnier, 1986, Sammouri et al., 1990; Exbrayat & Hraoui-Bloquet, 2006).

Intrauterine larvae of all viviparous caecilians feed on uterine secretions (carbohydrates and especially lipids), decomposed yolk from abortive eggs (oophagy), and cells (epitheliophagy), which together form the 'uterine milk.' To what extent the different sources are used by various species is unclear. For example, the very few cells found in the stomach of intrauterine *C. indistinctum* larvae suggest imbibition of uterine milk rather then grazing on the epithelium (Welsch et al., 1977). Epitheliophagy is closely associated with the ingestion of secretions of the uterine epithelium, and both secreted material and cells were found in the mouth and the pharynx of intrauterine larvae of other species (Wake, 1970; 1977).

Evidence for oophagy and adelphophagy is both indirect and direct. The numbers of embryos carried by pregnant females are considerably fewer than the average numbers of ova observed in pregnant females, and undeveloped, resorbed, or degenerate eggs can be found in the oviducts throughout pregnancy (Wake, 1968; 1980a; Exbrayat & Hraoui-Bloquet, 2006). Exbrayat (1984, 2006b) observed siblings in the stomach and intestine of intrauterine larvae.

Epitheliophagy appears to be facilitated by a special polystichous 'fetal' dentition and early ossification of jaw suspension elements (*D. mexicanus* (Wake & Hanken, 1982)). Functional fetal teeth differ from the first tooth generations and from transformed teeth by their variably shaped crowns. These teeth probably not only help to graze the uterine epithelium but also may stimulate secretion and cell turnover. Fetal teeth are shed at birth and replaced by transformed teeth (e.g., Wake, 1976; 1980b; Hraoui-Bloquet & Exbrayat, 1996).

'Fetal' teeth are also present in hatchlings of oviparous species. They are used here to peel the outermost layer of the lipid-rich epidermis (Figure 7.2(h)) of their attending mother. It was hypothesized that a 'fetal dentition' originated in a dermatotrophic, oviparous direct-developing ancestor of the viviparous taxa (Wilkinson & Nussbaum, 1998; Kupfer et al., 2006b; Wilkinson et al., 2008). This nursing obviously is costly, but mothers seem to also feed young other than their own (called alloparenting and seen in the caeciliid *B. taitanus* (Caeciliidae) (Kupfer, Wilkinson, Gower, Müller, & Jehle, 2008)). In *Siphonops annulatus* (Caeciliidae), young seem to imbibe fluid of unknown composition released from the cloaca (Wilkinson et al., 2008).

2.2.3.5. Parturition

In the aquatic *T. compressicauda*, parturition spans several days and a single birth lasts 10 to 40 minutes. At birth, young emerge head and gills first or occasionally tail first; the sac-like gills are lost within 36 hours of birth (Billo et al., 1985). Neurohypophysial peptides, especially AVT, promote contractions of the oviduct (Hilscher, Conklin, & Boyd, 1994). In the field, most species give birth at a time that is correlated with environmental variables such as precipitation and other climatic conditions.

2.3. Anura

In the Anura, oviductal incubators include members of the former '*Nectophrynoides* complex' (Bufonidae) from Africa (summarized in Xavier, 1986; Wake, 1980c) and *Eleutherodactylus jasperi* (Eleutherodactylidae) from Puerto Rico (Wake, 1978).

In the *Nectophrynoides* complex, currently three genera are distinguished. From these, *Altiphrynoides osgoodi* is oviparous, whereas *Altiphrynoides malcolmi* retains its eggs in the oviducts until the neurula stage (embryoparity). *Nectophrynoides* spp. include the larviparous, lecithotrophic *Nectophrynoides tornieri* and *Nimbaphrynoides* spp., the most derived being the pueriparous matrotrophic *Nimbaphrynoides* (formerly *Nectophrynoides*) *occidentalis*. Oviductal incubators in Anura achieve internal fertilization by cloacal apposition. There are some other anuran species that transfer sperm either by cloacal apposition or by a penis (see Sever, Hamlett, Slabach, Stephenson, & Verell, 2003) but that did not evolve oviductal incubation.

Most data on maternal adaptations are available from *N. occidentalis* and *N. tornieri*. Knowledge of *E. jasperi* is fragmentary.

2.3.1. The oviduct and its changes during reproduction

The oviduct of *N. occidentalis* is histologically separated into the ostium, a straight tube with subdivisions, the uterus, and the most distal common uterus (Xavier, 1973; 1986). Thus, it principally resembles the oviduct of oviparous toads such as *Bufo arenarum* (Winik et al., 1999; Fernandez & Ramos, 2003). The ostium and the first subdivision of the tube of *N. occidentalis* are equivalent to the pr. The pr is lined by a ciliated epithelium; glandular cells are lacking. This part does not change significantly during the reproductive cycle. In the straight tube, corresponding largely to the pc, four subdivisions were recognized; three of them are folded with glandular cells in the folds and ciliated cells at their apices. This part is quiescent for most of the gestation period, but becomes secretory under the influence of estrogens secreted by the theca interna several weeks before birth. Secretory activity reaches its maximum immediately before ovulation. Compared to the pc of *B. arenarum*, in which tubular acinous glands secrete the egg jelly, this part is simpler in *N. occidentalis*. Only a small subdivision secretes neutral mucopolysaccharides, which appear to form the reduced egg envelope, whereas the rest produces rather fluid acid mucopolysaccharides. Secretory cells at the base of the oviductal folds discharge their products continuously until the glands are depleted after two months of gestation.

Three distinct phases have been recognized in the pu and the common uterus during reproduction. The distended uterus becomes highly vascularized during gestation, and the non-ciliated epithelium secretes a 'mucoprotein' up to the end of gestation (phase of secretion and hyperemia triggered by P_4). After birth, the entire epithelium and capillaries degenerate and infiltration of macrophages is observed (necrotic phase). Then the tissue regenerates, ciliated cells appear again, and angiogenesis starts (proliferative phase up to the next ovulation). In females that become pseudopregnant in the absence of males, eggs are retained *in utero* for seven to eight months and are resorbed by the epithelium. However, changes of the uterus are less obvious in pseudopregnancy (Xavier, 1974).

In the larviparous *N. tornieri*, the organization of the oviduct corresponds to that of the pueriparous *N. occidentalis* described above. In the uterus, however, changes are not distinct during reproduction; vascularization is less intense and, thus, a regenerative phase is lacking (Xavier, 1986).

Wake (1978) distinguished macroscopically a narrow convoluted anterior part and a highly dilated posterior part in the oviducts of *E. jasperi*; the most posterior parts of both oviducts are fused terminally, forming a common uterus. Histology of the oviduct of a pregnant female reveals that the anterior portion is folded and lined by ciliated and secretory cells (no further specification is given), followed abruptly by the dilated posterior portion that is lined by cuboidal cells and is not notably vascularized.

2.3.2. Gestation time and endocrinological aspects

Gestation time in species of the *Nectophrynoides* complex ranges from two to three months in the larviparous *N. tornieri* to nine months in the pueriparous *N. occidentalis*. The long gestation period in *N. occidentalis* includes the dry season, in which the females are underground (approx. six months) and development of the young proceeds slowly, and three further months after emergence (reviewed in Wake, 1980c).

Compact CL without internal vascularization are present in the ovary of *N. occidentalis* and *N. tornieri*. In both pregnant and pseudopregnant females of *N. occidentalis*, decrease of the number of CL is correlated with the growth of oocytes. Luteal regression begins approximately two-thirds of the way through gestation, when females emerge from underground (e.g., Lamotte, Rey, & Vogeli, 1964; Xavier, 1986; 1987). In pseudopregnant females, CL degenerate one to two months earlier and the ovarian cycle is shorter (ca. nine months) instead of 12 months, as in normally pregnant females (Xavier, 1974). In females breeding for the first time, ovariectomy at the beginning of gestation leads to abortion, but, when performed at the end of gestation, development of the offspring is accelerated and birth takes place earlier.

Hypophysectomy of pregnant females gives similar results (Xavier, Zuber-Vogeli, & LeQuang-Trong, 1970).

High 3β-HSD activity was demonstrated histochemically in the granulosa cells of CL during the gestation period of *N. occidentalis* (Xavier et al., 1970). *In-vitro* studies on pregnant and pseudopregnant females revealed that the ovarian tissue metabolizes radioactively labeled pregnenolone mainly to progesterone. After parturition this steroid precursor is converted mainly to P_4, androstenedione (AND), and T. The amount of P_4 formed *in vitro* reaches a peak during the first two weeks of gestation (the ovary seems to be quasi on 'standby,' as suggested by Rastogi et al. (2005)). Then, the amount of P_4 gradually declines, but is relatively high during the underground period, decreases after emergence of the female, and reaches its minimum at the time of parturition. A similar activity was observed in the ovary of pseudopregnant females (Xavier & Ozon, 1971). Administration of P_4 causes a two to three month delay of parturition (Xavier, 1970). Progesterone is thought to elicit uterine secretions, promote uterine angiogenesis, and slow down development of the young when the female is underground, rather than to maintain gestation (Xavier, 1986).

Large but not very active CL are present also in the larviparous *N. tornieri* throughout a large part of gestation (gestation lasts up to 78 days). Steroidogenic activity analyzed by *in-vitro* incubation was weak during the first month of gestation and then increased progressively, maintaining a somewhat higher level until the next ovulation (the sexual cycle lasts from 90 to 150 days, according to the season). This increase corresponded to oocyte growth and vitellogenesis (Xavier, 1986; 1987). Xavier (1986) assumed that the low steroidogenic activity of persisting CL in this species does not play an important role in gestation.

As revealed by immunofluorescence, the pituitary of *N. occidentalis* shows abundant PRL cells and an increasing number of gonadotropic (LH) cells during the underground period (the first six months of gestation). The latter is linked to the presence of embryos in the uterus. In the last three months of gestation, PRL cells decrease in number and CL degenerate. After parturition, LH cells discharge their contents and the volume of the pituitary decreases. Then, a new generation of LH cells appears and PRL cells become abundant (Zuber-Vogeli, 1983). Hypophysectomy, but also castration or removal of embryos in early pregnancy, causes premature regression of the CL and atresia of young oocytes, suggesting interactions between the embryos and activity of the CL via the hypothalamus–pituitary level (Xavier 1986; 1987).

Gestation lasts approximately 33 days in *E. jasperi*. Wake (1978) did not find CL in three pregnant females with fully yolked eggs in their ovaries. Luteolysis is observed at various stages of the reproductive cycle in viviparous Amphibia; therefore, this observation cannot be taken as evidence for the total absence of CL in this species as the females studied contained oviductal froglets in an advanced stage of development (fore- and hindlimbs well developed).

2.3.3. Mother–offspring interactions

2.3.3.1. Respiration and gas exchange

There is no information on special adaptations of the offspring for gas exchange in *Nimbaphrynoides* spp. and *Nectophrynoides* spp. Tails of *E. jasperi* embryos still surrounded by the egg envelope in the dilated part of the oviduct, and the tails of froglets metamorphosing in the uterus are thin, broad, highly vascularized, and appressed to the uterine epithelium. In addition, the skin of embryos and froglets is well vascularized (Wake, 1978).

2.3.3.2. Osmoregulation and excretion

Not explored; intrauterine ureotelism is predicted.

2.3.3.3. Immunology

No data available.

2.3.3.4. Allocation of food

Eggs of *N. occidentalis* are small (0.5–0.6 mm in diameter) and poorly yolked. The increase of weight during the intrauterine growth is 300 times the egg mass. After a short lecithotrophic phase, young of *N. occidentalis* freely live in the uterus and, as the digestive tract differentiates early, ingest mucous substances secreted by the uterine wall ('uterine milk'). Uptake of these secretions may be facilitated by papillae surrounding the mouth, which may act as sponges (e.g., Lamotte & Xavier, 1972). Young are able to incorporate considerable amounts of amino acids supplied by the mother via the uterine secretions. In *N. tornieri*, eggs measure 3 mm and lecithotrophy is assumed (reviewed in Xavier, 1986).

In *El. jasperi*, young develop within one month. Lecithotrophy is assumed and newborn froglets still have a considerable amount of yolk (Wake, 1978).

2.3.3.5. Parturition

N. occidentalis females deliver 4–35 metamorphosed froglets per clutch; they are expelled by a complex interaction of the abdominal muscles and the lungs (Xavier, 1986). *N. tornieri* deliver 9–60 froglets and *Nectophrynoides viviparous* 114–135 larvae (Wake, 1980c). *E. jasperi* gives birth to three to five froglets (Wake, 1978).

3. SKIN INCUBATION

The basic organization of the skin is similar in all anurans. The epidermis is a stratified squamous epithelium

containing keratinized outer layer(s) (stratum corneum). The underlying dermis typically is composed of a superficial loose network of collagen and some elastic fibers (stratum spongiosum), in which most blood vessels and nerves can be found, and a deeper layer consisting of collagen bundles densely arranged in a criss-crossed manner (stratum compactum). At least two types of epidermis-derived glands, mucous and serous glands, are located in the stratum spongiosum and discharge their secretion on the body surface. Regional differences in the skin as well as seasonal or permanent sex dimorphic structures such as spines, nuptial pads, and specialized glands occur (e.g., summarized by Fox, 1994; Brizzi, Delfino, & Jantra, 2003).

The skin is a highly dynamic organ with a wide range of functions and is susceptible to various hormonal actions. For example, skin texture changes during the breeding season or when treated with PRL outside the breeding season in terrestrial and semiterrestrial species (e.g., Greven, 1987a) and in urodeles T stimulates proliferation and differentiation of mucus and granular glands (Norris, Austin, & Hijazi, 1989).

Skin incubation involves dorsal brood chambers (Pipidae), the unprotected skin (Hemiphractidae; Cryptobatrachidae), and more or less closed dorsal pouches (Amphignathodontidae) at the female's back. Members of some other anuran families such as Dendrobatidae, Sooglossidae, and Myobatrachidae practice 'tadpole brooding' *sensu* Lehtinen and Nussbaum (2003), but additional information on parental adaptations is generally unknown. In the Australian frog *Assa darlingtoni* (Myobatrachidae), the hatched larvae become incorporated in inguinal pouches of males several days after oviposition, from which froglets are released after 48 to 69 days. Comparison of dry weight of embryos at incorporation and of released froglets did not give evidence of paternal nutrition, but further details are unknown (Ehmann & Swan, 1985).

During the 'egg transport' of many Dendrobatidae, mother−offspring relationships appear largely unidirectional. Tadpoles wriggle onto the parent's back, becoming attached either simply by surface adhesion or by a conspicuous glycoprotein matrix secreted by special cells of the ventral epidermis of the larvae and the common mucous gland of the parent. Branched projections of a special larval cell type are embedded in this matrix. In *Ranitomeya* (*Minyobates*) *virolinensis*, the skin of the parent 'nurse frog' does not show significant alterations (De Pérez, Ruiz-Carranza, & Ramírez-Pinilla, 1992a; summarized in Brizzi et al., 2003).

All skin breeders have retained their ancestral mode of external fertilization. Hitherto, oviductal and egg jelly modifications such as the production of less resistant egg jelly layers, and so on, possibly associated with skin breeding are unknown.

3.1. Skin incubation in the Pipidae

Only the strictly aquatic South American members of the genus *Pipa* exhibit egg incubation in temporary brood chambers, one for each egg. These species are either larviparous, e.g., *Pipa parva* (Figure 7.3(b)), *Pipa myersi*, and *Pipa carvalhoi*; or pueriparous, e.g., *Pipa pipa*, *Pipa arrabali*, *Pipa snethlageae*, and *Pipa aspersa*. Duellman (1989) suggested that skin incubation in *Pipa* spp. is derived compared to egg laying of the related African genera, e.g., *Xenopus* and *Hymenochirus*, whereas Barthalmus (1994) hypothesized that *Xenopus laevis* and related species originated from a skin-brooding ancestor. Because female *X. laevis* have a thicker skin and more and larger glands in the dorsal skin, whereas in males the belly skin has fewer glands than the back (Fujikura, Kurabuchi, Tabuchi, & Inoue, 1988); Barthalmus (1994) assumed that the fewer ventral glands in the suggested skin-breeding ancestor might be an adaptation to prevent sticking of the eggs to the male's belly during transfer of eggs to the females back. Mechanical properties of the skin, namely extension load (ϵ_f) probably influenced by GTHs, are sexually dimorphic in *X. laevis* (Greven et al., 1995a; see Greven & Richter, 2009), but there is no information as to whether such sexually dimorphic traits as different numbers of glands or mechanical properties occur in other anurans.

The complex egg-laying behavior of *Pipa* spp. has been described repeatedly (*P. pipa*: Rabb and Rabb (1960); *P. carvalhoi*: Weygoldt (1976a; 1976b); *P. parva*: Greven (unpublished)) and is similar in all. After clasping a receptive female (inguinal amplexus) for hours, the male fertilizes the eggs, which emerge from the female's cloaca, and maneuvers them onto her back.

3.1.1. The skin and its changes during reproduction

Sexually receptive *Pipa* females can be identified by their swollen cloaca and swollen dorsal skin. The latter may be caused by enhanced accumulation of water, as suggested by Weygoldt (1976b), and is probably a consequence of the relatively high content of sialic acid in the dorsal skin (*P. carvalhoi* (Greven, Brenner, & Pyta, 1995b)). Swelling of the cloaca and skin is associated with ovulation and can be induced in *P. carvalhoi* by administration of human chorionic gonadotropin, hCG (Greven, unpublished). Water in which a receptive female has been held stimulates calling activity of males (*P. parva* (Greven & Woste, in prep.)), and water from a tank in which a mating pair has been held stimulates locomotor, calling, and clasping activity of unmated males (*P. pipa* (Rabb & Rabb, 1963)).

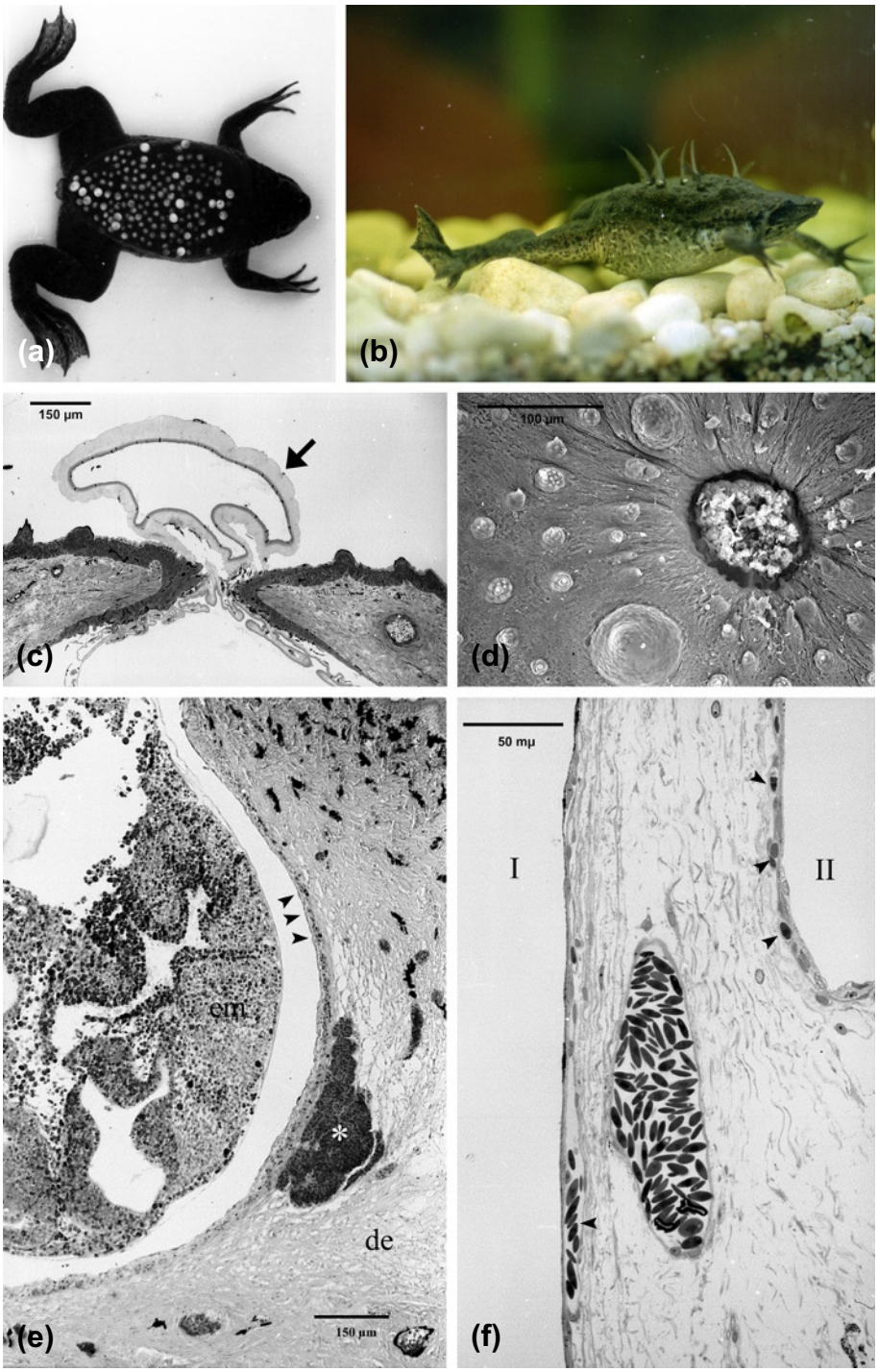

FIGURE 7.3 Skin incubation in *Pipa carvalhoi*. (a) Female a short time after mating with eggs on her back. (b) Birth of tadpoles tail ahead. (c) Pore of a brood chamber with a cup of egg jelly (arrow) (semithin epoxy section, toluidine blue borax). (d) Pore of the brood chamber closed by debris (SEM). (e) Brood chamber with embryo (em) and large dermal (de) blood vessel (asterisk); note thinness of the wall (arrowheads) (paraffin section, trichrome-Goldner). (f) Vascularized connective tissue between two chambers (I, II); note capillaries immediately beneath the very thin brood chamber epithelium (arrowheads)(Semithin epoxy section, toluidine blue borax). See color plate section at the end of the book.

In *P. carvalhoi* (Weygoldt, 1976b; Greven & Richter, 2009) and *P. parva* (Greven & Kretschmer, unpublished), eggs adhere to the female's back skin with their sticky outer jelly layer (Figure 7.3(a)) and are implanted within 24 to 36 hours. Implantation and the formation of the individual brood chambers, which resembles the nidation process in mammals, is caused by an unknown interaction of the egg and the skin surface. Part of the jelly becomes squeezed out

of the pore of the brood chamber and forms a cup, which drops off later (Figure 7.3(c)). Sites on which no eggs adhere exhibit detumescence.

The epidermis and dermis of brood chamber areas undergo considerable remodeling, which is associated with the growth of the larva. The depression containing the egg closes over the embryo. A small pore remains but is plugged with the egg jelly or its remnants (Figure 7.3(d)). In the dermis around and below the brood chamber, the number of collagen fibers decreases considerably and skin glands disappear. The distended epidermis forms the wall of the brood chambers, looses its keratinized layer, and becomes bilayered after complete implantation. Egg chambers become well-supplied with blood vessels, indicating extensive angiogenesis within a relatively short time (Figure 7.3(e–f)).

3.1.2. Gestation time and endocrinological aspects

Depending on the species and the stage at which the offspring are released, young are born after a gestation period of two to four weeks (*P. carvalhoi* (Weygoldt, 1976a; 1976b); *P. parva* (own observations)) or 105–145 days (*P. pipa* (Schütte & Ehrl, 1987)). There is some indirect evidence that suggests vitellogenesis is slowed down or even inhibited during skin incubation. In *P. carvalhoi*, postvitellogenic oocytes do not develop in the ovary during incubation (Greven & Richter, 2009). The presence of CL was mentioned for *P. pipa* (Lofts, 1974; Guillette, 1987), but has not been clearly demonstrated in the ovary of any *Pipa* spp.

3.1.3. Mother–offspring interactions

3.1.3.1. Respiration and gas exchange

The gills of the larvae during skin incubation are small; therefore, gas exchange may primarily take place across the well-vascularized larval yolk sac (*P. carvalhoi* (Weygoldt, 1976b)). A transparent, vascularized structure in the form of 'two rounded spiraling leaves' protruding from the cloaca of newborn froglets of *P. pipa* and their thin jaw flaps have been interpreted as respiratory organs, but further details are unknown (Rabb & Snedigar, 1960).

3.1.3.2. Osmoregulation and excretion

The cup of the dorsal brood chambers, consisting of egg jelly remnants and debris in advanced gestation, seems not to be tight. However, due to the smallness of the pore and the brood chamber, I suggest that ureotelism is the predominant mode of nitrogen excretion of the developing larvae.

3.1.3.3. Immunology

No information available

3.1.3.4. Allocation of food

Eggs of *Pipa* spp. contain considerable yolk. In *P. carvalhoi*, the dry weight of freshly hatched larvae was considerably lower than the dry weight of the deposited eggs, suggesting lecithotrophy (Weygoldt, 1976b). This contrasts with the report by Rabb and Snedigar (1960) that in *P. pipa* the weight of an emerging froglet is twice as much as that of a released egg, which information, however, might refer to the fresh weight of froglets.

3.1.3.5. Parturition

Nothing is known about the initiation of the birth process in skin-brooding *Pipa* spp. The first observable change is dilation of the brood chamber pore, followed by the release of larvae, mostly tail ahead. Larvae, including nonviable ones, appear to be pressed out. The mechanism of expulsion is not clear since there are no noticeable muscles around the brood chamber (Weygoldt, 1976b; Greven & Richter, 2009). Parturition appears to be associated with molting and spans a period of several days (in *P. pipa*, 77–134 (Rabb & Snedigar, 1960)). After birth, cellular debris, probably the lining of the brood chamber, is released and the female's dorsal skin is regenerated. This process resembles wound healing (Weygoldt, 1976b).

3.2. Skin incubation in the Hemiphractidae, Cryptobatrachidae, and Amphignathodontidae

In addition to the Pipidae, three families of skin-brooding anurans, previously subsumed under the Hemiphractinae (Hylidae), appear to be rather distantly related to hylids. They represent several clades not closely related to each other. However, the evolution of a partially or completely enclosed brood pouch probably occurred only once (Duellman, 1989).

Eggs are carried on the back either unprotected (no brood pouch)—e.g., in *Hemiphractus* spp. and the cryptobatrachids *Cryptobatrachus* spp. and *Stefania* spp. (Figure 7.4(a)—or in shallow basins; i.e., eggs and larvae are only bordered by lateral bulges of skin (e.g., some *Flectonotus* spp., formerly *Fritziana*) or brood pouches of increasing complexity, as in the Amphignathodontidae. Brood pouches range from partially closed with a middorsal opening to closed with a dorsal aperture anterior to the cloaca, e.g., in the genera *Flectonotus* and *Gastrotheca* (Figure 7.4(b)). The pouch is attached to the body by the dorsal septum (for a detailed description of the organization of the incubatory skin and different types of pouches, see del Pino (1980)). The more complex pouches are suggested to have originated from infoldings of the dorsal skin to protect the developing offspring in a terrestrial environment against predation and/or desiccation

FIGURE 7.4 Skin incubation in Hemiphractinae. (a) Egg brooding female of *Stefania evansi*. (b) Pregnant female of *Gastrotheca riobambae*. (c) Bell-gills in *Gastrotheca griswaldi*. (d) Birth of a froglet in *G. griswaldi* without the aid of the female. *Copyright of (a–c) retained by Prof. Dr. U. Sinsch, Koblenz, Germany.* See colr plate section at the end of the book.

(e.g., del Pino, 1980; 1989; Weygoldt & Potsch de Carvalho e Silva, 1991).

In *Flectonotus goeldii*, a species with an incubation site intermediate between no brood pouch and a closed brood pouch, the lateral skin folds that border the egg sac are absent in non-reproductive females. In *Gastrotheca* spp., however, pouches are formed during ontogeny by invaginations of the skin and are permanently present in sexually mature females (e.g., del Pino, Galarza, Albuja, & Humphries, 1975; Duellman & Maness, 1980). The formation of the pouch and its vascularization can be induced in juvenile female *Gastrotheca riobambae* by the administration of E_2 (Jones, Gerrard, & Roth, 1973).

In spite of the different pouch structures, mating behavior and oviposition seem to be similar. In species with pouches, the male distends the pouch aperture with his feet at the time of amplexus (e.g., Duellmann & Maness, 1980; Duellmann & Gray, 1983). Before the eggs are laid, females of *F. goeldii* (and perhaps of other species) extrude a mucous secretion from their oviducts that is beaten into foam by the male sitting on her back. The foam

subsequently surrounds all eggs and hardens to become a brood sac in which the eggs are embedded (Weygoldt & Potsch de Carvalhoi e Silva, 1991).

Young are released as larvae, e.g., *Flectonotus* spp. and some species of *Gastrotheca*, or as transformed froglets, e.g., the majority of *Gastrotheca* spp. (del Pino et al., 1975; Duellman & Maness, 1980; del Pino & Escobar, 1981). However, most studies on maternal adaptations involve *G. riobambae*, a species that produces free-living tadpoles. Data on other taxa with no pouches or with partially closed pouches are sketchy.

3.2.1. The skin and its changes during reproduction

Generally, the incubatory skin in species without a brood pouch has many mucous glands, appears more vascularized, and occasionally is folded. At incubation, the jelly of the deposited eggs adheres to the thickened and vascularized skin by means of small infoldings of the maternal epidermis and secretions of the mucous glands, The number of granular glands appears to be reduced and the number and size of mucous glands appear to be increased during reproduction (e.g., *Cryptobatrachus boulengeri* (De Pérez et al., 1992b); see also del Pino, 1980). There are slight partitions or depressions, each of which holds an egg or embryo (*Stefania* spp. and *F. goeldii*); they are formed passively by the shape of the eggs and disappear if the eggs are removed.

During the non-reproductive period, the lining of the pouch is less keratinized and folded; mucous glands, but not serous glands, are abundant. The dermis is well-vascularized.

Formation of the lateral folds in *F. goeldii* is probably induced during oviposition by the egg sac. The folds disappear when the egg sac is removed or if the egg sac does not reach the wall of the basin (e.g., *F. goeldii* (Weygoldt & Potsch de Carvalho e Silva, 1991)). This suggests that substances of the egg sac or eggs are involved in the maintenance of the basin. In addition, induction of angiogenesis by eggs and/or tadpoles is probable.

In *G. riobambae*, a species with a permanent pouch, the pouch aperture is open in non-reproductive females, but during vitellogenesis the pouch enlarges and the aperture narrows gradually. Dermal vascularization is increased in females with yolked eggs prior to ovulation (Jones et al., 1973). The pouch is closed prior to mating and ovulation. During oviposition the male opens it, but after that the pouch remains closed until birth (del Pino et al., 1975; del Pino & Escobar, 1981; del Pino, 1989).

During incubation, the pouch becomes distended by the growing offspring occupying the entire back and in most cases also the sides of the female's body (Figure 7.4(b)). The attenuated wall is highly vascularized and lateral projections form individual vascularized chambers for each egg. Further, the number of serous glands appears to be decreased, whereas the number of mucous glands appears to be increased (Jones et al., 1973; del Pino et al., 1975). After birth, reorganization of the brood pouch is connected with shedding of the epithelium (several larvi- and pueriparous *Gastrotheca* spp.: del Pino et al. (1975); del Pino (1980).

3.2.2. Gestation time and endocrinological aspects

The incubation period varies among species of anuran skin brooders. In *G. riobambae*, it lasts from 100 to 120 days. Development is slow and synchronous in a given clutch. In *Flectonotus* spp., incubation lasts only 17 to 29 days to produce an advanced tadpole with well-developed hind legs (e.g., del Pino et al., 1975; Duellman & Maness, 1980; del Pino & Escobar, 1981; Weygoldt & Potsch de Carvalho e Silva, 1991).

Maintenance of early incubation in *G. riobambae* and associated changes of the pouch seem to be mediated by CL. Del Pino and Sanchez (1977) described postovulatory follicles, not filled completely with granulosa cells, that proliferate in the first two or three weeks of incubation and degenerate after formation of the individual brood chambers. They may persist for approximately a third of the gestation time. Ovaries grew slightly during the second half of gestation. Although steroidogenic activity as well as plasma steroid levels have not been determined, there is indirect evidence that P_4 plays an important role in maintaining gestation. Ovariectomy 20 days after incubation results in abortion but has no effect later in gestation (del Pino & Sanchez, 1977). Intraperitoneal administration of P_4 induces closure of the pouch regardless of the size of oocytes and hCG elicits the same, but only in females with large ovaries. Progesterone also induces the formation of vascularized embryonic chambers, but only when inert plastic beads are inserted into the pouch of stimulated frogs, indicating the involvement of additional unknown stimuli. How much later pregnancy is maintained is unknown, and other sources for P_4 synthesis have not been examined. Exogenous P_4 administered to females with and without beads resulted in a behavior that females also show after mating: they move under a stone and try to accommodate the eggs (or the beads, if present) in one or two layers (del Pino, 1983). Generally, del Pino and coworkers suggested that (1) the ovarian follicle begins secretion of P_4 (or a P_4-like hormone) under the influence of GTH, resulting in changes to the pouch before ovulation; (2) GTHs synchronously stimulate and maintain CL; and (3) progesterone elicits the formation of embryonic chambers.

3.2.3. Mother–offspring interactions

3.2.3.1. Respiration and gas exchange

Embryos of marsupial frogs develop a single pair or two pairs of well-vascularized and stalked bell-shaped gills (Figure 7.4(c)), which in many cases envelop the offspring completely until birth. Gills of embryos, tadpoles, and froglets and the vascularized pouch epithelium are separated from each other only by the thin egg jelly (del Pino et al., 1975; del Pino, 1989).

3.2.3.2. Osmoregulation and excretion

G. riobambae is ureotelic and shows high arginase activity during the entire larval period; i.e., in the pouch and as a free-living tadpole. Urea is stored in the capsular fluid by the embryo and by larvae, which latter excrete it immediately after birth (Alcocer, Santacruz, Steinbeisser, Thierauch, & Del Pino, 1992; del Pino et al., 1994).

3.2.3.3. Immunology

No information available

3.2.3.4. Allocation of food

Eggs are considerably yolked and embryos from stage 19 (of 25 stages according to del Pino and Escobar (1981)) develop normally in amphibian physiological saline (del Pino et al., 1975). The dry weight of embryos does not decrease notably and remains rather constant during incubation with newborns still containing yolk. Therefore, transfer of maternal nutrition during gestation can be assumed (del Pino & Escobar, 1981; del Pino, 1989).

3.2.3.5. Parturition

The egg jelly breaks at birth. In some species the mother immerses the lower part of her body in the water and assists at birth by introducing the long toes of the hind legs inside the pouch (Figure 7.4(d)). The birth process may span a period of several days (e.g., del Pino et al., 1975; Duellman & Maness, 1980).

4. OTHER SITES OF INCUBATION

4.1. Gastric Incubation in the Myobatrachidae

Females of the two aquatic myobatrachids, *Rheobatrachus silus* and *Rheobatrachus vitellinus*, from Australia swallow their eggs or embryos (not seen yet), which develop in the stomach until metamorphosis (data for *R. silus* summarized in Tyler (1983a); *R. vitellinus*: Leong, Tyler, & Shearman, 1986). Gastric brooding may have evolved via cannibalism of eggs or young secreting considerable amounts of prostaglandins, which block the production of gastric hydrochloric acid at least in *R. silus* (Tyler, 1983c). Unfortunately, both species are now believed to be extinct (see McDonald & Alford, 1999; International Union for Conservation of Nature, 2008).

4.1.1. The stomach and its changes during reproduction

The incubation site is composed of the distal half to two thirds of the esophagus and the fundic region of the stomach. The esophagus is lined by an epithelium consisting of ciliated cells interspersed with goblet cells; the stomach has typical gastric pits and glands with mucous cells and hydrochloric acid-producing parietal (oxyntic) cells.

In *R. silus*, the stomach becomes greatly distended and thin-walled. Noticeable alterations are (1) the attenuation of the surface epithelium; (2) regression of the acid-secreting (oxyntic) glands (Fanning, Tyler, & Shearman, 1982; Fanning 1983); and (3) the disruption of the smooth muscle layers and muscular contraction in the underlying connective tissue. The partial dissolution of the connective tissue matrix surrounding the muscles allows the enormous distension of the stomach wall. An increased vascularization of the stomach during gestation has also been mentioned (O'Brien & Shearman, 1983). These alterations are entirely reversed eight days after birth (Gibbins & Tyler, 1983).

In *R. vitellinus*, there are no significant structural differences between the stomachs of non-brooding and brooding females. In the latter only an increased number of 'apoptotic bodies' was found in mucous and oxyntic cells. This probably suggests an independent evolution of gastric brooding in the two species (Leong et al., 1986).

4.1.2. Gestation time

Females of *R. silus* keep the developing young in the stomach for some eight weeks (Corben, Ingram, & Tyler, 1974). It probably will not be possible to obtain data on the occurrence and possible persistence of post-ovulatory follicles or steroid profiles in the plasma since these gastric-brooding species are probably extinct.

4.1.3. Mother–offspring interactions

Obviously, inhibition of acid and pepsin secretion in *R. silus* occurs once the eggs (or embryos) are swallowed. Water in which prematurely born tadpoles were held contained prostaglandin E_2 (PGE_2), which elicited a clear decrease in acid secretion of the gastric mucosa of the toad *Bufo marinus* and relaxed muscles of the gut. Sources of PGE_2 might be mucus threads the tadpoles release from their mouths and possibly secretions of their skin. In addition, the egg jelly as well as the skin and the buccal gland of transformed young have been suggested as possible sources (O'Brien & Shearman, 1983; Tyler et al.,

1983). Feeding tadpoles of several anuran species release prostaglandins, very probably from oral mucus glands, and PGE_2 may be used to regulate gill regression (Wassersug, 1986; Warkentin & Wassersug, 2001).

4.1.3.1. Respiration and gas exchange

Neither structural nor physiological data are available.

4.1.3.2. Immunology

No data available

4.1.3.3. Osmoregulation and excretion

Not explored, but ureotelism is expected.

4.1.3.4. Allocation of food

The mean diameters of oocytes from ovaries of two females of *R. silus* were 4.7 and 4.6 mm, respectively (Horton, 1983). Thus, offspring probably feeds exclusively on the yolk (lecithotrophy).

4.1.3.5. Parturition

Fully metamorphosed froglets emerge from the mother's mouth actively or are vomited, if the mother is restrained. In one birth, 25 froglets, 11.9 to 12.9 mm long, emerged (Corben et al., 1974; Tyler, 1983b). The trigger for parturition probably will remain unknown

4.2. Vocal Sac Incubation in the Cycloramphidae

The vocal sac(s) of anurans communicates with the buccal cavity through paired slits on both sides at the floor of the mouth. Vocal sacs are secondary sexual characteristics developing under the influence of gonadal hormones, and are permanently present in adult males of many species. The squamous epithelium lining the vocal sac is thin, non-keratinized, and non-ciliated, and its epithelial cells are secretory (Greven, 1987b).

Within the Cycloramphidae, there are two species of the genus *Rhinoderma* (Cycloramphinae), which are small, largely terrestrial frogs that live near cold streams in the southern beech forests of Chile and Argentina. These frogs use their enlarged unilobular vocal sacs for incubation (Jorquera, Pugin, & Goicoechea, 1972). Approximately 20 days after laying eggs in moist soil, male *Rhinoderna darwinii* pick up the embryos by mouth from their degrading egg jelly and deposit them in their vocal sacs until metamorphosis (e.g., Goicoechia, Garrido, & Jorquera, 1986; Crump, 2002). *Rhinoderma rufum* males pick up embryos approximately eight days after hatching and later (the exact period is unknown) release stage 3 larvae, characterized by differentiated horny teeth, into the water (stages according to Jorquera, Pugin, & Goicoechea, 1974; the species is named *R. darwinii* in the cited article).

4.2.1. The vocal sac and its changes during reproduction

Apical cells of the vocal sac epithelium of *R. darwinii* are highly secretory and secretory products obviously contribute to the viscous fluid found inside the sac. The most conspicuous changes during the time the male carries the developing offspring include enhancing vascularization in some regions; flattening of epithelial cells, especially near blood vessels; and changes in these cells that involve development of an irregular surface and prominent RER and a decrease of secretory products (Garrido, Pugin, & Jorquera, 1975).

4.2.2. Gestation time and endocrinological aspects

In *R. darwinii*, transformed juveniles emerge from their father's mouth after a gestation period of up to 52 days (Jorquera et al., 1972; Goicoechea et al., 1986). No further information is available.

4.2.3. Father–offspring interactions

4.2.3.1. Respiration and gas exchange

The skin and the cloaca of tadpoles are highly vascularized (Jorquera et al., 1972).

4.2.3.2. Osmoregulation and excretion

No data available, but ureotelism is expected.

4.2.3.3. Immunology

No data available.

4.2.3.4. Allocation of food

The eggs of *R. darwinii* are relatively large (diameter approximately 4 mm (e.g., Crump, 2002)). Larvae taken from the vocal sac develop more slowly than inside the vocal sac and do not reach metamorphosis (Jorquera et al., 1972). Goicoechia et al. (1986) injected peroxidase into the dorsal lymphatic sac of pregnant males. After that, peroxidase was cytochemically demonstrated in a few secretory granules of the vocal sac epithelium and in the skin (and some other organs) of the larvae up to stage 11, according to Jorquera et al. (1972). Peroxidase activity was also detected in the vocal sac fluid. After injection of labeled amino acids (^{3}H-leucine, ^{3}H-valine), radioactivity was detected in the 'inner wall' of the vocal sac and in different organs of the young. From these results it was concluded that tadpoles may receive nutrients from secretions of the

male's vocal sac (patrotrophy) and that presence of young may stimulate the discharge of secretory products (Garrido et al., 1975). Males feed while carrying larvae, but not more than non-brooding males (Crump, 2002).

4.2.3.5. Parturition

Males carry up to 19 tadpoles. Occurrence of different developmental stages in the vocal sac of a single male indicates brooding of more than one cohort. Parturition takes place at intervals of several days (for further reading see Crump, 2002). Signals for the father to release their offspring are unknown. Interestingly, in males of *Eleutherodactylus coqui*, an internally fertilizing egg-laying species with parental care, androgen levels are decreased while they are attending the terrestrial eggs, suggesting an (external) stimulus probably from the eggs (Townsend & Moger, 1987). Clearly, such aspects are worth being explored in *Rhinoderma* spp as well.

5. CONCLUSIONS

The present survey has summed up the very incomplete knowledge of physiological, endocrinological, and morphological adaptations of amphibians using the oviduct, skin, vocal sac, and stomach as incubation sites. Some challenges these incubating taxa, primarily the oviductal breeders, may face were outlined 20 years ago and some approaches have been suggested. In the absence of specific information, these suggestions are based on regulatory mechanisms known from mammals (Guillette, 1987; 1989).

In oviductal breeders, and probably also in some marsupial frogs, final differentiation of the genital tract, maintenance of gestation, gestation length, and inhibition of further growth of ovarian follicles is attributed to P_4 secreted by CL. However, in several species, CL are not active until term. This may imply that high levels of P_4 are not essential after a certain period of pregnancy or that there is another source of P_4 after luteolysis (e.g., the adrenal gland), but plasma levels of P_4 in at least one species (*S. salamandra*) were low during pregnancy.

A further unanswered question concerns the extent to which the tertiary egg jelly has to be reduced, if any, for sufficient gaseous exchange, which is complicated by the fact that egg jelly compounds are important for fertilization in amphibians (e.g., Guilllette, 1987; 1989; Greven, 2003a).

The relatively long gestation time in viviparous vertebrates gives the offspring the possibility of influencing maternal physiology to their own benefit and vice versa. This may result in an investment beyond the optimal investment that parents should make in their young (e.g., Trivers, 1974; Crespi & Semeniuk, 2004), and this also may be the case in species that are not 'viviparous' in the traditional sense.

Without any doubt, eggs, larvae, and the transformed offspring produce regulatory molecules; e.g., cytokines, to evade both local and systemic immune responses, and prostaglandins, to affect cell differentiation, proliferation, apoptosis, etc. Increase of vascularization seems to be a characteristic feature of incubation sites, except among salamanders. A quantitative approach of vascularization, however, is missing for any brooding amphibian species. Stimuli for angiogenesis and vasodilation such as local hypoxia produced by the developing embryo or specific chemical factors such as ovarian steroids (which, in turn, may activate a wide range of local factors such as prostaglandins, cytokines, etc.) have not been identified (see Augustin, Iruela-Arispe, Rogers, & Smith, 2002; Suzuki, Nakamura, Moriya, & Sasono, 2003). The same holds for the recognition of pregnancy, achieved probably by means of interferons, prostaglandins, and steroids (see Guillette, 1989).

Further, reproduction may be stressful in many cases (the few data on amphibians are reviewed in Moore and Jessop (2003)). This is reflected in higher metabolic rates and in a possibly altered immunocompetence (e.g., Davis & Maerz, 2008). The relatively long gestation times increase the likelihood for pregnant females to encounter stressful situations and, consequently, to respond to them. The stress response involves release of corticosterone (CORT) from the adrenal gland, influencing the physiology and behavior of the pregnant parent, which, in turn, may reinforce the parental−offspring conflict. However, nothing is known to date concerning this matter for the amphibian taxa.

The chances to learn more about all of this are relatively low. Some of the taxa treated herein appear to be extinct, e.g., *Rheobatrachus* spp.; others are critically endangered, e.g., *E. jasperi* and *R. rufum* (see IUCN, 2008); and others are not easy to study because of their rarity and/or threatened status (most are therefore protected by law) and yet others because of their burrowing lifestyles (e.g., Gymnophiona). Nearly all are unsuitable experimental animals, either because they do not easily breed in captivity, if at all, or because their reproductive cycles are considerably long.

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ABBREVIATIONS

ϵ_f	Extension load
3β-HSD	3β-hydroxysteroid dehydrogenase
AND	Androstenedione
AVT	Arginine vasotocin
CL	Corpus luteum
CORT	Corticosterone
E_2	Estradiol
FSH	Follicle-stimulating hormone
GTH	Gonadotropin
hCG	Human chorionic gonadotropin
IL	Interleukin
LH	Luteinizing hormone
P_4	Progesterone
pc	Pars convoluta
PGE_2	Prostaglandin E_2
pr	Pars recta
PRL	Prolactin
pu	Pars uterina
T	Testosterone

REFERENCES

Alcocer, I., Santacruz, X., Steinbeisser, H., Thierauch, K. H., & Del Pino, E. (1992). Ureotelism as the prevailing mode of nitrogen excretion in larvae of the marsupial frog *Gastrotheca riobambae* (Fowler) (Anura, Hylidae). *Comp. Biochem. Physiol., 101A*, 229−231.

Augustin, H. G., Iruela-Arispe, M., Rogers, P. A. W., & Smith, S. K. (Eds.). (2001). *Vascular morphogenesis in the female reproductive system*. (pp. 1-333). Basel: Birkhäuser.

Badet, M. T. (1984). Specific and non-specific immunosuppressive factors in salamander pregnancy serum. *J. Reprod. Immunol., 6*, 299−311.

Badet, M. T., Chateuareynaud-Duprat, P., Mouches, V., Bove, J. M., & Metivaud, J. (1980). Separation and study of two serum factors involved in the inhibition of the maternal cytotoxic reaction against embryonic cells in pregnant *Salamandra*. *An. Immunol., 131D*, 57−69.

Barthalmus, G. (1994). Biological roles of amphibian skin secretions. In H. Heatwole, & G. T. Barthalmus (Eds.), *"Amphibian Biology", Vol. 1* (pp. 382−410). New South Wales, Australia: Surrey Beatty and Sons.

Billo, R. R., Straub, J. O., & Senn, D. G. (1985). Vivipare Apoda (Amphibia: Gymnophiona), *Typhlonectes compressicaudus* (Dumeril and Bibron, 1841): Kopulation, Tragzeit und Geburt. *Amphibia-Reptilia, 6*, 1−9.

Blackburn, D. G. (2000). Classification on the reproductive patterns of amniotes. *Herpetological Monographs, 14*, 371−377.

Breckenridge, W. R., & Jayasinghe, S. (1979). Observation on the eggs and larvae of *Ichthyophis glutinosus*. *Ceylon J. Sci. (Biol. Sci.), 13*, 187−202.

Brizzi, R., Delfino, G., & Jantra, S. (2003). An overview of breeding glands. In B. G. M. Jamieson (Ed.), *"Reproductive Biology and Phylogeny of Anura"* (pp. 253−317). Enfield NH: Science Publishers.

Buckley, D., Alcobendas, M., García-Paris, M., & Wake, M. H. (2007). Heterochrony, cannibalism, and the evolution of viviparity in *Salamandra salamandra*. *Evolution & Development, 9*, 105−115.

Corben, C. J., Ingram, G. J., & Tyler, M. J. (1974). Gastric brooding: Unique form of parental care in an Australian frog. *Science, 186*, 946−947.

Crespi, B., & Semeniuk, C. (2004). Parent−offspring conflict in the evolution of vertebrate reproductive mode. *American Naturalist., 163*, 635−653.

Crump, M. L. (2002). Natural history of Darwin's frog, *Rhinoderma darwinii*. *Herpetological Natural History, 9*, 21−30.

Davis, A. K., & Maerz, J. C. (2008). Sex-related differences in hematological stress indices of breeding paedomorphic mole salamanders. *J. Herpetol., 42*, 197−201.

Degani, G., Sharon, R., & Warburg, M. (1997). Ovarian steroid levels in *Salamandra salamandra infraimmaculata* during the reproductive cycle. *Gen. Comp. Endocrinol., 106*, 356−360.

Del Pino, E. M. (1980). Morphology of the pouch and incubatory integument in marsupial frogs. *Copeia, 1980*, 10−17.

Del Pino, E. M. (1983). progesterone induces incubatory changes in the brooding pouch of the frog *Gastrotheca riobambae (Fowler)*. *J. Exptl. Zool., 227*, 159−163.

Del Pino, E. M. (1989). Modifications of oogenesis and development in marsupial frogs. *Development, 107*, 169−187.

Del Pino, E. M., & Escobar, B. (1981). Embryonic stages of *Gastrotheca riobambae* (Fowler) during maternal incubation and comparison of development with that of other egg-brooding hylid frogs. *J. Morphol., 167*, 277−296.

Del Pino, E. M., & Sanchez, G. (1977). Ovarian structure of the marsupial frog *Gastrotheca riobambae* (Fowler). *J. Morphol., 153*, 153−162.

Del Pino, E. M., Alcocer, I., & Grunz, H. (1994). Urea is necessary for the culture of embryos of the marsupial frog *Gastrotheca riobambae*, and is tolerated by embryos of the aquatic frog, *Xenopus laevis*. *Develop. Growth & Differ., 36*, 73−80.

Del Pino, E. M., Galarza, M. L., Albuja de, C. M., & Humphries, A. A., Jr. (1975). The maternal pouch and development in the marsupial frog *Gastrotheca riobambae (Fowler)*. *Biol. Bull., 149*, 480−491.

De Pérez, G., Ruiz-Carranza, P. M., & Ramírez-Pinilla, M. P. (1992a). Modificaciones tegumentarias de larvas y adultos durante el cuidado parental en *Minyobates virolinensis* (Amphibia: Anura: Dendrobatidae). *Caldasia, 17*, 75−86.

De Pérez, G., Ruiz-Carranza, P. M., & Ramírez-Pinilla, M. P. (1992b). Especializaciones del tegumento de incubacion de la hembra de *Cryptobatrachus boulengeri* (Amphibia: Anura: Hylidae). *Caldasia, 17*, 87−94.

Delsol, M., Exbrayat, J.-N., Flatin, J., & Gueydan-Baconnier, M. (1986). Nutrition embryonnaire chez *Typhlonectes compressicaudus* (Dumeril et Bibron, 1841), amphibien apode vivipare. *Mem. Soc. Zool. Fr., 43*, 39−54.

Duellman, W. E. (1989). Alternative life-history styles in anuran amphibians: Evolutionary and ecological implications. In M. N. Bruton (Ed.), *"Alternative life-history styles of animals"* (pp. 101−126). Dordrecht: Kluwer Academic.

Duellman, W. E., & Maness, S. J. (1980). The reproductive behavior of some hylid marsupial frogs. *J. Herpetol., 14*, 213−222.

Duellman, W. E., & Gray, P. (1983). Developmental biology and systematics of the egg-brooding hylid frogs, genera *Flectonotus* and *Fritziana*. *Herpetologica, 39*, 333−359.

Ehmann, H., & Swan, G. (1985). Reproduction and development in the marsupial frog, *Assa darlingtoni* (Leptodactylidae, Anura). In G. Grigg, R. Shine, & H. Ehmann (Eds.), *"Biology of Australasian frogs and reptiles"* (pp. 279−285). Chipping Norton: Surrey Beatty & Sons.

Exbrayat, J.-M. (1984). Quelques observations sur l'evolution des voies genitales femelles de *Typhlonectes compressicaudus* (Dumeril et Bibron, 1841), Amphibien Apode vivipare, au cours du cycle de reproduction. *C. R. Acad. Sci. Paris Ser., 298*, 13−18, III.

Exbrayat, J.-M. (1988). Croissance et cycle des voies génitales femelles de *Typhlonectes compressicaudus* (Duméril et Bibron, 1841), amphibien apode vivipare. *Amphibia-Reptilia, 9*, 117−134.

Exbrayat, J.-M. (1989). Quelques observations sur les appareils génitaux de trois gymnophiones; *hypothéses sur le mode de reproduction de Microcaecilia unicolor (Amphibia, Gymnophiona)*. *Bull. Soc. Herp. Fr., 52*, 34−44.

Exbrayat, J.-M. (2006a). Endocrinology of reproduction in Gymnophiona. In J.-M. Exbrayat (Ed.), *"Biology and phylogeny of Gymnophiona (Caecilians)"* (pp. 184−227). Enfield NH: Science Publishers.

Exbrayat, J.-M. (2006b). Modes of parity and oviposition. In J.-M. Exbrayat (Ed.), *"Biology and phylogeny of Gymnophiona (Caecilians)"* (pp. 303−323). Enfield NH: Science Publishers.

Exbrayat, J. M., & Collenot, G. (1983). Quelques aspects de l'évolution de ĺovaire de *Typhlonectes compressicaudus* (Duméril et Bibron, 1841), batracien apode vivipare.—Etude quantitative et histochimique des corps jaunes. *Reprod. Nutr. Développement, 23*, 889−898.

Exbrayat, J. M., & Hraoui-Bloquet, S. (2006). Viviparity in *Typhlonectes compressicauda*. In J.-M. Exbrayat (Ed.), *"Biology and phylogeny of Gymnophiona (Caecilians)"* (pp. 325−357). Enfield NH: Science Publishers.

Exbrayat, J.-M., & Morel, G. (1990/1991). The cytological modifications of the distal lobe of the hypophysis in *Typhlonectes compressicaudus* (Duméril and Bibron, 1841), Amphibia Gymnophiona, during the cycles of seasonal activity. II. In adult females. *Biol. Struct. Morphol., 23*, 129−138.

Exbrayat, J.-M., & Morel, G. (1995). Prolactin (PRL)-coding mRNA in *Typhlonectes compressicaudus*, a viviparous gymnophionian amphibian: an *in situ* hybridization study. *Cell Tissue Res., 280*, 133−138.

Exbrayat, J.-M., & Morel, G. (2003). Visualization of gene expression of prolactin-receptor (PRL-R) by *in situ* hybridization in reproductive organs of *Typhlonectes compressicauda*, a gymnophionan amphibian. *Cell Tissue Res., 312*, 361−367.

Exbrayat, J.-M., Pujol, P., & Hraoui-Bloquet, S. (1995). First observations on the immunological materno−foetal relationships in *Typhlonectes compressicaudus*, a viviparous gymnophionan Amphibia. In G. A. Lorente, A. Montori, A. X. Santos, X., & M. A. Carretero (Eds.), *"Scientia Herpetologica"* (pp. 271−273). Barcelona: Asociacion Herpetologica Espanola.

Fanning, J. J. (1983). In M. J. Tyler (Ed.), *"The gastric brooding frog"* (pp. 93−115). London & Canberra: Croom Helm.

Fanning, J. L., Tyler, M. J., & Shearman, D. J. C. (1982). Converting a stomach to a uterus: the microscopic structure of the stomach of the gastric brooding frog, *Rheobatrachus silus*. *Gastroenterology, 82*, 62−70.

Fernández, S. N., & Ramos, I. (2003). Endocrinology of Reproduction. In B. G. M. Jamieson (Ed.), *"Reproductive Biology and Phylogeny of Anura"*. 174−117. Enfield NH: Science Publishers.

Fox, H. (1994). The structure of the integument. In H. Heatwole, & G. T. Barthalmus (Eds.), *"Amphibian Biology", Vol. 1* (pp. 1−32). Chipping Norton, New South Wales, Australia: Surrey Beatty and Sons.

Frost, D. R. (2008). *Amphibian species of the world: an online reference. Version 5.2. (15 July 2008)*. New York, USA: American Museum of Natural History. Electronic database accessible at. http://research.amnh.org/herpetology/amphibian/index.php.

Fujikura, K., Kurabuchi, S., Tabuchi, M., & Inoue, S. (1988). Morphology and distribution of the skin glands in *Xenopus laevis* and their response to experimental stimulation. *Zool. Sci., 5*, 415−430.

Garlick, R. L., Davis, B. J., Farmer, M., Fyhn, H. J., Fyhn, U. E. H., Noble, R. W., Powers, D. A., Riggs, A., & Weber, R. E. (1979). A fetal−maternal shift in the oxygen equilibrium of hemoglobin from the viviparous caecilian, *Typhlonectes compressicauda*. *Comp. Biochem. Physiol., 62A*, 239−244.

Gasche, P. (1942). Beeinflussung der Larvenablage von *Salamandra salamandra* L. *Verhandlungen der Naturforschenden Gesellschaft Basel, 53*, 246−264.

Garrido, O., Pugin, E., & Jorquera, B. (1975). Correspondance ultrastructurale entre la bourse gutterale du *Rhinoderma darwini* et le tegument des larves. *Boll. Zool., 42*, 133−134.

Gibbins, I. L., & Tyler, M. J. (1983). Changes in the organization and ultrastructure of smooth muscle cells in the stomach of the gastric brooding frog, *Rheobatrachus silus*, during brooding. *Cell Tissue Res., 231*, 451−456.

Goicoechea, P., Garrido, O., & Jorquera, B. (1986). Evidence for a trophic paternal−larval relationship in the frog *Rhinoderma darwini*. *J. Herpetol., 20*, 168−178.

Gower, D. J., Giri, V., Dharne, M. S., & Shouche, Y. S. (2008). Frequency of independent orgins of viviparity among caecilians (Gymnophiona): evidence from the first 'live bearing' Asian amphibian. *J. Evol. Biol., 21*, 1220−1226.

Greven, H. (1987a). Skin texture and specific gravity of three amphibian species during their aquatic and terrestrial phase. In J. J. van Gelder, H. Strijbosch & P. J. M. Bergers, (Eds.), *"Proceedings of the Fourth Ordinary General Meeting of the S.E.H. Nijmegen 1987"* (pp. 55−58). Faculty of Sciences, Nijmegen.

Greven, H. (1987b). The vocal sacs of *Rana ridibunda*: a TEM and SEM study. In J. J. van Gelder, H. Strijbosch & P. J. M. Bergers, (Eds.), *"Proceedings of the Fourth Ordinary General Meeting of the S.E.H. Nijmegen 1987"* (pp. 159−162). Faculty of Sciences, Nijmegen.

Greven, H. (1998). Survey of the oviduct of salamandrids with special reference to the viviparous species. *J. Exptl. Zool., 282*, 507−525.

Greven, H. (2002). The urodele oviduct and its secretions in and after G. von Wahlert's doctoral thesis "Eileiter, Laich und Kloake der Salamandriden". *Bonner zoologische Monographien, 50*, 25−61.

Greven, H. (2003a). Oviduct and egg jelly. In D. M. Sever (Ed.), *"Reproductive biology and phylogeny of Urodela"* (pp. 151−181). Enfield NH: Science Publishers.

Greven, H. (2003b). Larviparity and pueriparity. In D. M. Sever (Ed.), *"Reproductive biology and phylogeny of Urodela"* (pp. 447−475). Enfield NH,: Science Publishers.

Greven, H., & Guex, G. -D. (1994). Structural and physiological aspects of viviparity in *Salamandra salamandra*. In H. Greven, & B. Thiesmeier, (Eds). "*Biology of Salamandra and Mertensiella*" *Mertensiella 4*, 139−160.

Greven, H., & Richter, S. (2009). Morphology of skin incubation in *Pipa carvalhoi (Anura Pipida)*. *J. Morphol, 270*, 1311−1319.

Greven, H., & Thiesmeier, B., Eds. (1994). Biology of *Salamandra* and *Mertensiella. Mertensiella*, 4, 1−454.

Greven, H., Brenner, M., & Pyta, B. (1995). Sialic acid in the skin of some Amphibia. In G. A. Lorente, A. Montori, X. Santos, & M. A. Carretero (Eds.), *"Scientia Herpetologica"* (pp. 265−267). Barcelona: Asociacion Herpetologica Espanola.

Greven, H., Zanger, K., & Schwinger, G. (1995a). Mechanical properties of the skin of *Xenopus laevis* (Anura, Amphibia). *J. Morphol., 224*, 15−22.

Guex, G.-D. (1994). Zur Fortpflanzungsbiologie von *Salamandra atra aurorae* Trevisan 1982, *S. salamandra bernadezi* Wolterstorff, 1928, *S. salamandra fastuosa*, Schreiber 1892 und *Mertensiella luschani helversenii* Piper, 1963. *Abhandlungen und Berichte für Naturkunde, Magdeburg, 17*, 143−150.

Guex, G.-D., & Chen, P. S. (1986). Epitheliophagy: intrauterine cell nourishment in the viviparous alpine salamander *Salamandra atra (Laur.) Experientia, 42*, 1205−1218.

Guex, G.-D. & Greven, H. (1994). Structural and physiological aspects of viviparity in *Salamandra atra*. In H. Greven & B. Thiesmeier, (Eds.). "*Biology of Salamandra and Mertensiella*". *Mertensiella 4*, 139−160.

Guillette, L. R. Jr. (1987). The evolution of viviparity in fishes, amphibians, and reptiles: an endocrine approach. In D. O. Norris & R.E. Jones, (Eds.), *"Hormones and Reproduction in Fishes, Amphibians and Reptiles"* (pp. 523−562). New York, Plenum Press.

Guillette, L. R. Jr. (1989). The evolution of vertebrate viviparity: Morphological modifications and endocrine control. In D. B. Wake, D. B. & G. Roth, (Eds.), *"Complex Organismal Functions: Integration and Evolution in Vertebrates"* (pp. 219−233). Chichester: John Wiley & Sons.

Häfeli, H.-P. (1971). Zur Fortpflanzungsbiologie des Alpensalamanders (*Salamandra atra* Laur.). *Rev. Suisse Zool., 78*, 235−293.

Heller, H., Ferreri, E., & Leathers, D. H. G. (1970). The effect of neurohypophysial hormones on the amphibian oviduct *in vitro*, with some remarks on the histology of this organ. *J. Endocrinol., 47*, 495−509.

Hilscher, C. A., Conklin, D. J., & Boyd, S. K. (1994). Neurohypophysial peptides control of oviduct contraction in a caecilian amphibian. *Amer. Zool., 34*, 20A.

Horton, P. (1983). Reproductive system. In M. J. Tyler (Ed.), *"The gastric brooding frog"* (pp. 84−92). London & Canberra: Croom Helm.

Hraoui-Bloquet, S., & Exbrayat, J.-M. (1994). Développement des branchies chez les embryons de *Typhlonectes compressicaudus*, amphibien gymnophione vivipare. *Ann. Sci. Nat. Zool., Paris 13^{e} Série, 15*, 33−46.

Hraoui-Bloquet, S., & Exbrayat, J.-M. (1996). Les dents de *Typhlonectes compressicaudus* (Amphibia, Gymnophiona) au cours du développement. *Ann. Sci. Nat. Zool., Paris 13^{e} Série, 17*, 11−23.

Hraoui-Bloquet, S., Scudie, G., & Exbrayat, J.-M. (1994). Aspects ultrastructuraux de l'évolution de la muqueuse utérine au cours de la gestation chez *Typhlonectes compressicaudus*, Amphibien Gymnophione vivipare. *Bull. Soc. Zool. Fr., 119*. 237−234.

International Union for Conservation of Nature (IUCN). Read list of threatened species, 2008: www.iucnredlist.org

Jantra, S., Bigliardi, E., Brizzi, R., Ietta, F., Bechi, N., & Paulescu., L. (2007). Interleukin1 in oviductal tissue of viviparous, oviparous, and ovuliparous species of amphibians. *Biol. Reprod., 76*, 1009−1015.

Joly, J. (1986). La reproduction dé la salamandre terrestre (*Salamandra salamandra* L.). In P. P. Grasse, & M. Delsol (Eds.), *"Traite de zoologie", Vol. 14* (pp. 471−486). Paris: Masson, fasc. IB: Batraciens.

Joly, J., & Boisseau, C. (1973). Localisation des spermatozoïdes dans l'oviducte de la salamandre terrestre *Salamandra salamandra* (L.) (Amphibien, Urodèle) au moment de la fécondation. C. *r. hebd. séance. Acad. Sci. Paris Ser.* D, Sci. Nat*., 277*, 2537−2540.

Joly, J., & Picheral, B. (1972). Ultrastructure, histochimie et physiologie du follicule préovulatoire et du corps jaune de l'urodèle ovo-vivipare *Salamandra salamandra* (L.). *Gen. Comp. Endocrinol, 18*, 235−259.

Joly, J., Chesnel, F., & Boujard, D. (1994). Biological adaptations and reproductive strategies in the genus Salamandra. In H. Greven, & B. Thiesmeier, (Eds.) "Biology of *Salamandra and Mertensiella*". Mertensiella 4, 255−269.

Jones, R. E., Gerrard, A. M., & Roth, J. J. (1973). Estrogen and brood pouch formation in the marsupial frog *Gastrotheca riobambae*. *J. Exptl. Zool., 184*, 177−184.

Jorquera, B., Pugin, E., & Goicoechea, O. (1972). Tabla de desarrollo normal de *Rhinoderma darwini*. *Archivos de Medicina Veterinaria, 4*, 1−15.

Jorquera, B., Pugin, E., & Goicoechea, O. (1974). Tabla de desarrollo normal de *Rhinoderma darwini* (Concepcion). *Bol. Soc. Biol. De Concepcion, 48*, 127−146.

Kikuyama, S., Tanaka, S., & Moore, F. (2003). Endocrinology of reproduction. In D. M. Sever (Ed.), *"Reproductive biology and phylogeny of Urodela"*. 1275−321. Enfield NH: Science Publishers.

Kupfer, A., Kramer, A., Himstedt, W., & Greven, H. (2006a). Copulation and egg retention in an oviparous caecilian (genus *Ichthyophis*). *Zool. Anz., 244*, 223−228.

Kupfer, A., Müller, H., Antoniazzi, M. M., Jared, C., Greven, H., Nussbaum, R. A., & Wilkinson, M. (2006b). Parental investment by skin feeding in a caecilian amphibian. *Nature, 440*, 926−929.

Kupfer, A., Wilkinson, M., Gower, D. J., Müller, H., & Jehle, R. (2008). Care and parentage in a skin-feeding caecilian amphibian. *J. Exptl. Zool., 309A*, 460−467.

Lamotte, M., & Xavier, F. (1972). Les amphibiens anoures a développement direct d'africque. Observations sur la biologie de *Nectophrynoides tornieri* (Roux). *Bull. Soc. Zool. Fr., 97*, 413−418.

Lamotte, M., Rey, P., & Vogeli, M. (1964). Recherches sur l'ovaire de *Nectophrynoides occidentalis*, batracien anoure vivipare. *Arch. Anat. Microsc. Morphol. Exp., 53*, 173−224.

Lehtinen, R. M., & Nussbaum, R. A. (2003). Parental care: a phylogenetic perspective. In B. G. M. Jamieson (Ed.), *"Reproductive Biology and Phylogeny of Anura"* (pp. 343−385). Enfield NH,: Science Publishers.

Leong, A. S.-Y., Tyler, M. J., & Shearman, D. J. C. (1986). Gastric brooding: A new form in a recently discovered Australian frog of the genus *Rheobatrachus*. *Austral. J. Zool., 34*, 205−209.

Lewinson, D., Rosenberg, M., & Warburg, M. R. (1984). 'Chloride-cell-like mitochondria-rich cells of salamander larva gill epithelium. *Experientia, 40*, 956−958.

Lofts, B. (1974). Reproduction. In B. Lofts (Ed.), *"Physiology of the Amphibia", Vol. II* (pp. 309−521). New York: Academic Press.

Lostanlen, D., Boisseau, C., & Joly, J. (1976). Donneés ultrastructurales et physiologiques sur l'utérus d'un amphibien ovovivipare *Salamandra salamandra* (L.) (Amphibia, Urodela). *Ann. Sci. Nat., Zool. Biol. animale, Paris, 18*, 113−144.

Massood-Parveez, U., & Nadkarni, V. B. (1991). Morphological, histological, histochemical and annual cycle of the oviduct in *Ichthyophis beddomei* (Amphibia: Gymnophiona). *J. Herpetol., 25*, 234−237.

McDonald, K. R., & Alford, R. A. (1999). A review of declining frogs in Northern Queensland. In Campbell, A (ed), *"Declines and*

Disappearances of Australian frogs", (pp.14−22). (Environment Australia, Department of the Environment and Heritage: Canberra).

Moore, I. T., & Jessop, T. S. (2003). Stress, reproduction, and adrenocortical modulation in amphibians and reptiles. *Hormones and Behavior, 43*, 39−47.

Mossman, H. W. (1937). Comparative morphogenesis of the fetal membranes and accessory uterine structures. *Carnegie Institute Contributions in Embryology, 26*, 129−246.

Niederl, H. (1981). Anatomische und histologische Untersuchungen des weiblichen und männlichen Geschlechtszyklus von Salamandra atra (Laur.). *Zool. Jb. Anat. Ontogenie, 106*, 46−63.

Norris, D. O., & Lopez, K. H. (2005). Anatomy of the amphibian endocrine system. In H. Heatwole (Ed.), *"Amphibian biology". Endocrinology, Vol. 6* (pp. 2021−2044). Chipping Norton: Surrey, Beatty & Sons.

Norris, D. O., Austin, H. B., & Hijazi, A. S. (1989). Induction of cloacal and dermal skin glands of Tiger Salamander larvae, (*Ambystoma tigrinum*): Effects of testosterone and prolactin. *Gen. Comp. Endocrinol, 73*, 194−204.

O'Brien, P., & Shearman, D. (1983). Inhibition of gastric secretion. In M. J. Tyler (Ed.), *"The gastric brooding frog"* (pp. 116−128). London & Canberra: Croom Helm.

Packard, G. C. (1989). How are reproductive systems integrated and how has viviparity evolved? In D. B. Wake, D.B.., & G. Roth (Eds.), *"Complex Organismal Functions: Integration and Evolution in Vertebrates"* (pp. 281−293) Chichester: John Wiley & Sons.

Paulesu, L. (1997). Cytokines in mammalian reproduction and speculation about their possible involvement in nonmammalian viviparity. *Microsc. Res. Tech., 38*, 188−194.

Rabb, G. B., & Rabb, M. S. (1960). On the mating and egg-laying behavior of the Surinam toad, *Pipa pipa*. *Copeia, 1960*, 271−276.

Rabb, G. B., & Rabb, M. S. (1963). Additional observations on breeding behavior of the Surinam toad, *Pipa pipa*. *Copeia, 1960*, 636−642.

Rabb, G. B., & Snedigar, R. (1960). Observations on breeding and development of the Surinam toad, *Pipa pipa*. *Copeia, 1960*, 40−44.

Rastogi, R. K., Iela, L., di Meglio, M., di Fiore, M. M., ĎAniello, B., Pinelli, C., et al. (2005). Hormonal regulation of reproductive cycles in amphibians. In H. Heatwole. (Ed.), *"Amphibian biology". Endocrinology, Vol. 6* (pp. 2045−2177). Chipping Norton: Surrey, Beatty & Sons.

Sammouri, R., Renous, S., Exbrayat, J.-M., & Lescure, J. (1990). Développement embryonnaire de *Typhlonectes compressicaudus* (Amphibia, Gymnophiona). *Ann. Sci. Nat. Zool., Paris 13^{e} Série, 11*, 135−163.

Sever, D. M. (2002). Female sperm storage in amphibians. *J. Exptl. Zool., 291*, 165−179.

Sever, D. M., Hamlett, W. C., Slabach, R., Stephenson, B., & Verell, P. A. (2003). Internal fertilization in the Anura with special reference to mating and female sperm storage in *Ascaphus*. In B. G. M. Jamieson (Ed.), *"Reproductive Biology and Phylogeny of Anura"* (pp. 319−341). Enfield NH: Science Publishers.

Schindelmeiser, J. (1985). Structure and activity of the thyroid gland in intra- and extrauterine larvae of the ovoviviparous *Salamandra salamandra* (L.) (Amphibia, Urodela). *Z. mikrosk. anat. Forsch., 99*, 475−497.

Schindelmeiser, J., & Greven, H. (1981). Nitrogen excretion in intra- and extrauterine larvae of the ovoviviparous salamander, *Salamandra salamandra* (L.) (Amphibia, Urodela). *Comp. Biochem. Physiol., 75B*, 471−473.

Schindelmeiser, J., Schindelmeiser, I., & Greven, H. (1983). Hepatic arginase activity in intra- and extrauterine larvae of the ovoviviparous salamander, *Salamandra salamandra* (L.) (Amphibia, Urodela). *Comp. Biochem. Physiol., 75B*, 471−473.

Schütte, F., & Ehrl, A. (1987). Zur Haltung und Zucht der großen südamerikanischen Wabenkröte *Pipa pipa* (Linnaeus, 1758) (Anura: Pipidae). *Salamandra., 23*, 256−268.

Smith, H. M., & Chiszar, D. (2006). Dilemma of name-recognition: Why and when to use new combinations of scientific names. *Herpetol. Conserv. Biol., 1*, 6−8.

Stiffler, D. F., DeRuyter, M. L., & Talbot, C. R. (1994). Osmotic and ionic regulation in the aquatic caecilian *Typhlonectes compressicauda* and the terrestrial caecilian. *Ichthyophis kohtaoensis. Physiol. Zool., 67*, 1077−1086.

Suzuki, T., Nakamura, Y., Moriya, T., & Sasono, H. (2003). Effects of steroid hormones on vascular function. *Microsc. Res. Tech., 60*, 70−84.

Toews, D., & Macintyre, D. (1977). Blood respiratory properties of a viviparous amphibian. *Nature, 266*, 464−465.

Townsend, D. S., & Moger, W. (1987). Plasma androgen levels during male parental care in a tropical frog (*Eleutherodactylus*). *Hormones and Behavior, 21*, 93−99.

Trivers, R. L. (1974). Parent-offspring conflict. *Amer. Zool., 14*, 249−264.

Tyler, M. J. (Ed.). (1983a). *The gastric brooding frog*. London & Canberra: Croom Helm.

Tyler, M. J. (1983b). Oral birth and perinatal behaviour. In M. J. Tyler (Ed.), *"The gastric brooding frog"* (pp. 36−43). London & Canberra: Croom Helm.

Tyler, M. J. (1983c). Evolution of gastric brooding. In M. J. Tyler (Ed.), *"The gastric brooding frog"* (pp. 129−135). London & Canberra: Croom Helm.

Tyler, M. J., Shearman, D. J., Franco, R., ÓBrien, P., Seamark, R. F., & Kelly, R. (1983). Inhibition of gastric acid secretion in the gastric brooding frog, *Rheobatrachus silus*. *Science, 220*, 609−610.

Veith, M., Steinfartz, S., Zardoya, R., Seitz, A., & Meyer, A. (1998). A molecular phylogeny of "true" salamanders (family Salamandridae) and the evolution of terrestriality of reproductive modes. *J. Zool. Syst. Evol. Res., 36*, 7−16.

Vilter, V. (1986). La reproduction de la salamandre noire (*Salamandra atra*). In P. P. Grasse, & M. Delsol (Eds.), *"Traite de zoologie", Vol. 14*. fasc. IB: Batraciens (pp. 487−495). Paris: Masson.

Vilter, V., & Vilter, A. (1964). Sur Ívolution des corps jaunes ovariens chez *Salamandra atra* Laur. des Alpes vaudoises. *Comptes rendus des séances de la Société de Biologie, 158*, 457−461.

Wake, M. H. (1968). Evolutionary morphology of the caecilian urogenital system. Part I: The gonads and fat bodies. *J. Morphol., 126*, 291−332.

Wake, M. H. (1970). Evolutionary morphology of the caecilian urogenital system. Part II. The kidneys and urogenital ducts. *Acta Anat., 75*, 321−332.

Wake, M. H. (1976). The development and replacement of teeth in viviparous caecilians. *J. Morph., 148*, 33−64.

Wake, M. H. (1977). Fetal maintenance and its evolutionary significance in the Amphibia: Gymnophiona. *J. Herpetol., 11*, 379−386.

Wake, M. H. (1978). The reproductive biology of *Eleutherodactylus jasperi* (Amphibia, Anura, Leptodactyilidae), with comments on the evolution of live-bearing systems. *J. Herpetol., 12*, 121−133.

Wake, M. H. (1980a). Reproduction, growth, and population structure of *Dermophis mexicanus* (Amphibia: Gymnophiona). *Herpetologica, 36*, 244–256.

Wake, M. H. (1980b). Fetal tooth development and adult replacement in *Dermophis mexicanus* (Amphibia: Gymnophiona): Fields versus clones. *J. Morphol., 166*, 203–216.

Wake, M. H. (1980c). The reproductive biology of *Nectophrynoides malcolmi* (Amphibia: Bufonidae), with comments on the evolution of reproductive modes in the genus *Nectophrynoides*. *Copeia, 1980*, 193–209.

Wake, M. H. (1993). Evolution of oviductal gestation in amphibians. *J. Exptl. Zool., 266*, 394–413.

Wake, M. H., & Dickie, R. (1998). Oviduct structure and function and reproductive modes in amphibians. *J. Exptl. Zool., 282*, 477–506.

Wake, M. H., & Hanken., J. (1982). Development of the skull of *Dermophis mexicanus* (Amphibia: Gymnophiona) with comments on skull kinesis and amphibian relationships. *J. Morphol., 173*, 203–222.

Warkentin, K. M., & Wassersug, R. J. (2001). Do prostaglandins regulate external gill regression in anurans? *J. Exptl. Zool., 289*, 366–373.

Wassersug, R. (1986). How does a tadpole know when to metamorphose? A theory linking environmental and hormonal cues. *J. Theor. Biol., 118*, 171–181.

Welsch, U., Müller, W., & Schubert, C. (1977). Electron microscopical and histochemical observations on the reproductive biology of viviparous caecilians (*Chtonerpeton indistinctum*). *Zool. Jb. Anat. Ontogenie, 97*, 532–549.

Weygoldt, P. (1976a). Beobachtungen zur Biologie und Ethologie von *Pipa* (*Hemipipa*) *carvalhoi* Mir. Rib. 1937 (Anura, Pipidae). *Z. Tierpsychol., 40*, 80–99.

Weygoldt, P. (1976b). Beobachtungen zur Fortpflanzungsbiologie der Wabenkröte *Pipa carvalhoi* Miranda Ribeiro. *Zeitschrift des Kölner Zoo, 19*, 77–84.

Weygoldt, P., and Potsch de Carvalho e Silva, S. (1991). Observations on mating, oviposition, egg sac formation and development in the egg-brooding frog, *Fritziana goeldii*. *Amphibia-Reptilia* 12, 67–80.

Wilkinson, M., & Nussbaum, R. A. (1998). Caecilian viviparity and amniote origins. *J. Nat. Hist., 32*, 1403–1409.

Wilkinson, M., Kupfer, A., Marques-Porto, R., Jeffkins, H., Antoniazzi, M. M., & Jared, C. (2008). One hundred million years of skin feeding? Extended parental care in a Neotropical caecilian (Amphibia: Gymnophiona). *Biology Letters, 4*, 358–362.

Winik, B. C., Alcaide, M. F., Crespo, C. A., Medina, M. F., Ramos, I., & Fernandez, S. N. (1999). Ultrastructural changes in the oviduct mucosa throughout the sexual cycle in *Bufo arenarum*. *J. Morphol., 239*, 61–73.

Xavier, F. (1970). Action modératrice de la progestérone sur le développement des embryons chez *Nectophrynoides occidentalis* Angel. C. *r. hebd. séance. Acad. Sci. Paris Ser. D, Sci. Nat., 270*, 2115–2117.

Xavier, F. (1973). Le cycle des voies génitales femelles de *Nectophrynoides occidentalis* Angel, Amphibien Anoure vivipare. *Z. Zellforsch. Mikrosk. Anat., 140*, 509–534.

Xavier, F. (1974). La pseudogestation chez *Nectophrynoides occidentalis* Angel. *Gen. Comp. Endocrinol, 22*, 98–116.

Xavier, F. (1986). La reproduction des Nectophrynoides. In P.P. Grasse & M. Delsol, (Eds), *"Traite de zoologie"*, Vol. 14, fasc. IB: Batraciens (pp. 497–513). Masson, Paris.

Xavier, F. (1987). Functional morphology and regulation of the corpus luteum. In D. O. Norris, & R. E. Jones (Eds.), *"Hormones and Reproduction in Fishes, Amphibians and Reptiles"* (pp. 241–282). New York: Plenum Press.

Xavier, F., & Ozon, R. (1971). Recherches sur l'activité endocrine de l'ovaire de *Nectophyrnoides occidentalis* Angel (Amphibien Anoure vivipare). II. Synthese *in vitro* des steroides. *Gen. Comp. Endocrinol, 16*, 30–40.

Xavier, F., Zuber-Vogeli, M., & LeQuang-Trong, Y. (1970). Recherches sur l'activité endocrine de l'ovaire de *Nectophyrnoides occidentalis* Angel (Amphibien Anoure vivipare). I. Etude histochimique. *Gen. Comp. Endocrinol, 15*, 425–431.

Zakrzewski, M. (1976). Development of the female reproductive organ and nominal form of the spotted salamander, *Salamandra s. salamandra* (L.) in the West Beskid region (Poland) in the annual cycle. *Acta Biologica Cracoviensia, 19*, 23–38.

Zuber-Vogeli, M. (1983). L'hypophyse de *Nectophrynoides occidentalis* Angel (Amphibien Anoure vivipare). *Arch. Biol. (Bruxelles)., 94*, 109–154.

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Chapter 8

Hormones and Reproductive Behavior in Amphibians

Sarah K. Woodley
Duquesne University, Pittsburgh, PA, USA

SUMMARY
As modern representatives of basal tetrapods, amphibians can provide much insight into conserved endocrine mechanisms of vertebrate behaviors. As a vertebrate class encompassing a diversity of mating systems, amphibians can provide insight into the evolution and ecology of behavioral mechanisms. This review discusses the endocrine basis of amphibian mating behaviors, male acoustic and olfactory advertisement behaviors, female attraction to male social signals, and mate searching behaviors. Despite great advances in the understanding of the endocrine basis of male salamander mating behavior and male anuran calling behavior, large gaps in our understanding of the endocrine basis of other amphibian reproductive behaviors exist. In particular, the endocrine basis of female reproductive behaviors has been neglected until recently. Many promising future directions in the field of amphibian behavioral endocrinology are described.

1. INTRODUCTION

For successful reproduction, animals must coordinate internal physiological events with external climatic and social environments. There are several types of environmental cue that influence the expression of reproductive behaviors (Wingfield, Hahn, Levin, & Honey, 1992). Predictable cues such as photoperiod or temperature signal the approach of environmental conditions suitable for breeding. Supplementary cues such as rainfall provide more short-term predictive cues for reproduction. Synchronizing cues from conspecifics coordinate reproductive activities of mating partners. Finally, modifying cues such as inclement weather or predation may disrupt reproduction. These environmental cues are transduced by the neuroendocrine system to coordinate the expression of reproductive behaviors at the appropriate time and context. Understanding the role of hormones in reproductive behavior involves identifying the hormones involved in a particular behavior, determining mechanistically where and how hormones modulate behavioral expression, and determining how environmental cues regulate hormone release and action. It is also of great interest to understand how hormone–behavior relationships vary within evolutionary and ecological frameworks.

There are more than 6400 species of amphibian (Frost, 2009). More than 85% are frogs and toads (order Anura) and the rest are salamanders (order Urodela) and caecilians (order Gymnophiona). Despite the large number of species, the study of the hormonal regulation of amphibian reproductive behavior is restricted to a few species and a few behaviors. The role of hormones in caecilian reproductive behavior has not been studied. Salamanders and anurans last shared a common ancestor more than 250 million years ago, and rapid evolution followed the initial origins of the amphibian orders (San Mauro, Vences, Alcobendas, Zardoya, & Meyer, 2005). Given the ancient origins of salamanders and anurans, the neuroendocrine mechanisms contributing to salamander and anuran reproductive behaviors may be very different.

This review places previous studies in context, incorporates new findings, and identifies areas of study and questions that have been neglected. The review focuses on experimental studies of the role of hormones in reproductive behaviors in the last 25 years or so. This review is not comprehensive, and the reader is referred to several recent reviews (Moore & Rose, 2002; Yamaguchi & Kelley, 2003; Kelley, 2004; Moore, Boyd, & Kelley, 2005; Wilczynski, Lynch, & O'Bryant, 2005).

2. APPROACHES

The study of hormones and reproductive behavior in amphibians is characterized by two primary approaches (Wilczynski et al., 2005). The first approach is exemplified by Frank L. Moore and his many talented colleagues, who studied amphibian reproductive behavior to discern conserved principles of vertebrate neuroendocrinology. To

this end, Moore emphasized the conserved nature of the mechanisms found in amphibians. He promoted amphibian models because many of their reproductive behaviors are straightforward and stereotyped, and the amphibian nervous system is relatively simple and tractable to experimental studies (Moore & Rose, 2002). Moore was one of the first to demonstrate the behavioral effects of arginine vasotocin (AVT), which led the way to the discovery of widespread behavioral effects of AVT and vasopressin in many vertebrates, (Donaldson & Young, 2008). As another example, Moore's work on the membrane receptor for the steroid hormone corticosterone (CORT) contributed to a growing recognition of the importance of membrane receptors for steroid hormones (Moore & Evans, 1999). Also using this approach, studies by Darcy Kelley on the calling behavior of South African clawed frogs (*Xenopus laevis*) have revealed many conserved principles of vertebrate sexual differentiation (Kelley, 1996).

The second approach focuses on how variation in endocrine mechanisms contributes to variation in behavior. Amphibian systems have long been the subject of studies on sexual selection, mate choice, and mating systems evolution (Houck, Arnold, & Thisted, 1985; Ryan, 1985; Sullivan, Ryan, & Verrell, 1995). With this behavioral and ecological background, behavioral neuroendocrinologists are well positioned to identify neuroendocrine mechanisms contributing to sexual selection and the evolution of mating systems. To this end, recent studies have examined the neuroendocrine basis of female mate choice (Lynch, Crews, Ryan, & Wilczynski, 2006), alternative mating tactics (Marler, Boyd, & Wilczynski, 1999; Emerson, 2001; Leary, Jessop, Garcia, & Knapp, 2004; Leary, Garcia, & Knapp, 2006; Leary, Garcia, & Knapp, 2008), and species variation in the role of steroid hormones in male behavior (Emerson & Hess, 1996). In order to capture natural endocrine and behavioral variation, such studies are typically conducted in the field.

3. BACKGROUND TO REPRODUCTIVE BEHAVIORS

This review focuses on three general classes of reproductive behaviors about which the most is known: mating behavior, male advertisement/female attraction, and mate searching behaviors. Before discussing the hormonal regulation of reproductive behaviors, a brief overview of reproductive behaviors and mating systems is provided. The reader is encouraged to refer to reviews of amphibian mating systems for a more thorough discussion (Wells, 1977; Duellman & Trueb, 1986; Halliday, 1990; Stebbins & Cohen, 1995; Sullivan et al., 1995; Houck & Arnold, 2003; Wells, 2007).

3.1. Mating

An important difference between anurans and salamanders is the mode of fertilization (Duellman & Trueb, 1986). In most anurans, fertilization is external and mating behavior, ovulation, spermiation, oviposition, and fertilization typically occur around the same time. Receptive female anurans allow males to clasp them in a behavior called amplexus. While in amplexus, females oviposit their eggs and males release sperm on or near eggs that have been oviposited. In anurans, breeding seasons range from very short and explosive to more prolonged (Wells, 1977). In species with explosive mating, males may remain in amplexus with females for several hours, perhaps as a form of mate guarding.

In salamanders, fertilization is internal. Sperm is transferred externally via a spermatophore (a gelatinous mass that contains spermatozoa) that is deposited on the substrate by a male. A female picks up the sperm from the spermatophore with her cloaca, and the sperm are stored in a sperm storage organ in the female reproductive tract (Sever, 2002). Fertilization occurs when oocytes are ovulated and pass through the oviducts on their way to oviposition. Due to the ability of females to store sperm, mating may be dissociated in time from ovulation, fertilization, and oviposition. Males of some species lay down many spermatophores per night, while males of other species deposit only a few per night (Arnold, 1976). In some species, females pick up the sperm without any guidance from males. In other species, spermatophore deposition is preceded by courtship behaviors or amplectic clasping, during which males deliver pheromonal, tactile, and other cues to females to increase female receptivity and the probability that a female will pick up a spermatophore (Arnold, 1976; Halliday, 1990). Females may mate with multiple males and store sperm for several months (Adams, Jones, & Arnold, 2005; Sever, 2002).

3.2. Male Advertisement and Female Attraction

Amphibians communicate primarily with acoustic and chemosensory social signals. In many anuran species, males form territories or leks, and call to attract receptive females. Sounds are produced when air is squeezed out of the lungs, over the larynx, and into a vocal sac which amplifies the call (not all species have a vocal sac). Multiple species using the same site for breeding can be distinguished by the nature of their calls, which are characterized by temporal features such as the frequency or duration of call components, and/or in spectral traits such as the intensity of different sound frequencies making up the call (Figure 8.1) (Stebbins & Cohen, 1995). The calls of some species can be heard at great distances and can be

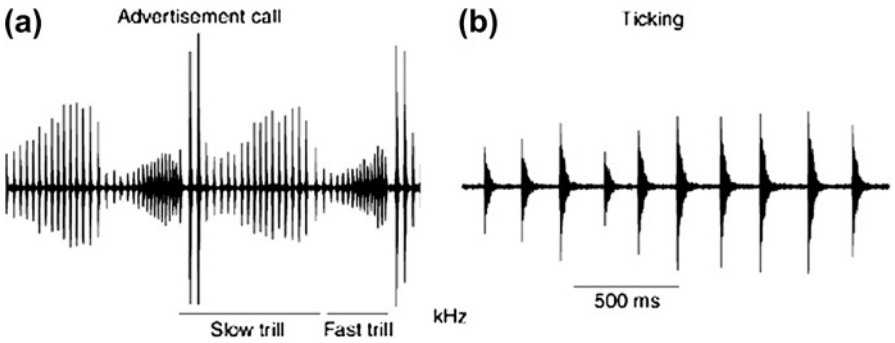

FIGURE 8.1 Temporal organization of African clawed frog (*Xenopus laevis*) calls emitted by males and females. (a) Sound waveform of the male advertisement call, which consists of alternating slow and fast clicking trills. (b) Sound waveform of female release call (ticking), which consists of slow repetitions of clicks. *Adapted from Zornik and Yamaguchi (2008).*

energetically costly (Taigen & Wells, 1985). Calling in most species is sexually dimorphic, with females calling much less than males, if at all.

Sexually receptive anuran females are attracted to calling males and will move towards them, a behavior called phonotaxis. Male advertisement calling may also signal the presence of competitors to rival males. If clasped by a male, unreceptive females and males typically emit a release call. Males of some species emit aggressive calls that function in agonistic interactions. The sensitivity of the auditory system is matched to species-specific aspects of calls (Gerhardt & Schwartz, 2001). The sensitivity of the auditory system to call traits can also vary depending on season and sex (Narins & Capranica, 1976; Hillery, 1983; Goense & Feng, 2005).

Chemosensory cues are used by some amphibian species to signal mating readiness. Males of many amphibian species possess breeding glands or skin secretions with pheromonal properties (Duellman & Trueb, 1986; Houck & Sever, 1994; Largen & Woodley, 2008). In Japanese newts (*Cynops*), species-specific peptide pheromones of males attract reproductive females, but not nonreproductive females or other males (Kikuyama et al., 1995; Iwata et al., 1999). In plethodontid salamanders, males possess a submandibular gland called the mental gland. Secretions from the mental gland are applied to a female during courtship and act to increase her receptivity (Houck & Reagan, 1990; Rollmann, Houck, & Feldhoff, 1999; Houck et al., 2008). Males also respond to conspecific chemosensory cues. Male red-legged salamanders (*Plethodon shermani*) chemoinvestigated body rinses from females more than did females (Schubert, Houck, Feldhoff, Feldhoff, & Woodley, 2008). Male newts are attracted to female-scented water (Thompson & Moore, 2000) but are repelled by male-scented water (Park & Propper, 2001). Although pheromone production is probably less costly than calling behavior, males may express behaviors that could possibly incur energetic costs, such as tail fanning to distribute chemosensory cues (Green, 1991). Chemical communication is also important in some species of frog in which males produce potent female-attracting chemosensory cues (Wabnitz, Bowie, Tyler, Wallace, & Smith, 1999; Pearl et al., 2000; Wabnitz, Bowie, Tyler, Wallace, & Smith, 2000).

3.3. Mate Searching

In many species of amphibian, breeding occurs after movement from terrestrial to aquatic habitats. Breeding may thus entail changes in locomotory activity. Further, males and females may express specific investigation behaviors to locate suitable mating partners.

4. MALE MATING BEHAVIORS

The hormonal basis of male mating behavior has been studied in only a few amphibian species. Much of what is known is derived from studies of salamanders, in particular the rough-skinned newt, *Taricha granulosa*. Below, the roles of androgens, AVT, prolactin (PRL), CORT, and other hormones in male mating behavior are discussed.

4.1. Androgens

4.1.1. Androgens maintain expression of salamander mating behavior

Circulating androgens are necessary for the expression of male reproductive behaviors in most vertebrates (Nelson, 2005). Androgens are released from the testes in response to luteinizing hormone (LH). Luteinizing hormone is secreted from the pituitary gland in response to gonadotropin-releasing hormone (GnRH) (Norris, 2007). In seasonally breeding animals, androgens typically coordinate the temporal expression of male mating behavior with other androgen-dependent aspects of reproduction, namely sperm production and the development of secondary sexual characteristics. Species in which

elevated plasma androgens, sperm production, and the expression of male mating behavior coincide in time were labeled as having associated reproductive patterns by Crews (1984). However, in many species of urodele, male mating behavior is dissociated in time from sperm production. For example, in dusky salamanders (*Desmognathus ochrophaeus* and *Desmognathus ocoee*) sperm production occurs over several months throughout the warm summer months, whereas male mating behavior begins when temperatures are cooler, in the fall and early spring (Woodley, 1994). The reproductive pattern of such species was termed a dissociated reproductive pattern (Crews, 1984) and it was proposed that male mating behavior would be androgen-independent in species with dissociated reproductive patterns (Crews, 1984; Crews & Moore, 1986).

Studies of dusky salamanders found that the reproductive patterns were neither associated nor dissociated. The seasonal profile of plasma androgens in *D. ocoee* was characterized by elevated androgens in spring and fall, coincident with the mating season, and low plasma androgens during the summer when the majority of spermatogenesis occurs (Woodley, 1994). These data challenged the assumption of the associated/dissociated reproductive pattern framework (Crews, 1984) that maximal levels of plasma androgens are found during the period of peak spermatogenesis. Further, a study of *D. ochrophaeus* (a sister species to *D. ocoee* with a similar reproductive pattern) found that male mating behavior was indeed dependent on androgens (Benner & Woodley, 2007). Castration almost completely eliminated spermatophore deposition and female insemination. Castrated males receiving testosterone proprionate implants had levels of spermatophore deposition and female insemination that were similar to sham males receiving blank implants and to intact males (Benner & Woodley, 2007). Therefore, although mating behavior and peak spermatogenetic events were dissociated in time, male mating behavior was androgen-dependent.

The reproductive pattern found in dusky salamanders is common among salamanders. In the aquatically breeding salamanders *T. granulosa*, *Triturus cristatus*, and *Cynops pyrrhogaster*, mating occurs during the winter–spring season whereas most of spermatogenesis occurs during the summer months after mating has ceased (Tso & Lofts, 1977; Specker & Moore, 1980; Tanaka & Takikawa, 1983; Zerani et al., 1991; Zerani & Gobbetti, 1993). As with the dusky salamanders described above, androgens are elevated during much of the mating season, and castration and androgen-replacement experiments indicate that androgens activate the expression of mating behavior in these three species of salamandrid salamander (Andreoletti, Malacarne, & Vellano, 1983; Moore, 1978; Toyoda, Ito, Tanaka, & Kikuyama, 1993). Thus, studies in four species of salamander representing two families indicate that male mating behavior requires circulating androgens for expression.

4.1.2. Androgens activate male mating behavior in some but not all anurans

The role of androgens in male amplexus behavior has been directly examined in only three species of anuran amphibian. Remarkably, androgen activation of male mating behavior has been demonstrated in only one anuran species. The most studied amphibian in terms of the role of androgens in male mating behavior is the African clawed frog, *X. laevis* (Kelley, 1996). The African clawed frog is originally from South Africa but has been introduced into habitats all over the world and is currently a major model in developmental biology. In the wild, the African clawed frog breeds opportunistically when temperatures and humidity permit (Kalk, 1960). In laboratory populations, production of spermatozoa, development of nuptial pads, and male amplectic clasping behavior occur several months after metamorphosis (Kelley, 1996). Amplectic clasping of females is facilitated by spines on the nuptial pads located on male forearms. Males also produce advertisement calls that attract females.

Experiments involving castration and androgen replacement in adult male *X. laevis* indicated that androgens promote development of nuptial pads and activate male amplectic clasping of females. Treatment with estradiol (E_2) had no effect on these characteristics in castrated males (Kelley & Pfaff, 1976; Wetzel & Kelley, 1983; Kelley, 1996). Surprisingly, androgens failed to activate male amplectic clasping studies in the leopard frog (*Rana pipiens*). The leopard frog breeds seasonally throughout North America and injection of pituitary extracts stimulates male mating behavior (Rugh, 1962). One interpretation of the stimulatory effects of pituitary extracts is that pituitary gonadotropins induce testicular secretion of androgens which, in turn, promote the expression of male mating behavior. This is supported by the observation that treatment with pituitary extracts does not evoke amplexus in castrated leopard frogs (Palka & Gorbman, 1973). However, injection of testosterone (T) failed to evoke male amplexus in pituitary-treated castrates (Palka & Gorbman, 1973). Kelley and Pfaff (1976) suggested that the animals were not observed for a long enough period in the study by Palka and Gorbman (1973) and that androgens most likely do activate male amplectic clasping in leopard frogs. A subsequent study found that implantation of T in the preoptic area of the brain stimulated amplexus in male leopard frogs (Wada & Gorbman, 1977). Clearly, more studies are warranted to establish the role of androgens in the activation of male amplexus in anurans other than African clawed frogs.

Finally, a study of the spadefoot toad (*Scaphiopus couchii)* found no evidence for androgen activation of male amplexus (Harvey & Propper, 1997). The spadefoot toad is a desert-breeding amphibian that breeds explosively when ponds fill with water during the summer monsoon season. In animals sampled in the field, androgens were elevated in males that were calling and mating compared to males sampled before and after the brief breeding season (Harvey, Propper, Woodley, & Moore, 1997). However, reducing plasma androgens by castration did not reduce levels of male amplectic clasping (Harvey & Propper, 1997). Hence, the elevated androgens found in clasping males may have been a result of male clasping behavior rather than a cause of the behavior. Harvey, Propper, Woodley, & Moore (1997) proposed that androgens may act too slowly to initiate the rapid onset of mating behaviors that occurs in explosively breeding species. This idea should be tested in additional species of explosively breeding anuran.

4.1.3. Sex steroid hormone brain receptors and mating

Androgen and estrogen receptors are found throughout the brain in *T. granulosa* and *X. laevis* (Kelley, Morrell, & Pfaff, 1975; Kelley, 1980; Davis & Moore, 1996). Lesions to the preoptic area (POA) inhibited mating behavior in the crested newt *T. cristatus* (Malacarne & Giacoma, 1980). Implantation of T into the POA induced sexual behavior in *R. pipiens* (Wada & Gorbman, 1977). Thus, steroid hormone action in the POA appears to be central to the expression of male mating behavior.

4.1.4. Conclusions and future directions

The associated/dissociated framework developed by Crews (Crews, 1984; Woolley, Sakata, & Crews, 2004; Crews & Moore, 2005) to explain the role of androgens in male mating behavior does not apply to many species of amphibian. In many amphibian species, plasma levels of androgens do not parallel levels of testicular androgens (Moore et al., 2005). The mechanism for uncoupling plasma androgens from testicular androgens should be examined. In amphibians and reptiles, it has been proposed that there are two testicular sources of androgens: the Leydig cells and the Sertoli cells (Lofts, 1972; Licht, 1982; Licht, 1984; Lofts, 1984; Dubois, Pudney, & Callard, 1988; Mesner, Mahmoud, & Cyrus, 2005). Thus, in some species, androgen production by Sertoli and Leydig cells could be temporally asynchronous. Androgen production by Sertoli cells could sustain spermatogenesis whereas androgen production by Leydig cells could elevate plasma androgens to modulate secondary sexual characteristic development and sexual behavior. It is also unclear how testes can be spermatogenically inactive in the presence of elevated plasma androgens. It is possible that sperm development could be shielded from elevated androgens via down-regulation of testicular androgen receptors or via sequestering of androgens by binding proteins. Further investigation of androgen secretion in these salamander species could reveal important vertebrate endocrine mechanisms and relationships.

Although studies in multiple salamander species indicate that androgens are required for the expression of male mating behavior, evidence for a similar relationship in anurans exists for only one species, the African clawed frog. It is important to confirm that androgens modulate the expression of male amplexus in additional species of anuran.

4.2. Arginine Vasotocin (AVT)

Arginine vasotocin (AVT) is the nonmammalian functional homolog of arginine vasopressin (AVP). Arginine vasotocin and AVP are made by magnocellular neurons in the POA and are released by the posterior pituitary to regulate water balance (Boyd, 2006). However, AVT and AVP are also produced by other areas of the brain, where they act as neurohormones, binding to receptors in the brain to influence social and reproductive behaviors in a variety of species (Rose & Moore, 2002). Frank L. Moore and colleagues demonstrated the crucial role of AVT in male sexual behavior in the rough-skinned newt, *T. granulosa*. Many aspects of the AVT system are conserved across vertebrates, and the studies in the rough-skinned newt reviewed below have provided much insight into general vertebrate mechanisms of AVP/AVT action (Goodson & Bass, 2001).

4.2.1. Arginine vasotocin (AVT) promotes expression of male sexual behavior in the rough-skinned newt

In the rough-skinned newt, male sexual behavior consists of a prolonged period of male clasping of the female, a behavior called amplexus (Propper, 1991). During this time, the male rubs his chin against the female's nares, presumably delivering pheromones to the female. The prolonged clasping is necessary to make the female sexually receptive. After a while, the male releases the female, deposits a spermatophore, guides her over it, and reclasps her after she has picked up the sperm and stored it in her cloaca. Post-mating clasping is thought to be a form of mate guarding (Propper, 1991).

Although T is required for mating in rough-skinned newts, it is not sufficient. Testosterone restored male amplectic clasping in castrated males during the breeding season, but T had no effect on amplexus in males tested outside of the breeding season (Moore, 1978). Further,

there was no correlation between plasma androgen levels and incidence of amplectic clasping during the breeding season (Moore & Muller, 1977).

Moore and colleagues suspected that androgens could interact with a neuromodulator and they therefore systematically tested several neuromodulators (Moore & Zoeller, 1979b). Systemic and intracerebroventricular injection with AVT activated male amplectic clasping behavior within a few moments of injection in intact males and in castrated males implanted with androgens (Moore & Miller, 1983; Moore & Zoeller, 1979a). Treatment with an AVP antagonist or anti-AVT immune serum reduced the amount of male amplectic clasping (Moore & Miller, 1983). The success of intracerebroventricular injections indicated that AVT was acting on receptors in the brain rather than on peripheral targets. Finally, concentrations of AVT in the dorsal POA, optic tectum, ventral infundibulum, and cerebrospinal fluid were higher in sexually responsive males compared to unresponsive males (Zoeller & Moore, 1988). A role for AVT was also found in tail fanning, an important sexual behavior in the Japanese red-bellied newt, *C. pyrrhogaster*. In the red-bellied newt, intracerebroventricular injections of AVT increased tail fanning behavior and AVP receptor antagonists decreased tail fanning behavior (Toyoda et al., 2003). Although AVT is clearly involved in salamander mating behaviors, manipulations of AVT did not alter amplexus in the Great Plains toad (*Bufo cognatus*) (Propper & Dixon, 1997).

4.2.2. Arginine vasotocin (AVT) neurons and receptors in the urodele brain

Compared to other vertebrates, including anurans, the urodele AVT system is very extensive. In two species of urodeles from two different families, more than 20 populations of AVT-synthesizing neurons were found in the brain (Lowry et al., 1997; Hollis et al., 2005). Arginine vasotocin-synthesizing neurons were found in sensory, neuroendocrine, motor, and sensorimotor integrating areas, and AVT fibers were found in limbic forebrain areas as well as in the medulla, a brainstem area involved in motor output (Lowry et al., 1997). Out of the multiple populations of AVT neurons, three populations were sexually dimorphic. Males had more AVT neurons in the bed nucleus of the stria terminalis (BNST), amygdala (AMG), and anterior POA than did females (Moore, Richardson, & Lowry, 2000). The sex difference was particularly prominent in the anterior POA. The BNST, AMG, and anterior POA are all implicated in male sexual behavior in vertebrates. Thus, AVT may play an important role in regulating male mating behavior by acting at these sites.

Androgens regulate male amplexus behavior, in part by interacting with the AVT system of the brain (Moore, Lowry, & Rose, 1994). Although numbers of AVT-immunoreactive neurons in males did not change seasonally (Moore et al., 2000), the concentration of AVT in the optic tectum was highest during the breeding season (Zoeller & Moore, 1986). It is possible that androgens regulate the seasonal change in AVT levels in the optic tectum, but experimental studies confirming this have yet to be done. Androgens may regulate levels of AVT receptors because castration reduced AVT binding in the AMG, although not in the three other areas examined (olfactory bulb, pallium, and medulla oblongata) (Boyd & Moore, 1991). It is unknown whether replacement with T in castrated males would restore AVT binding in the AMG. More studies examining the androgen regulation of AVT concentrations and receptors are warranted in this system to better understand the interaction between androgens and the AVT system in the regulation of male mating behavior. Three types of AVT receptors were recently cloned in the red-bellied newt (*C. pyrrhogaster*) (Hasunuma et al., 2007), providing useful tools for investigating possible androgen regulation of AVT receptor expression.

4.2.3. Mechanisms of arginine vasotocin (AVT) action in rough-skinned newts

How does AVT affect mating behavior in male rough-skinned newts? Arginine vasotocin could alter general arousal, motivation for sexual stimuli, and/or sensorimotor mechanisms linking species-specific releasing stimuli to species-specific motor outputs (Thompson & Moore, 2000; Moore & Rose, 2002).

Initial studies focused on sensorimotor processing because AVT fiber terminals and AVT receptors were found in the medulla, a brainstem area involved in clasping behavior (Boyd & Moore, 1991; Lowry et al., 1997). Clasping behavior is hypothesized to be mediated by a central pattern generator for clasping (Rose, 2000; Moore & Rose, 2002). In this model, the clasping generator, located in the spinal cord, provides input to motor neurons innervating the limbs to regulate flexion and extension of the limbs. The clasping generator is modulated by medullary reticulospinal neurons that control onset, duration, quality, and termination of clasping (Figure 8.2(a)) (Moore & Rose, 2002).

Tactile stimulation of the male cloaca elicited male clasping behavior and also increased electrical activity of neurons located in the spinal cord and in the medulla (Rose, Kinnaird, & Moore, 1995). Application of AVT directly to the medullary neurons increased electrical responsiveness of medullary neurons to cloacal stimulation (Rose et al., 1995). Arginine vasotocin also increased medullary neuronal responsiveness to tactile stimulation of other areas of the body. These data are consistent with the hypothesis that AVT acts in sensorimotor processing related to male

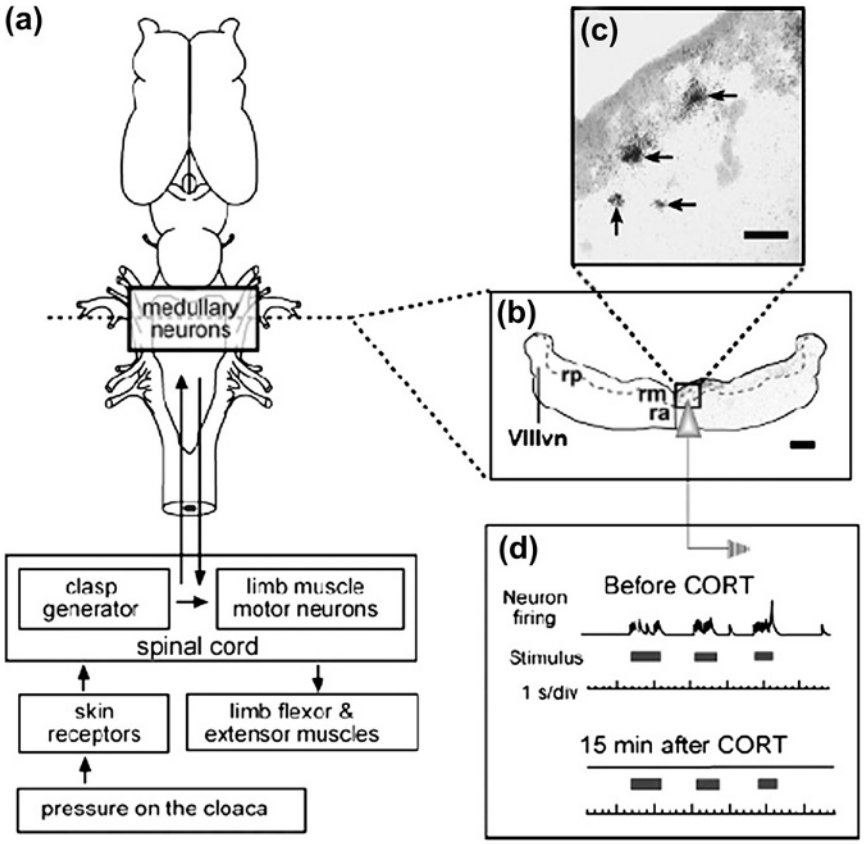

FIGURE 8.2 Schematic of the principal neural system that controls courtship clasping behavior in the rough-skinned newt (*Taricha granulosa*). (a) Somatosensory pressure of the cloaca initiates hindlimb flexion. Increased firing of rostromedial medullary neurons is coincident with somatosensory stimulation of the cloaca, and descending input from the rostromedial medulla controls clasp characteristics. (b) Transverse section through the rostromedial medulla. VIII vn, VIIIth cranial nerve; rp, parvocellular reticular nucleus; rm, middle reticular nucleus; ra, raphe nucleus. Bar = 200 µm. (c) Light microscope photographs of CB1 receptor mRNA expression (arrows) from the middle reticular nucleus, in the same region in which electrophysiological recordings in (d) were made. Bar = 20 µm. (d) Output from a single medullary neuron illustrating CORT-induced suppression of response to cloacal pressure. Bars represent duration of pressure applied to the cloaca. *Adapted from Coddington, Lewis, Rose and Moore (2007).*

sexual behaviors by affecting somatosensory responsiveness of medullary neurons that modulate clasping behavior.

Subsequent studies found that AVT also altered appetitive responses to sexually relevant sensory stimuli (Thompson & Moore, 2000). Males use both visual and chemosensory cues to locate females and, once males have clasped females, pheromones are transmitted between males and females. Males spent more time in proximity to female-derived visual cues than females did (Thompson & Moore, 2003). Males injected systemically with AVT spent more time near a beaker containing a live female or live worms (a food source) than to a beaker containing a model of a female, suggesting that AVT increases behavioral responses to movement (Thompson & Moore, 2000). The neural basis of the effect of AVT on responses to visual cues is unknown. However, AVT concentrations increased during the breeding season in the optic tectum (Zoeller & Moore, 1986), an important site for processing visual information.

Arginine vasotocin also increased responsiveness to chemosensory cues. Males spent more time in close proximity to female-derived chemosensory cues than did females (Thompson & Moore, 2003). Injection of males with AVT increased time spent in proximity to female-derived chemosensory cues (Thompson & Moore, 2000). Likewise, injection of AVT in T-treated ovariectomized females increased the amount of time spent in proximity to female-derived chemosensory cues and also increased clasping of a model scented with female chemosensory cues (Thompson & Moore, 2003). The neural basis of the effects of AVT on chemosensory responsiveness is unknown, but could possibly involve the AMG, an area that contains AVT neurons and also receives olfactory information in amphibians (Schmidt & Roth, 1990; Laberge, Muhlenbrock-Lenter, Grunwald, & Roth, 2006). Although AVT increased appetitive responses to sexual stimuli, it is not clear whether AVT increased sensitivity to species-specific releasing stimuli or increased motivation to

respond to sexual stimuli. However, the increased responsiveness to visual cues, including worms, suggests an effect on sensory sensitivity rather than increased motivation for sexually relevant stimuli. Finally, AVT decreased locomotion as measured by line crossings, indicating that AVT does not increase responsiveness to sexually relevant sensory cues simply by increasing general arousal and activity (Thompson & Moore, 2000).

Until recently, the sensory cues inducing AVT release in the medulla were unknown. Thompson et al. (2008) provided evidence that chemosensory cues from females trigger AVT release in the medulla of males. Thompson et al. (2008) found that exposure to female-scented water increased neuronal responsiveness of medullary neurons to tactile stimuli in males. This response was blocked by perfusion of the medulla with an AVP receptor V1a antagonist (Thompson et al., 2008). Since the AMG receives chemosensory information (Schmidt & Roth, 1990; Laberge et al., 2006) and contains AVT neurons, female chemosensory cues may cause release of AVT from vasotocinergic neurons located in the AMG that project to the medulla. The AMG also contains sex steroid hormone receptors (Davis & Moore, 1996), and could be a site for androgen regulation of male clasping behavior. It is not known whether additional sensory cues (such as visual cues) from females induce AVT release in the medulla. Thompson et al. (2008) proposed that AVT links multimodal sensory information to allow appropriate behavioral expression. The work of Thompson et al. (2008) lends further support to the hypothesis that AVT facilitates sensorimotor processing of species-specific sensory cues.

4.2.4. Conclusions and future directions

Arginine vasotocin neurons and fibers are found throughout the forebrain, midbrain, and brainstem in rough-skinned newts. Arginine vasotocin increased behavioral and neuronal responsiveness to sexually relevant sensory stimuli, including olfactory, visual, and tactile stimuli. Arginine vasotocin levels and/or receptors may be regulated by androgens in some areas of the brain. Arginine vasotocin affects many aspects of sensorimotor integration, although an additional role for motivation cannot be ruled out (Moore & Rose, 2002). Outstanding issues to be resolved include determining 1) which populations of AVT neurons contribute to the sensorimotor processing of male sexual behavior, 2) the role of the sexually dimorphic populations of AVT neurons, 3) the sensory cues that cause AVT release into the medulla to trigger male clasping behavior, and 4) the locations of AVT receptors related to mating behaviors (Rose and Moore, 2002). Finally, AVT is released from the pituitary gland into the bloodstream and is known to have peripheral effects (Boyd, 2006). The potential role of peripheral effects of AVT should be considered (Wilczynski et al., 2005).

4.3. Corticosterone (CORT)

Vertebrates respond to stressful stimuli in a highly stereotypical manner conserved across taxa (Sapolsky, 2002). An important response to a stressor is activation of the hypothalamic–pituitary–adrenal axis and release of glucocorticoid hormones from the adrenal gland into the circulation (Romero & Reed, 2005; Norris, 2007). Plasma glucocorticoids coordinate a suite of physiological and behavioral responses that allow an animal to cope with stressors (Romero, 2002). These bodily responses to elevated glucocorticoids represent an emergency stage and allow an animal to survive life-threatening situations such as predator attacks, infection, or unexpected changes in weather (Landys, Ramenofsky, & Wingfield, 2006). Although plasma glucocorticoids increase within a few minutes of the onset of a stressor (Romero & Reed, 2005), few studies have linked increased glucocorticoids to rapid changes in behavior (Orchinik, Gasser, & Breuner, 2002). The main glucocorticoid in amphibians is corticosterone (CORT). Virtually all that is known about the effects of CORT on male mating behavior in amphibians is derived from studies of the rough-skinned newt.

4.3.1. Corticosterone (CORT) rapidly suppresses amplectic clasping

Moore, Rose, and colleagues described the effects of rapid increases in CORT on rough-skinned newt clasping behavior. Males that were confined in a small container with other males for one hour exhibited increased plasma CORT and reduced amplectic clasping of females (Moore & Miller, 1984). Males that were injected with metyrapone, an inhibitor of CORT synthesis, before crowding with males exhibited normal levels of clasping of females. Injection of males with CORT (but not vehicle) suppressed clasping in a dose-dependent manner. Some doses of CORT suppressed clasping rapidly, within 15 minutes of injection (Moore & Miller, 1984). In another study, males injected with CORT exhibited no clasping of females even though males injected with vehicle began to clasp females within three minutes of injection (Orchinik, Murray, & Moore, 1991). Finally, injection with CORT suppressed reflexive clasping of a probe pressed upon the cloaca in 5 to 25 minutes after CORT injection (Rose, Marrs, & Moore, 1998). Thus, both endogenous and exogenous elevation of plasma CORT rapidly suppressed male clasping.

The rapid behavioral effects of CORT are unlikely to be mediated by the classical intracellular receptors that exert their effects via changes in gene expression. Instead, evidence suggests that CORT suppresses newt courtship

behavior rapidly by acting through a G-protein-coupled membrane receptor (see Moore & Rose (2002) for a review). The membrane receptor has similar properties to the κ-opioid receptor, and the expression of clasping is sensitive to injections of κ-opioid receptor agonists and antagonists (Deviche & Moore, 1987). Thus, it is hypothesized that the membrane CORT receptor is a κ-opioid-like receptor (Moore & Rose, 2002).

4.3.2. Context modulates the effects of corticosterone (CORT) on amplectic clasping

The effects of CORT on behavior are context-dependent. Although CORT prevented the expression of clasping in reproductive males exposed to females, CORT did not suppress clasping behavior in males that were already clasping. For example, males that had clasped a female for 60 minutes before injection with CORT continued to clasp females (Coddington & Moore, 2003). Thus, the ability of CORT to suppress clasping depended on the prior experience of the male. It was hypothesized that sensory stimuli associated with clasping might trigger the release of AVT (see Section 4.2), which might override the suppressive effects of CORT on clasping. To test this hypothesis, males were pretreated with AVT or a saline vehicle and then injected 60 minutes later with CORT or a saline vehicle. As expected from previous studies, males treated with AVT followed by saline exhibited high levels of clasping, and males pretreated with saline followed by CORT exhibited much less clasping behavior. The novel finding was that males treated with AVT followed by CORT still exhibited clasping, indicating that pretreatment with AVT prevented CORT from fully suppressing clasping behavior (Coddington & Moore, 2003). Thompson et al. (2008) demonstrated that female odors induced release of AVT in the medulla, providing a mechanism whereby interactions with a female may alter the effects of CORT on mating.

4.3.3. Corticosterone (CORT) modulates excitability of clasp-controlling neural circuits

The rapid behavioral effects of CORT are likely due, in part, to the effects of CORT on neuronal excitability. Clasping behavior in the rough-skinned newt is mediated by a hypothetical clasp generator located in the spinal cord (Rose, 2000; Moore & Rose, 2002). The clasp generator is believed to be modulated by input from reticulospinal neurons of the medulla that control various aspects of clasping behavior such as onset, duration, quality, and termination of the clasp (Figure 8.2(a)) (Moore & Rose, 2002). In one experiment, the activity of reticulospinal and nonreticulospinal neurons of the medulla was recorded. Nonreticulospinal neurons included local interneurons and neurons with ascending projections. Neuronal activity was measured in immobilized newts in response to application of cloacal pressure and also in freely behaving newts that engaged in clasping behavior. The precise effects of CORT on neuronal activity were diverse, but consistent with a reduction in activity of both reticulospinal and nonreticulospinal neurons. First, in immobilized newts, intraperitoneal injection of CORT reduced or stopped both spontaneous activity of medullary neurons and neuronal activity elicited by pressure to the cloaca (Figure 8.2(d)). These effects were also found in newts that had a transection anterior to the medulla, indicating that the effects of CORT were not on forebrain or rostral brainstem areas that provide input to the medulla (Rose et al., 1995). Corticosterone (CORT) had similar effects on neuronal activity in the medulla in freely behaving newts. For example, neuronal activity that accompanied clasping was reduced, although neuronal activity that accompanied head movements or locomotion was not usually affected by CORT (Rose et al., 1998).

Corticosterone also altered the activity of spinal cord neurons involved in clasping behavior (Lewis & Rose, 2003). Males in which the connection between the medulla and the first cervical vertebra of the spinal cord was cut typically expressed sustained clasping behavior in response to pressure on the cloaca. Clasping was maintained even after removal of the pressure on the cloaca. Intraperitoneal injection with CORT rapidly reduced the quality of the clasp as well as the time spent in the clasp behavior (Lewis & Rose, 2003). Thus, although the spinal cord receives modulatory input from the medulla, components of the clasp such as clasp duration are intrinsic to the spinal cord, and CORT acts directly on the spinal cord to affect clasp duration. The rapidity of the response suggests that CORT acts on the spinal cord via a rapid nongenomic mechanism.

The context-dependent effects of CORT on clasping behavior are mirrored by context-dependent effects of CORT on neural activity of medullary neurons (Rose et al., 1995). As stated earlier, treatment of the medulla with AVT increased spontaneous neural activity as well as increased neuronal responsiveness to cloacal stimulation. An intraperitoneal injection of CORT 30 minutes before exposure to AVT suppressed the ability of AVT to increase neuronal activity. Conversely, intraperitoneal injection of CORT did not suppress neuronal activity if the CORT injection was preceded by 10–17 minutes by exposure to AVT (Rose et al., 1995). Thus, the precise hormonal milieu may modulate rapid behavioral transitions between clasping and nonclasping behaviors. It is proposed that, in the presence of predators, responding to female-derived sensory cues with clasping may be maladaptive. However, once clasping has begun, males may be less responsive to stressors such as interactions with rival males (Rose et al., 1995). If so, sensory cues from predators or conspecific males may influence levels of CORT and/or AVT in the newt brain.

4.3.4. Corticosterone (CORT) and endocannabinoid signaling

Endocannabinoids are a group of signaling molecules, including the active compound of cannabis, derived from membrane phospholipids. Endocannabinoids signal in a retrograde fashion by binding to cannabinoid receptors located on neurons presynaptic to the neuron secreting the endocannabinoids (Kreitzer & Regehr, 2002). Retrograde signaling suppresses neurotransmitter release by presynaptic neurons. The main cannabinoid receptor in the brain is cannabinoid receptor type 1 (CB1). The distribution of CB1 receptors has been described in rough-skinned newts as well as in African clawed frogs (Cottone, Guastalla, Mackie, & Franzoni, 2003; Hollis, Coddington, & Moore, 2006).

Studies in rats showed that endocannabinoids act in various areas of the brain to mediate different stress-related responses (Coddington, Lewis, Rose, & Moore, 2007; Denver, 2007), suggesting the possibility that endocannabinoids could also mediate the rapid effects of CORT on mating behavior in the rough-skinned newt. Indeed, in rough-skinned newts, intraperitoneal injections with an endocannabinoid agonist rapidly suppressed clasping behavior (Soderstrom, Leid, Moore, & Murray, 2000). Further, mRNA of CB1 in newts was found in multiple areas of the brain, including the medulla (Figure 8.2(b–c)) (Hollis et al., 2006).

To determine whether CORT suppressed male clasping behavior via endocannabinoid signaling in newts, males were treated with CB1 antagonists (Coddington et al., 2007). Cannabinoid receptor type 1 antagonists blocked the suppressive effects of stress and CORT on male amplectic clasping. Application of a CB1 antagonist to the medulla of male newts also prevented the suppressive effects of CORT on spontaneous activity of medullary neurons and neuronal activity evoked by cloacal stimulation (Coddington et al., 2007). These data are consistent with the hypothesis that CORT suppresses clasping behavior via endocannabinoid signaling in the neural circuitry controlling clasp behavior in the medulla.

Given the widespread distribution of CB1 receptors in the *T. granulosa* brain and evidence that endocannabinoids affect diverse aspects of the stress response, Coddington et al. (2007) proposed that endocannabinoids coordinate physiological and behavioral responses to acute stressors. Corticosterone may act as a global signal to coordinate endocannabinoid signaling across diverse neural sites. This work suggests that examining the effects of CORT on endocannabinoid signaling in other parts of the brain would be a fruitful line of investigation. Examining the role of endocannabinoid signaling in other aspects of reproductive behavior in amphibians may also be rewarding. Much is known about endocannabinoid signaling in anurans (Cottone et al., 2008). Some GnRH neurons co-label for CB1, suggesting an interaction between reproductive function and endocannabinoids (Cottone, Salio, Conrath, & Franzoni, 2003; Meccariello et al., 2008).

4.3.5. Conclusions and future directions

Studies of the rough-skinned newt indicate that acute increases in plasma CORT can dramatically suppress the expression of male clasping behavior in a context-dependent manner. Acute increases in CORT are hypothesized to facilitate rapid behavioral transitions, depending on the current context, via changes in sensory processing (Orchinik et al., 2002). Although work with the rough-skinned newt focused on somatosensory processing by the medulla, CORT may affect sensory responsiveness of additional brain areas. For example, there is evidence that CORT affects visual processing by the optic tectum in rough-skinned newts (Moore & Rose, 2002).

The effects of acute increases in plasma CORT on mating behavior should be examined in additional amphibian species. The tiger salamander, *Ambystoma tigrinum*, has a membrane CORT receptor with properties similar to those of the membrane CORT receptor in *T. granulosa* (Orchinik, Matthews, & Gasser, 2000). The rapid suppression of mating behavior by CORT that occurs in rough-skinned newts may also occur in other salamander species. Alternatively, whether CORT suppresses mating behavior may depend on the length of the breeding season. In species with prolonged breeding seasons, like the rough-skinned newt (Specker & Moore, 1980), CORT may rapidly suppress mating behavior. In species with explosive mating patterns, males cannot afford to forgo mating even for a short period; thus, mating behavior might be resistant to the suppressive effects of CORT.

Despite evidence that CORT has suppressive effects on reproduction in some species, it is hypothesized that CORT could support breeding activities via its metabolic actions (Romero, 2002; Moore & Jessop, 2003). In many vertebrate species including amphibians, baseline levels of plasma CORT increase during the breeding season. In some toads, males expressing amplectic behavior had elevated plasma CORT (Orchinik, Licht, & Crews, 1988; Harvey et al., 1997). Exposure of male red-legged salamanders to courtship pheromones resulted in a rapid increase in plasma CORT (Schubert et al., 2009). Male mountain dusky salamanders (*D. ochrophaeus*) treated with T implants had significantly elevated plasma CORT (Benner & Woodley, 2007). This unusual finding in mountain dusky salamanders suggests that androgens may regulate CORT levels, perhaps to support breeding activities via energy mobilization.

Finally, nothing is known about the effects of chronic elevations of CORT on amphibian mating behavior. In

contrast, studies in other vertebrate classes have found that chronic elevation of CORT has clear behavioral effects (e.g.,Wingfield & Silverin, 1986; DeNardo & Sinervo, 1994; French, McLemore, Vernon, Johnston, & Moore, 2007). It seems likely that chronically elevated glucocorticoids would suppress reproductive behaviors by binding to classical glucocorticoid receptors found in the anterior POA (Yao, Hu, & Denver, 2008). The anterior POA is linked to the expression of male reproductive behavior in amphibians (Malacarne & Giacoma, 1980; Wada & Gorbman, 1977).

4.4. Prolactin (PRL)

4.4.1. Prolactin promotes male mating behavior in salamanders

In vertebrates, PRL has disparate roles ranging from lactation in mammals to water balance in fish to mating in newts (Cooke et al., 2004). In salamanders of the family Salamandridae, breeding occurs in water and animals undergo a remarkable transformation from a terrestrial form to an aquatic form. The size of the male tail fin increases, the skin becomes smooth and mucous-covered, osmotic permeability of the skin decreases, locomotion (i.e., the water drive) increases, and animals prefer an aquatic environment over a terrestrial environment (Mazzi & Vellano, 1987). These changes are triggered by low temperatures and are under the influence of the pituitary hormone PRL (Duvall & Norris, 1977; Moore, Seide, Specker, & Swanson, 1978; Toyoda, Matsuda, Yamamoto, & Kikuyama, 1996; Iwata, Toyoda, Yamamoto, & Kikuyama, 2000).

In newts exhibiting a water drive, PRL is also involved in mating behavior. In Japanese red-bellied newts (*C. pyrrhogaster*), courtship begins with the male vibrating or fanning his tail in front of the female, presumably to waft pheromones from his pheromone-producing cloacal glands towards the nares of the female. Prolactin is released in red-bellied newts in response to cold temperatures (Yazawa, Yamamoto, Kikuyama, & Abe, 1999). Systemic injection of anti-PRL serum in males reduced tail fanning whereas injection with PRL increased tail fanning (Toyoda et al., 1996). The effects of PRL on tail fanning required concurrent plasma androgens (Toyoda et al., 1993).

Prolactin induces tail fanning by acting on receptors in the brain. Intracerebroventricular injections of low doses of ovine PRL induced tail fanning in males primed with gonadotropins (Toyoda, Hasunuma, Yamamoto, Yamashita, & Kikuyama, 2005). Intracerebroventricular injections of an anti-newt PRL receptor antibody suppressed mating behavior (Figure 8.3) (Toyoda et al., 2005). Immunocytochemistry using an antibody generated against newt PRL receptor revealed PRL receptor in the choroid plexus and several forebrain areas including the medial AMG, anterior POA, magnocellular preoptic nucleus, suprachiasmatic nucleus, and ventral hypothalamic nucleus (Hasunuma, Toyoda, Yamamoto, Yamashita, & Kikuyama, 2005). There is no evidence that PRL is synthesized by neurons in the brain, and it has been suggested that PRL accesses the brain via the choroid plexus (Hasunuma et al., 2005). Many of the forebrain areas containing PRL receptors also express androgen receptors and estrogen receptors (Davis & Moore, 1996), but it is not known whether sex steroid hormones regulate PRL receptor levels, analogous to androgenic regulation of AVT receptors. In addition, PRL receptors were found on AVT-synthesizing neurons of the magnocellular POA, suggesting that PRL may influence AVT secretion.

Finally, PRL may act in the brainstem on the pair of Mauthner neurons (Matsumoto, Arai, Kouki, & Kikuyama, 1995; Kikuyama et al., 2000). Mauthner neurons are large paired cells in the medulla of fishes and amphibians that are involved in tail-flip escape responses (Will, 1991). The

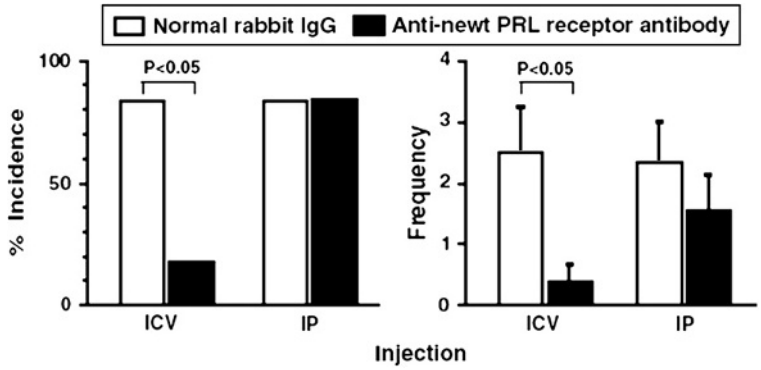

FIGURE 8.3 The effect of anti-newt prolactin (PRL) receptor antibody on spontaneously occurring courtship behavior (tail fanning) in male Japanese red-bellied newts (*Cynops pyrrhogaster*). A single injection of normal rabbit gamma globulin (IgG) or anti-newt PRL receptor antibody intracerebroventricularly (ICV) but not intraperitoneally (IP) reduced incidence and frequency of tail fanning. Each group consisted of six animals. *Adapted from Toyoda, Hasunuma, Yamamoto, Yamashita, Kikuyama (2005).*

sizes of the nucleus and cell bodies of Mauthner cells are larger in sexually active males compared to sexually inactive males. Treatment with PRL and gonadotropin (human chorionic gonadotropin (hCG)) increased the size of Mauthner cells (Matsumoto et al., 1995). It would be interesting to determine whether PRL promotes processing of neural input by Mauthner cells, reminiscent of the actions of AVT on brainstem processing in rough-skinned newts.

4.4.2. Conclusions and future directions

Prolactin increases male mating behavior in Japanese red-bellied newts and is an important hormonal regulator of behavioral, morphological, and physiological changes associated with the water drive. Future studies should determine whether PRL promotes male mating behavior in amphibian species without a water drive. In species with a water drive, PRL may have been evolutionarily co-opted to coordinate the expression of mating behaviors with the development of traits required for an aquatic lifestyle. Thus, PRL may only regulate courtship behavior in species that experience a water drive. For example, PRL did not regulate amplexus of African clawed frogs (*X. laevis*), a fully aquatic species (Taylor & Boyd, 1991). Alternatively, the link between PRL and amphibian mating behaviors may represent a conserved mechanism and may be widespread in amphibians.

4.5. Other Factors

4.5.1. Nitric oxide

Nitric oxide (NO) is a gaseous neurotransmitter produced by the actions of nitric oxide synthase (NOS). Nitric oxide is implicated in sexual behavior in mammals (Hull & Dominguez, 2006). In male crested newts, progression through the stages of courtship was associated with increased levels of whole brain NOS activity (Zerani & Gobbetti, 1996). Nitric oxide synthase activity dropped quickly when mating culminated with spermatophore deposition or when courtship ceased due to female disinterest. There was no relationship between whole brain NOS and the expression of mating behavior in females. It is unknown whether the change in NOS activity is a cause or a result of the expression of male courtship behavior, or what aspect of male courtship behavior is associated with NO. However, in other vertebrates, NO synthesis in the POA is upregulated by androgens and appears to coordinate responses to cues from females (Hull & Dominguez, 2006; Sanderson, Le, Zhou, & Crews, 2008).

4.5.2. Gonadotropins

In contrast to salamanders, the hormonal basis of anuran amplexus does not appear to involve AVT or PRL, but may be regulated by other hormones such as GnRH (Propper & Dixon, 1997), thyrotropin-releasing hormone (TRH) (Taylor & Boyd, 1991), or gonadotropins. In some anurans, LH levels are elevated in amplexing males compared to solitary males (Mendonça, Licht, Ryan, & Barnes, 1985; Itoh & Ishii, 1990). It is well known that injection of hCG and/or injections of pituitaries trigger male amplectic clasping and release of sperm from the testes in anurans (Rugh, 1935; Rugh, 1962). Gonadotropins also increase androgen production, and it was assumed that gonadotropins increased male amplexus by affecting androgen release by the testes. However, studies with *X. laevis* indicated that gonadotropins may have a direct effect on male amplexus: although treatment of castrated males with androgens restored amplexus compared to treatment with sham controls, levels of amplexus were still far less than those found in intact males injected with hCG (Kelley & Pfaff, 1976). Yang, Nasipak, & Kelley (2007) provided evidence that gonadotropins increased male advertisement calling by acting in the brain (Yang et al., 2007). Studies should determine whether gonadotropins, acting directly in the brain, increase male amplectic clasping as well as calling. It should be noted, however, that in *Bufo japonicus* tactile stimuli associated with amplexus triggered increases in LH and FSH, suggesting that changes in gonadotropins may be the result and not the cause of amplexus in some species (Ishii & Itoh, 1992).

5. MALE ADVERTISEMENT BEHAVIOR

5.1. Androgens and Auditory Advertisement

Acoustic communication in male anurans is dependent on androgens. As reviewed below, plasma levels of androgens fluctuate seasonally during adulthood, activate male advertisement calling, and may modulate auditory sensitivity. Androgens also act during development to organize the structure of the voice box and associated muscles, motorneurons, and central nervous system structures that together generate calls.

5.1.1. Seasonal activation of calling

In seasonally breeding adult male frogs, the seasonal increase in plasma androgens activates advertisement calling. Injection of male African clawed frogs with hCG increased plasma androgen levels as well as advertisement calling (Wetzel & Kelley, 1983). Likewise, castration of adult male African clawed frogs and green treefrogs reduced advertisement calling and replacement with androgens restored calling, indicating that androgens

support the expression of male advertisement calls (Wetzel & Kelley, 1983; Burmeister & Wilczynski, 2001).

The seasonal increase in plasma androgens is due to gonadal recrudescence in response to initial predictive cues such as temperature, rainfall, humidity, and photoperiod (Lofts, 1984). However, once breeding has begun, social cues may synchronize timing of breeding among conspecifics. In male green treefrogs (*Hyla cinerea*), exposure to the sounds of a mating chorus every night for 10–20 days increased plasma androgens and the number of GnRH-immunoreactive neurons in the septo-preoptic area of the brain (Burmeister & Wilczynski, 2000; 2005). Similar results were found in southern leopard frogs, *Rana sphenocephala* (Chu & Wilczynski, 2001). Social regulation of androgen levels is important in maintaining calling and other androgen-dependent traits when the social environment is favorable to successful mating.

A comparison of seven species of tropical frog representing four families revealed a positive association between plasma androgen levels and call rate (Emerson & Hess, 1996). Because elevated androgen levels are required to support the size, strength, and endurance of muscles involved in calling, Emerson and Hess (1996) proposed that species variation in plasma androgens may reflect species variation in call rate. However, within a species, calling effort is not always correlated with plasma androgen levels (Mendonça et al., 1985), perhaps because male advertisement calling is also modulated by social context. Hearing a mating chorus evokes calling in males. Although plasma androgen levels were correlated with spontaneous call rate in green treefrogs (*H. cinerea*), androgens were not correlated with calling evoked by the sound of a mating chorus (Burmeister & Wilczynski, 2001). Thus, aspects of a mating chorus must activate calling independently of changes in androgen levels.

5.1.2. Sex steroid hormone receptors in vocal and auditory brain areas

The effects of androgens on calling are mediated through sex steroid hormone receptors. The larynx and areas of the central nervous system involved in vocalization bind sex hormones including T (Kelley, Lieberburg, McEwen, & Pfaff, 1978; Kelley, 1980; Boyd, Wissing, Heinsz, & Prins, 1999). An important brain area is the POA, in which intracranial implants of T elicited male calling behavior (Wada & Gorbman, 1977). Although work thus far has only examined the androgen basis of male calling, androgens could also influence auditory perception of calls by males. A brain area of particular interest is the laminar nucleus of the torus semicircularis, a midbrain area that integrates auditory information (Wilczynski & Endepols, 2007) and concentrates androgens in both African clawed frogs and leopard frogs (Kelley et al., 1978; Kelley, 1980).

5.1.3. Development of the vocal system

The development of the anuran vocal system is best understood in the African clawed frog (*X. laevis*) (reviewed in Kelley, 1996). Male advertisement calling in the African clawed frog consists of rapid patterns of clicking trills. Adult female African clawed frogs produce clicks at much slower rates (Figure 8.1). Clicks are produced by the larynx. The larynx and the associated laryngeal muscles and motorneurons are masculinized by androgens during development. The larynx is 3–4 times larger in males than in females. Males have fast-twitch laryngeal muscles that allow males to click rapidly. Females have slow-twitch laryngeal muscles such that they do not produce the rapid male-like clicking. Likewise, firing patterns and cell body sizes of the motorneurons that innervate the laryngeal muscles are sexually dimorphic. Vocal production relies on a neural network located in the brainstem called the vocal pattern generator, which is also sexually differentiated (Zornik & Yamaguchi, 2008). The dorsal tegmental area of the medulla (also called the pretrigeminal area in some species) is an important premotor component of the vocal pattern generator.

Despite the organizational effects of androgens on the vocal system early in development, there is considerable adult plasticity in the morphological and neural bases of calling behavior. Initial studies in *X. laevis* found that treatment of ovariectomized adult females with androgens did not consistently induce male-like advertisement calls (Hannigan & Kelley, 1986; Watson & Kelley, 1992). However, a careful assessment of the effects of T in ovariectomized adult females determined that many aspects of the vocal system were defeminized and masculinized after only a few weeks of T treatment (Potter, Bose, & Yamaguchi, 2005). In fact, T completely masculinized the contractile properties of the muscles and the laryngeal motorneuron sizes of females by four weeks of treatment. Testosterone treatment began to alter female-typical calls within a few weeks and all females produced a male-like advertisement call by three months. Although females never produced advertisement calls identical to those of males, this study indicates that there is considerable plasticity in adulthood in the vocal control system.

5.1.4. Conclusions

Although androgens are required for the expression of male advertisement calling in anurans, other cues such as exposure to a mating chorus are also involved. This is reminiscent of the actions of androgens on salamander clasping, in which androgens are permissive but not sufficient for expression of the behavior. As with salamander clasping behavior, the expression of anuran calling

behavior may require hormones in addition to androgens, such as gonadotropins (GTHs), AVT, and CORT.

5.2. Gonadotropins (GTHs) and Auditory Advertisement

Studies of the androgen control of male advertisement calling indicated that androgens were necessary but not always sufficient to elicit male advertisement calling. An early study with African clawed frogs (*X. laevis*) hinted that GTHs may play a role beyond simply increasing plasma androgen levels (Wetzel & Kelley, 1983). Levels of calling achieved by injecting intact males with hCG exceeded levels obtained in androgen-treated castrated males (Wetzel & Kelley, 1983). Indeed, Yang et al. (2007) provided several lines of evidence indicating that GTHs act in the brain to directly stimulate calling. First, injection of hCG into the cerebral ventricles of androgen-treated castrated males elicited male calling behavior (Yang et al., 2007). Next, the *Xenopus* LH receptor (xLHR) was cloned and shown to be expressed in ventral forebrain areas implicated in vocalization. Finally, *in-vitro* treatment with hCG increased neuronal activation in ventral forebrain areas, as indexed by the presence of the protein product of the immediate-early gene, *egr 1* (Yang et al., 2007). Together, these studies indicate that GTHs regulate the expression of male calling behavior via direct effects on the brain.

5.3. Arginine Vasotocin (AVT) and Auditory Advertisement

5.3.1. Behavioral effects of arginine vasotocin (AVT)

The effects of AVT on male advertisement calling in anurans have been studied in both temperate and tropical species, in both laboratory and field settings. Injection with AVT increased various aspects of male advertisement calling in multiple species (reviewed in Wilczynski et al., 2005). Treatment with a vasopressin receptor antagonist blocked AVT-induced calling in the Great Plains toad (*B. cognatus*) (Propper & Dixon, 1997). Increased calling by males might have important fitness consequences. For example, treatment with AVT induced calling in nonresident and satellite males such that these males had increased access to important calling sites (Semsar, Klomberg, & Marler, 1998; Ten Eyck, 2005). Arginine vasotocin might also make male advertisement calls more attractive to females (Marler, Chu, & Wilczynski, 1995), but studies have not directly examined female preferences for the calls of males injected with AVT.

Several hypotheses have been advanced to better understand the precise effects of AVT on male advertisement calling. First, AVT could block stress responses in frogs. Injection with saline vehicle often suppresses male calling, possibly due to the stress of handling and injection. In contrast, injection with AVT induces calling, perhaps by blocking the effects of CORT, a hormone released when animals are stressed. However, Burmeister, Somes, and Wilczynski (2001) found that AVT injections actually increased CORT levels, and AVT potentiated corticosteroid secretion by the frog interrenal gland (Larcher, Delarue, Idres, & Vaudry, 1992). Second, AVT might mediate the effects of social context on calling. For example, in male gray treefrogs (*Hyla versicolor*), AVT altered calling only if other males were close by (Trainor, Rouse, & Marler, 2003). However, AVT increased calling regardless of the presence of conspecific calls in túngara frogs (*Physalaemus pustulosus*) (Kime, Whitney, Davis, & Marler, 2007). Third, AVT might simply increase motivation to call, as suggested by multiple studies in which AVT increased call production and/or decreased latency to calling (Chu, Marler, & Wilczynski, 1998; Tito, Hoover, Mingo, & Boyd, 1999; Klomberg & Marler, 2000; Burmeister et al., 2001; Kime et al., 2007). Finally, AVT might modulate sensorimotor processing (Moore & Rose, 2002). Studies of salamander mating behavior indicate that social cues trigger AVT release, and AVT enhances sensorimotor processing related to amplectic clasping by cross-modal amplification of sensory signals (Thompson et al., 2008). There are interesting parallels between the neurochemistry and neurocircuitry of salamander clasping behavior and anuran vocalization. It will be interesting to determine whether AVT modulates sensorimotor processing of components of the anuran vocal pattern generator. Ultimately, to resolve the role of AVT in male advertisement calling, the stimuli that induce endogenous AVT release and the neurophysiological effects of AVT must be determined.

5.3.2. Distribution of arginine vasotocin (AVT) cells, fibers, and receptors in the anuran brain

Arginine vasotocin-synthesizing neurons have been found throughout the bullfrog brain, including areas that concentrate sex hormones (Boyd & Moore, 1992; Boyd, Tyler, & De Vries, 1992; Boyd, 1997). A review of AVT cell bodies and fibers in the frog brain can be found in Acharjee et al. (2004). The most prominent group of AVT-synthesizing cells was found in the magnocellular portion of the preoptic nucleus. These cells project to the posterior pituitary and secrete AVT peripherally to regulate water balance (Boyd, 2006). Other populations of AVT cells were found in brain areas involved in vocal production and auditory perception. Some of these areas (the pretrigeminal nucleus, the AMG, and the POA) also concentrated sex steroid hormones in the African clawed frog (Kelley et al., 1978; Kelley, 1980). Further, AVT fibers were found in the torus semicircularis, a midbrain auditory processing area

that binds androgens. Thus, sex hormones may interact with AVT in specific areas of the brain.

An AVT receptor (vasotocin receptor (VTR)) with sequence similarity to the mammalian AVP receptor V1a has been cloned in the bullfrog (*Rana catesbeiana*) and European green frog (*Rana esculenta*) (Acharjee et al., 2004). Vasotocin receptors were moderately to highly expressed in several telencephalic areas including the lateral AMG, pallium, lateral septum, and nucleus accumbens. In the diencephalon, VTRs were expressed in the magnocellular POA, anterior POA, and dorsal hypothalamus. In the brainstem, VTRs were expressed in tegmental areas, the optic tectum, and the torus semicircularis. Many of these areas also had AVT-synthesizing cell bodies and/or AVT fibers (for a review see Acharjee et al., 2004). These findings were consistent with those described using autoradiography (Boyd, 1997). A VTR with sequence similarity to the mammalian AVP receptor V2R has been cloned in the Japanese treefrog (*Hyla japonica*) and was expressed in the brain (Kohno, Kamishima, & Iguchi, 2003).

Male advertisement calling evokes sexually dimorphic responses: calling in other males and approaches by females. Sex differences in the behavioral responses to male calls in bullfrogs (*R. catesbeiana*) were reflected in sex differences in the AVT system of the brain (reviewed in Boyd, 1997). Males had more AVT cells and fibers in the lateral AMG, septum, habenula, optic tectum, pretrigeminal nucleus, and tegmentum than did females. Females had more AVT in the dorsolateral nucleus, an auditory area, than did males. A sex difference was also found in cricket frogs (*Acris crepitans*), in which more AVT staining in the nucleus accumbens was found in males compared to females (Marler et al., 1999). Sex differences in AVT binding also exist in bullfrogs. Arginine vasotocin binding was greater in the lateral AMG and hypothalamus in breeding females compared to breeding males, but greater in the pretrigeminal nucleus and the dorsolateral nucleus in breeding males compared to breeding females (Boyd, 1997). Some of these sex differences were altered with gonadectomy and hormone treatments. Boyd hypothesized that sex differences in AVT content and binding could contribute to sex differences in calling behavior. However, it is still unknown where in the anuran brain AVT influences male calling behavior or female phonotaxis to male calls.

5.4. Corticosterone and Auditory Advertisement

5.4.1. The energetics–hormone–vocalization model

One of the first field studies of the hormonal correlates of calling behavior in frogs found that calling male bullfrogs had higher CORT and lower androgens than noncalling males (Mendonça et al., 1985). These researchers hypothesized that the energetic demands of calling induced a stress response and release of the stress hormone CORT, which inhibited the hypothalamic–pituitary–gonadal (HPG) axis to block androgen production. Consistent with this hypothesis, plasma CORT levels in calling males were positively associated with qualitative measures of calling effort in multiple species of frog (Emerson & Hess, 1996; 2001). Further, a study in male túngara frogs showed that treatment with CORT inhibited both plasma androgen levels and calling behavior (Marler & Ryan, 1996).

To formally explain the dynamic hormonal regulation of male advertisement calling, Emerson proposed the energetics–hormone–vocalization model (Emerson, 2001). Emerson proposed that: (1) plasma androgens activate calling behavior and, in turn, rise in response to calling; (2) with increased calling effort, plasma CORT levels rise in response to the energetic demands of calling; (3) when CORT becomes sufficiently elevated, CORT feeds back on the HPG axis to inhibit androgen secretion; (4) as plasma androgen levels drop, calling decreases and animals begin foraging to regain energetic stores; and (5) once animals have restored energetic stores, plasma CORT decreases, androgens levels rise, and calling begins again. This cycle could repeat within a night or across several nights within a breeding season.

5.4.2. Testing the energetics–hormone–vocalization model in explosive-breeding toads

Predictions of the energetics–hormone–vocalization model were tested in two species of explosive-breeding toad in which male *Bufo cognatus* and *B. woodhousii* alternate between calling behavior and noncalling satellite behavior. Satellite males attempt to intercept receptive females that are attracted to calling males. Leary et al. (2004) measured steroid hormone levels in calling and satellite males and found that androgens levels were similar between the satellite and calling males, but calling males had higher CORT compared to satellite males (Leary et al., 2004). Thus, differences in calling behavior were more closely associated with plasma CORT than with plasma androgens. To determine whether CORT had a direct effect on calling behavior, Leary, Garcia, and Knapp (2006a) injected calling males with CORT, which elevated plasma CORT. After injection, call duration decreased and males switched to noncalling satellite behavior within one hour (Figure 8.4) (Leary et al., 2006a; 2006b). Androgen levels were not altered by CORT injections, suggesting that the behavioral effects of CORT were direct and not due to changes in androgen levels. Leary et al. (2004) proposed that CORT may exert its

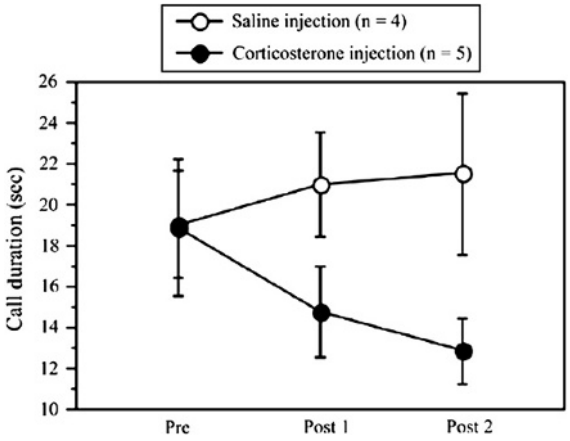

FIGURE 8.4 The effects of corticosterone and saline injections on call duration of male Great Plains toads (*Bufo cognatus*) either immediately before treatment (Pre), 10 minutes post-injection (Post 1) or 30 minutes postinjection (Post 2). Injection with corticosterone resulted in decreased call duration. *Adapted from Leary, Garcia and Knapp (2006b).*

rapid effects on calling via a nongenomic mechanism involving AVT action in vocal control areas of the anuran brain.

Leary, Garcia, Knapp, & Hawkins (2008b) also examined changes in plasma androgens, plasma CORT, calling effort, and body condition (an index of energy stores) over the course of a night and across several nights of mating activity in Woodhouse's toads (*B. woodhousii)*. Contrary to predictions of the energetics–hormone–vocalization model, it was found that androgen levels, calling effort, and body condition did not change within a night or over several nights. Also contrary to prediction, CORT decreased rather than increased over the course of a night (Leary et al., 2008b). Within a male, CORT was positively correlated with calling effort but not with body condition. Thus, although CORT was associated with calling effort, increased CORT did not appear to be due to increased energy expenditure during calling. A subsequent study of Woodhouse's toads found that CORT was positively correlated with chorus density (males calling per meter squared), providing an interesting density-dependent mechanism for switching from calling to satellite tactics as chorus density increases (Leary et al., 2008a).

Finally, variation in calling effort, a sexually selected trait (Gerhardt & Huber, 2002), appears to be more closely related to variation in plasma CORT rather than plasma androgens. First, females were attracted to calls of long duration, typical of those expressed by calling males with relatively low plasma CORT (Leary et al., 2006b). Second, satellite males were more often associated with males emitting longer calls and possessing lower CORT. Satellite males may seek out males emitting calls that are most attractive to females (Leary et al., 2006b). Thus, the physiological basis of this sexually selected trait may involve CORT rather than androgens.

5.4.3. Conclusions and future directions

The energetics–hormone–vocalization model has stimulated important research on the hormonal and physiological mechanisms underlying male advertisement calling, a sexually selected trait. Tests of the model in explosive-breeding toads found that the variation in male advertisement calling may be due to variation in plasma CORT rather than to plasma androgens (Leary et al., 2006b). The uncoupling of plasma CORT and plasma androgens in explosive-breeding toads is intriguing. An uncoupling of CORT and androgens may be advantageous in explosively breeding toads because the stress of calling would not compromise androgen-dependent behaviors that are critical to reproductive success (Leary et al., 2004). In species with prolonged breeding seasons, such as the bullfrog or the túngara frog, calling-related increases in plasma CORT may indeed suppress plasma androgens, as predicted by the energetics–hormone–vocalization model. Thus, future studies should examine the energetics-hormone-vocalization model in species with prolonged breeding seasons to better understand diversity in the endocrine correlates of calling.

5.5. Hormones and Olfactory Advertisement

A number of male amphibians release female-attracting pheromones. In male Japanese newts (*Cynops*), several different hormones together regulate the production, release, and behavioral transmission of the female-attractant pheromones (sodefrin and silefrin). A cloacal gland called the abdominal gland produces female attractant pheromones under the influence of both androgens and PRL (Kikuyama, Nakano, & Yasumasu, 1975). The abdominal gland expresses androgen and PRL receptors, and treatment with T and PRL increased pheromone mRNA expression and pheromone content in the gland (Iwata et al., 2000a; Yamamoto, Toyoda, Tanaka, Hayashi, & Kikuyama, 1996; Toyoda et al., 2004). Testosterone and PRL have, in addition, been implicated in the development of glands that potentially produce pheromones in other salamanders (Norris, Austin, & Hijazi, 1989). Secretion of pheromones from the abdominal gland was induced by AVT, which also increased contractility of the gland. Incidentally, AVT also increased spermatophore release in the absence of females. Males of some salamander species fan or vibrate their tails to direct chemosensory cues towards females in early stages of courtship. Tail fanning behavior required androgen and PRL for maximal expression (Toyoda et al., 1993; 1996) and was also modulated by AVT acting in the brain (Toyoda et al., 2003).

6. MALE MATE-SEARCHING BEHAVIORS

Many species of amphibian experience a water drive in which animals return to an aquatic habitat in order to successfully breed (Mazzi & Vellano, 1987). At the end of the breeding season, animals return to a more terrestrial habitat. In addition, animals may alter levels of locomotion, activity, specific mate-searching behaviors such as chemo-investigation, and/or become attuned to specific sensory information in order to find mates.

6.1. Habitat Preferences

In species of terrestrial salamander that return to water to breed, PRL increased locomotion and preferences for aquatic environments (Grant & Grant, 1958; Duvall & Norris, 1977; Toyoda et al., 1996). Treatment with thyroxine increased locomotion related to preference for a terrestrial habitat in tiger salamanders (*A. tigrinum*) (Duvall & Norris, 1980).

6.2. Locomotion

There is evidence for hormonal regulation of locomotory activity related to mating in Japanese red-bellied newts (*C. pyrrhogaster*). In this species, males are reported to move more than females, as males search for and court females (Matsunaga, Ukena, Baulieu, & Tsutsui, 2004; Haraguchi, Matsunaga, Koyama, Do Rego, & Tsutsui, 2009). This increase in activity is associated with a seasonal increase in levels of a neurosteroid, 7α-hydroxypregnenolone, in the male brain (Matsunaga et al., 2004; Haraguchi et al., 2009). Levels of 7α-hydroxypregnenolone in the brain were highest during the spring mating season, and intracerebroventricular injection of 7α-hydroxypregnenolone in nonbreeding males increased locomotor activity. As with other vertebrates, dopamine is involved with aspects of motor behavior in amphibians (Chu & Wilczynski, 2007). 7α-hydroxypregnenolone increased dopamine release by the brain, and co-administration of a dopamine D2-like receptor antagonist (haloperidol) prevented the 7α-hydroxypregnenolone-induced increase in locomotion. Thus, a neurosteroid, acting via the dopamine system, may regulate seasonal changes in locomotor activity related to mate searching (Matsunaga et al., 2004). In addition, 7α-hydroxypregnenolone may also mediate diurnal changes in locomotor activity (Koyama, Haraguchi, Vaudry, & Tsutsui, 2009).

It is possible that androgens also contribute to the seasonal change in locomotion. The dopaminergic system has been well described in anuran amphibians and is sexually dimorphic and steroid-sensitive (Wilczynski & Chu, 2001). Using tyrosine hydroxylase (TH), the rate-limiting enzyme in the production of catecholamines, as a marker for potential dopaminergic cells, three main groups of putative dopaminergic cells were found in the forebrain. All three groups were sensitive to the actions of T: castration reduced, and T implants restored, the number of immunoreactive TH cells in male Northern leopard frogs (*R. pipiens*) (Chu & Wilczynski, 2002). Further, the numbers of TH immunoreactive cells depended on gonadal sex and circulating levels of androgens and estrogens (Wilczynski, Yang, & Simmons, 2003). Thus, it will be important to determine whether the actions of 7α-hydroxypregnenolone on locomotion are modulated by androgenic effects on catecholaminergic neurons.

Several other hormones also have been demonstrated to alter levels of locomotion. For example, treatment with thyrotropin-releasing hormone increased locomotion in male African clawed frogs (*X. laevis*) (Taylor & Boyd, 1991). Treatment with AVT increased locomotion in female bullfrogs (Boyd, 1991). The function of increased locomotion is not always clear and may not be related to changes in reproductive activity. In the rough-skinned newt, treatment with corticotropin-releasing factor (CRF) increased swimming and walking, effects believed to be mediated through extrahypothalamic actions of CRF (Moore et al., 1984). Treatment with an antagonist of CRF (α-helical $CRF_{9\text{-}41}$) prevented handling-induced increases in locomotion (Lowry & Moore, 1991). These changes in locomotion are suggested to be components of the flight-or-fight response rather than to be related to reproductive activities (reviewed in Lowry and Moore, 2006).

6.3. Chemo-investigation

In red-legged salamanders (*P. shermani*), animals express a behavior called nose tapping that functions to transfer chemosensory cues from the substrate to the nasal cavity (Dawley & Bass, 1989). Animals nose tapped substrates moistened with chemosensory cues from conspecifics more than they nose tapped control substrates moistened with water alone. Nose tapping is facilitated by fleshy structures called cirri, which are larger in males than in females (Schubert et al., 2008). Further, males nose tapped substrates containing chemosensory cues derived from females more than did other females (Schubert et al., 2008), suggesting that males may be searching for females by nose tapping.

To determine the role of T in chemo-investigative behavior, Schubert, Houck, Feldhoff, Feldhoff, and Woodley (2006) elevated T levels of nonbreeding males with T implants. Compared to blank-implanted control males, T-implanted males had larger cirri and increased levels of nose tapping. Testosterone increased levels of nose tapping in response to chemosensory cues derived from conspecifics as well as the control cue of water (Figure 8.5). Finally, T also increased preferences of males

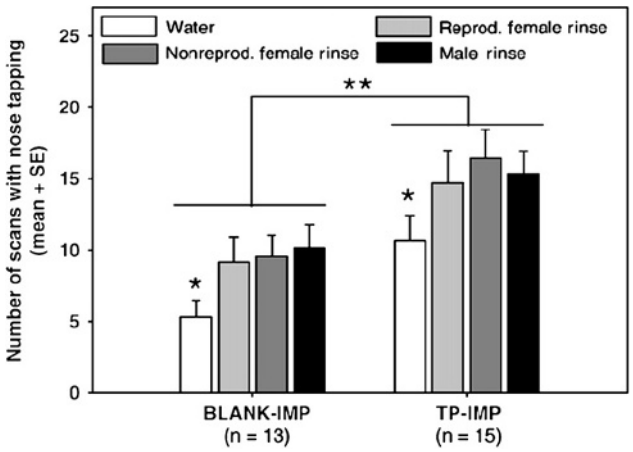

FIGURE 8.5 Levels of chemoinvestigative behavior (nose tapping) observed in blank-implanted (IMP) or testosterone (TP)-implanted male red-legged salamanders (*Plethodon shermani*) in response to substrates moistened with water or with body rinses from conspecifics. Levels of chemoinvestigation are increased in the presence of chemosensory cues from conspecifics. Treatment with testosterone increased chemoinvestigation of all chemosensory stimuli tested. **, significant overall effects of implant type on nose tapping. *, significantly different from the other chemosensory stimuli within an implant type. *Adapted from Schubert, Houck, Feldhoff P.W., Feldhof R.C., and Woodley (2006).*

for substrates that had been marked by females (Schubert et al., 2006).

6.4. Sensitivity to Sensory Information

Nonvolatile chemosensory cues are detected by the vomeronasal organ (VNO) in the red-legged salamander (Wirsig-Wiechmann, Houck, Feldhoff, & Feldhoff, 2002). The VNO is almost twice as large in males compared to females in both absolute size and size relative to body length (Dawley, 1992; Dawley & Crowder, 1995; Woodley, 2007). A sex difference in VNO size was found in juveniles and was not altered by changes in T level or female reproductive condition (Schubert et al., 2006; Woodley, 2007). The responsiveness of the VNO to chemosensory cues was measured in males treated with T implants or blank implants. Testosterone treatment had no effect on the responsiveness of the VNO to male pheromones (Schubert et al., 2006). Thus, although T enhanced morphological (cirri) and behavioral (nose tapping) traits that bring salamanders into contact with socially relevant chemosensory cues, there is, as yet, no evidence that hormones modulate sensitivity of the VNO in this species (Schubert et al., 2006; 2008). However, it should be noted that males were tested with male pheromones rather than female pheromones. Future studies should examine the responsiveness of the male VNO to additional pheromones, especially those derived from reproductive females.

7. FEMALE REPRODUCTIVE BEHAVIORS

Studies of female amphibian reproductive behaviors have considered the hormonal basis of female receptivity to mating, female attraction to social signals emitted by males, and other behaviors such as egg-laying behavior in salamanders and female mating vocalizations in anurans. Until very recently, study of the neuroendocrine basis of female amphibian reproductive behaviors has been relatively neglected.

7.1. Female Receptive Behaviors

7.1.1. Salamander mating behavior

During courtship, female rough-skinned newts (*T. granulosa*) are clasped by males for many hours both before and after insemination. Pre-insemination clasping increases female receptivity, and post-insemination clasping reduces female receptivity (Propper, 1991). Surprisingly, the endocrine basis of changes in female receptivity is unknown. Although plasma E_2 is elevated in females after insemination (Propper & Moore, 1991), a causal relationship between E_2 and female receptivity has not been experimentally determined.

7.1.2. Anuran mating behavior

When receptive, female anurans allow males to clasp them in amplexus. Sexually unreceptive females can avoid being clasped by males by leg extension, rolling behavior, or emitting release calls (Kelley, 1982). Injection of female African clawed frogs (*X. laevis*) with hCG prevented female leg extensions in response to clasping by a male (Kelley, 1982). Treatment with a combination of E_2 and progesterone (P_4) was required to prevent leg extensions in ovariectomized females. Injection with GnRH potentiated the effects of E_2 and P_4 on leg extensions in ovariectomized females (Kelley, 1982).

Female release calls are also modulated by hormones. In one of the earliest studies on the behavioral actions of AVT, release calls by female leopard frogs (*R. pipiens*) were suppressed by AVT injections (Diakow, 1978b). The effects of AVT injection could be mimicked by experimentally increasing fluid retention, suggesting that AVT acts peripherally to increase water uptake, perhaps by potentiating prostaglandin actions (Diakow & Nemiroff, 1981). In addition, release calls were suppressed in female bullfrogs (*R. catesbeiana*) after injection with AVT and prostaglandins (Boyd, 1992). Surprisingly, treatment of ovariectomized females with E_2 and/or P_4 did not inhibit the release calls (Diakow, 1978a). On the basis of these results, Diakow (1978a) concluded that female anuran reproductive behavior is not modulated by ovarian hormones and is the result of peripheral actions of AVT.

7.2. Attraction to Male Social Signals

7.2.1. Sensitivity of the salamander vomeronasal sensory epithelium

In salamanders, males produce pheromones that attract females and persuade females to mate with males (Houck, 1986; Kikuyama, Yamamoto, Iwata, & Toyoda, 2002). In female Japanese red-bellied newts (*C. pyrrhogaster*), attraction to the male pheromone, sodefrin, was enhanced by injection with PRL plus hCG (Toyoda, Tanaka, Matsuda, & Kikuyama, 1994). Increased attraction may have been due to the increased sensitivity of the VNO following treatment with PRL and E_2 (Toyoda & Kikuyama, 2000). Although PRL and E_2 increased vomeronasal sensitivity to female-attracting pheromones in the Japanese newt, female hormones did not modulate vomeronasal sensitivity to male courtship pheromones in red-legged salamanders (*P. shermani*): in female red-legged salamanders there was no difference in responsiveness of the female VNO to male courtship pheromones in reproductive vs. nonreproductive females (Schubert et al., 2008), despite differences in plasma E_2 levels (Woodley, 2007).

7.2.2. Anuran phonotaxis

In many anuran species, receptive females approach the sound of a calling male, a behavior called phonotaxis. The role of gonadotropins and ovarian steroid hormones in the expression of phonotaxis has been studied in female túngara frogs (*P. pustulosus*). In this species, levels of E_2 and P_4 were elevated when females were expressing phonotactic responses (Lynch & Wilczynski, 2005). Treatment of female túngara frogs with E_2 or hCG increased phonotaxis (Lynch et al., 2006; Chakraborty & Burmeister, 2009). Injection with hCG also increased the range of calls that a female would approach, suggesting that hormones could modulate aspects of female mate choice (Lynch et al., 2006). Progesterone may also be involved in some species, as a regimen of prostaglandin injection in P_4-primed females also induced phonotaxis (Schmidt, 1985; Gordon & Gerhardt, 2009). There is also evidence that AVT modulates phonotaxis; in female bullfrogs (*R. catesbeiana*), treatment with AVT increased phonotactic responses to male calls (Boyd, 1994). Arginine vasotocin also facilitated female phonotaxis in the American toad (*Bufo americanus*) (Schmidt, 1985).

Hormones may modulate female phonotaxis by altering sensitivity to the sounds of male calls. Neural responses of the auditory midbrain (torus semicircularis) were reduced in female green treefrogs that had recently mated compared to unmated females, suggesting that hormonal changes associated with changes in receptivity modulate phonotaxis by altering auditory sensitivity (Miranda & Wilczynski, 2009). Lynch and Wilczynski (2008) proposed that gonadotropins and E_2 modulate sensory processing by the auditory midbrain in female túngara frogs (Lynch & Wilczynski, 2008). They found that the sounds of a male chorus increased both plasma E_2 levels and expression of the activity-dependent gene egr-1 in the torus semicircularis of females. Thus, by elevating E_2 in females, male calling may manipulate female choice by exploiting a pre-existing sensitivity to E_2 by the female auditory system.

7.2.3. Salamander chemo-investigation

Red-legged salamanders (*P. shermani*) express a chemo-investigative behavior called nose tapping to transfer substrate-borne chemosensory stimuli to the nasal cavity (Dawley & Bass, 1989). In female red-legged salamanders, levels of nose tapping were higher in reproductive females compared to nonreproductive females when tested on substrates moistened with water and male body rinses but not on substrates moistened with female body rinses (Schubert et al., 2008). Reproductively active females also had higher levels of plasma E_2 than did nonreproductive females (Woodley, 2007). Thus, it is possible that E_2 increases nose tapping in reproductive females, especially in response to male-derived chemosensory cues.

7.3. Other Female Reproductive Behaviors

7.3.1. Salamander egg-laying behavior

Several days after mating and insemination, female rough-skinned newts (*T. granulosa*) ovulate and oviposit a few eggs at a time onto submerged plants or twigs. Egg-laying behavior involves clasping of vegetation, a behavior very similar in appearance to male clasping of females. Clasping behavior by females requires E_2 and can be triggered by injection of AVT (Moore, Wood, & Boyd, 1992). Treatment of ovariectomized females with androgens and AVT caused females to clasp other females instead of vegetation and to spend more time near female-scented models relative to ovariectomized females treated with androgens and saline (Thompson & Moore, 2003). These results suggest that AVT works in concert with sex steroid hormones to trigger clasping behavior in response to the appropriate releasing stimuli.

A role for AVT in salamander female behavior is supported by neuroanatomical evidence: there are four populations of AVT-synthesizing neurons that have more AVT-immunoreactive cells in female newts compared to male newts (Moore et al., 2000). The sex difference was largest in the pars dorsalis hypothalamus and ventromedial hypothalamus from animals collected during the breeding season. This area is implicated in female mating behavior and stress responses. Further, ovariectomy reduced AVT

binding in the AMG of females although not in the olfactory bulb, pallium, or medulla oblongata (Boyd & Moore, 1991). Thus, ovarian hormones might regulate expression of AVT receptors in females. Clearly, more studies of the role of AVT in female behaviors are warranted.

7.3.2. Anuran mating vocalizations of females

In some species of anuran, females call to signal receptivity (Emerson & Boyd, 1999). These vocalizations are not release calls because they are not elicited after clasping by a male. Males respond strongly to female mating vocalizations by approaching and calling and may engage in duets with females (Tobias, Viswanathan, & Kelley, 1998). Although the hormonal basis for female vocalizations is hypothesized to be due to androgens, experimental tests of this hypothesis are lacking (Emerson & Boyd, 1999).

7.4. Conclusions and Future Directions

Despite recent studies, there are still large gaps in our understanding of the hormonal basis of female amphibian reproductive behaviors. Although ovarian hormones are required for female receptivity to mating in African clawed frogs, the role of ovarian hormones in female receptivity in other anurans and in salamanders is not well understood. It is also unclear whether findings regarding PRL, AVT, or CORT derived from studies of male amphibians apply to females. In particular, the role of AVT in female behaviors warrants additional attention. Clearly, future study of the hormonal bases of female amphibian reproductive behaviors will provide important insight into endocrine mechanisms of behavior. As one example, recent studies examining the role of hormones in auditory sensitivity and female mate choice in anurans are promising and represent an important future direction (Lynch & Wilczynski, 2008).

8. FINAL CONCLUSIONS

As modern representatives of basal tetrapods, amphibians can provide much insight into the hormonal bases of vertebrate reproductive behaviors. Amphibians are easily studied in the laboratory as well as the field, and amphibian behaviors and neural systems are relatively simple and experimentally tractable. To date, the majority of studies have focused on only a few amphibian species such as *X. laevis*, *T. granulosa*, and *C. pyrrhogaster*. Studies of these species have revealed important endocrine mechanisms that are likely to be conserved across vertebrates. However, researchers are beginning to capitalize on the diversity of amphibian mating systems to test evolutionary hypotheses about alternative reproductive tactics, female mate choice, and sexual selection. It is hoped that this review will stimulate additional interest in amphibians as subjects for studies of the hormonal bases of reproductive behavior.

ABBREVIATIONS

AMG	Amygdala
AVP	Arginine vasopressin
AVT	Arginine vasotocin
BNST	Bed nucleus of the stria terminalis
CB1	Cannabinoid receptor type 1
CORT	Corticosterone
CRF	Corticotropin-releasing factor
E_2	Estradiol
GnRH	Gonadotropin-releasing hormone
hCG	Human chorionic gonadotropin
HPG	Hypothalamic−pituitary−gonadal
LH	Luteinizing hormone
LHRH	Luteinizing hormone-releasing hormone
NO	Nitric oxide
NOS	Nitric oxide synthase
P_4	Progesterone
POA	Preoptic area
PRL	Prolactin
T	Testosterone
TH	Tyrosine hydroxylase
TRH	Thyrotropin-releasing hormone
VNO	Vomeronasal organ
VTR	Vasotocin receptor
xLHR	*Xenopus* luteinizing hormone receptor

REFERENCES

Acharjee, S., Do-Rego, J. L., Oh, D. Y., Moon, J. S., Ahn, R. S., Lee, K., Bai, D. G., Vaudry, H., Kwon, H. B., & Seong, J. Y. (2004). Molecular cloning, pharmacological characterization, and histochemical distribution of frog vasotocin and mesotocin receptors. *J. Mol. Endocrinol., 33*, 293−313.

Adams, E. M., Jones, A. G., & Arnold, S. J. (2005). Multiple paternity in a natural population of a salamander with long-term sperm storage. *Mol. Ecol., 14*, 1803−1810.

Andreoletti, G. E., Malacarne, G., & Vellano, C. (1983). Androgen control of male sex behavior in the crested newt (*Triturus cristatus carnifex* Laur.): castration and sex steroid administration. *Horm. Behav., 17*, 103−110.

Arnold, S. J. (1976). Sexual behavior, sexual interference, and sexual defense in the salamanders *Amybstoma maculatum, Amybystoma tigrinum* and *Plethodon jordani*. *Z. Tierpsychol., 42*, 247−300.

Benner, S. L., & Woodley, S. K. (2007). The reproductive pattern of male dusky salamanders (genus *Desmognathus*) is neither associated nor dissociated. *Horm. Behav., 51*, 542−547.

Boyd, S. K. (1991). Effect of vasotocin on locomotor activity in bullfrogs varies with developmental stage and sex. *Horm. Behav., 25*, 57−69.

Boyd, S. K. (1992). Sexual differences in hormonal control of release calls in bullfrogs. *Horm. Behav., 26*, 522−535.

Boyd, S. K. (1994). Arginine vasotocin facilitation of advertisement calling and call phonotaxis in bullfrogs. *Horm. Behav., 28*, 232−240.

Boyd, S. K. (1997). Brain vasotocin pathways and the control of sexual behaviors in the bullfrog. *Brain. Res. Bull., 44*, 345–350.

Boyd, S. K. (2006). Amphibian neurohypophysial peptides. In *"Handbook of Biologically Active Peptides"* (pp. 327–332). Burlington: Academic Press: Elsevier.

Boyd, S. K., & Moore, F. L. (1991). Gonadectomy reduces the concentrations of putative receptors for arginine vasotocin in the brain of an amphibian. *Brain. Res., 541*, 193–197.

Boyd, S. K., & Moore, F. L. (1992). Sexually dimorphic concentrations of arginine vasotocin in sensory regions of the amphibian brain. *Brain. Res., 588*, 304–306.

Boyd, S. K., Tyler, C. J., & De Vries, G. J. (1992). Sexual dimorphism in the vasotocin system of the bullfrog (*Rana catesbeiana*). *J. Comp. Neurol., 325*, 313–325.

Boyd, S. K., Wissing, K. D., Heinsz, J. E., & Prins, G. S. (1999). Androgen receptors and sexual dimorphisms in the larynx of the bullfrog. *Gen. Comp. Endocrinol., 113*, 59–68.

Burmeister, S., & Wilczynski, W. (2000). Social signals influence hormones independently of calling behavior in the treefrog (*Hyla cinerea*). *Horm. Behav., 38*, 201–209.

Burmeister, S. S., & Wilczynski, W. (2001). Social context influences androgenic effects on calling in the green treefrog (*Hyla cinerea*). *Horm. Behav., 40*, 550–558.

Burmeister, S. S., & Wilczynski, W. (2005). Social signals regulate gonadotropin-releasing hormone neurons in the green treefrog. *Brain. Behav. Evol., 65*, 26–32.

Burmeister, S., Somes, C., & Wilczynski, W. (2001). Behavioral and hormonal effects of exogenous vasotocin and corticosterone in the green treefrog. *Gen. Comp. Endocrinol., 122*, 189–197.

Chakraborty, M., & Burmeister, S. S. (2009). Estradiol induces sexual behavior in female tungara frogs. *Horm. Behav., 55*, 106–112.

Chu, J., Marler, C. A., & Wilczynski, W. (1998). The effects of arginine vasotocin on the calling behavior of male cricket frogs in changing social contexts. *Horm. Behav., 34*, 248–261.

Chu, J., & Wilczynski, W. (2001). Social influences on androgen levels in the southern leopard frog, *Rana sphenocephala*. *Gen. Comp. Endocrinol., 121*, 66–73.

Chu, J., & Wilczynski, W. (2002). Androgen effects on tyrosine hydroxylase cells in the northern leopard frog, *Rana pipiens*. *Neuroendocrinology, 76*, 18–27.

Chu, J., & Wilczynski, W. (2007). Apomorphine effects on frog locomotor behavior. *Physiol. Behav., 91*, 71–76.

Coddington, E., Lewis, C., Rose, J. D., & Moore, F. L. (2007). Endocannabinoids mediate the effects of acute stress and corticosterone on sex behavior. *Endocrinology, 148*, 493–500.

Coddington, E., & Moore, F. L. (2003). Neuroendocrinology of context-dependent stress responses: vasotocin alters the effect of corticosterone on amphibian behaviors. *Horm. Behav., 43*, 222–228.

Cooke, P. S., Holsberger, D. R., Witorsch, R. J., Sylvester, P. W., Meredith, J. M., Treinen, K. A., & Chapin, R. E. (2004). Thyroid hormone, glucocorticoids, and prolactin at the nexus of physiology, reproduction, and toxicology. *Toxicol. Appl. Pharmacol., 194*, 309–335.

Cottone, E., Guastalla, A., Mackie, K., & Franzoni, M. F. (2008). Endocannabinoids affect the reproductive functions in teleosts and amphibians. *Mol. Cell. Endocrinol., 286*, S41–S45.

Cottone, E., Salio, C., Conrath, M., & Franzoni, M. F. (2003). *Xenopus laevis* CB1 cannabinoid receptor: molecular cloning and mRNA distribution in the central nervous system. *J. Comp. Neurol., 464*, 487–496.

Crews, D. (1984). Gamete production, sex hormone secretion, and mating behavior uncoupled. *Horm. Behav., 18*, 22–28.

Crews, D., & Moore, M. C. (1986). Evolution of mechanisms controlling mating behavior. *Science, 231*, 121–125.

Crews, D., & Moore, M. C. (2005). Historical contributions of research on reptiles to behavioral neuroendocrinology. *Horm. Behav., 48*, 384–394.

Davis, G. A., & Moore, F. L. (1996). Neuroanatomical distribution of androgen and estrogen receptor-immunoreactive cells in the brain of the male roughskin newt. *J. Comp. Neurol., 372*, 294–308.

Dawley, E. M. (1992). Sexual dimorphism in a chemosensory system: the role of the vomeronasal organ in salamander reproductive behavior. *Copeia., 1992*, 113–120.

Dawley, E. M., & Bass, A. H. (1989). Chemical access to the vomeronasal organ of a plethodontid salamander. *J. Morphol., 200*, 163–174.

Dawley, E. M., & Crowder, J. (1995). Sexual and seasonal differences in the vomeronasal epithelium of the red-backed salamander (*Plethodon cinereus*). *J. Comp. Neurol., 359*, 382–390.

DeNardo, D. F., & Sinervo, B. (1994). Effects of corticosterone on activity and home-range size of free-ranging male lizards. *Horm. Behav., 28*, 53–65.

Denver, R. J. (2007). Endocannabinoids link rapid, membrane-mediated corticosteroid actions to behavior. *Endocrinology, 148*, 490–492.

Deviche, P., & Moore, F. L. (1987). Opioid kappa-receptor agonists suppress sexual behaviors in male rough-skinned newts (*Taricha granulosa*). *Horm. Behav., 21*, 371–383.

Diakow, C. (1978a). Female frog reproductive behavior elicited in the absence of ovaries. *Horm. Behav., 11*, 183–189.

Diakow, C. (1978b). Hormonal basis for breeding behavior in female frogs: vasotocin inhibits the release call of *Rana pipiens*. *Science, 199*, 1456–1457.

Diakow, C., & Nemiroff, A. (1981). Vasotocin, prostaglandin, and female reproductive behavior in the frog, *Rana pipiens*. *Horm. Behav., 15*, 86–93.

Donaldson, Z. R., & Young, L. J. (2008). Oxytocin, vasopressin, and the neurogenetics of sociality. *Science, 322*, 900–904.

Dubois, W., Pudney, J., & Callard, I. P. (1988). The annual testicular cycle in the turtle, *Chysemys picta*: a histochemical and electron microscopic study. *Gen. Comp. Endocrinol., 71*, 191–204.

Duellman, W. E., & Trueb, L. (1986). *"Biology of Amphibians."* New York: McGraw-Hill Book Company.

Duvall, D., & Norris, D. O. (1977). Prolactin and substrate stimulation of locomotor activity in adult tiger salamanders (*Ambystoma tigrinum*). *J. Exp. Zool., 200*, 103–106.

Duvall, D., & Norris, D. O. (1980). Stimulation of terrestrial-substrate preferences and locomotor activity in newly transformed tiger salamanders (*Ambystoma tigrinum*) by exogenous or endogenous thyroxine. *Anim. Behav., 28*, 116–123.

Emerson, S. B. (2001). Male advertisement calls: behavioral variation and physiological processes. In M. J. Ryan (Ed.), *"Anuran Communication"* (pp. 36–44). Washington, DC: Smithsonian Institution Press.

Emerson, S. B., & Boyd, S. K. (1999). Mating vocalizations of female frogs: control and evolutionary mechanisms. *Brain. Behav. Evol., 53*, 187−197.

Emerson, S. B., & Hess, D. L. (1996). The role of androgens in opportunistic breeding, tropical frogs. *Gen. Comp. Endocrinol., 103*, 220−230.

Emerson, S. B., & Hess, D. L. (2001). Glucocorticoids, androgens, testis mass, and the energetics of vocalization in breeding male frogs. *Horm. Behav., 39*, 59−69.

French, S. S., McLemore, R., Vernon, B., Johnston, G. I., & Moore, M. C. (2007). Corticosterone modulation of reproductive and immune systems trade-offs in female tree lizards: long-term corticosterone manipulations via injectable gelling material. *J. Exp. Biol., 210*, 2859−2865.

Frost, D. R. (2009). Amphibian Species of the World: an Online Reference. Version 5.3 (12 February, 2009). *Electronic Database accessible at http://research.amnh.org/herpetology/amphibia/American Museum of Natural History, New York, USA.*

Gerhardt, H. C., & Huber, F. (2002). *"Acoustic Communication in Insects and Anurans."* Chicago: University of Chicago Press.

Gerhardt, H. C., & Schwartz, J. J. (2001). Auditory tuning and frequency preferences in anurans. In M. J. Ryan (Ed.), *"Anuran Communication"* (pp. 73−85). Washington: Smithsonian Institution Press.

Goense, J. B., & Feng, A. S. (2005). Seasonal changes in frequency tuning and temporal processing in single neurons in the frog auditory midbrain. *J. Neurobiol., 65*, 22−36.

Goodson, J. L., & Bass, A. H. (2001). Social behavior functions and related anatomical characteristics of vasotocin/vasopressin systems in vertebrates. *Brain. Res. Rev., 35*, 246−265.

Gordon, N. M., & Gerhardt, H. C. (2009). Hormonal modulation of phonotaxis and advertisement-call preferences in the gray treefrog (*Hyla versicolor*). *Horm. Behav., 55*, 121−127.

Grant, W. C., & Grant, J. A. (1958). Water drive studies on hypophysectomized efts of Demyctylus viridescens. Part I. The role of the lactogenic hormone. *Biol. Bullet., 114*, 1−9.

Green, A. (1991). Competition and energetic constraints in the courting great crested newt, *Triturus cristatus* (Amphibia: Salamandridae). *Ethology, 87*, 66−78.

Halliday, T. R. (1990). The evolution of courtship behavior in newts and salamanders. *Adv. Study. of. Beh., 19*, 137−169.

Hannigan, P., & Kelley, D. B. (1986). Androgen-induced alterations in vocalizations of female *Xenopus laevis*: modifiability and constraints. *J. Comp. Physiol. [A], 158*, 517−527.

Haraguchi, S., Matsunaga, M., Koyama, T., Do Rego, J. L., & Tsutsui, K. (2009). Seasonal changes in the synthesis of the neurosteroid 7alpha-hydroxypregnenolone stimulating locomotor activity in newts. *Ann. N.Y. Acad. Sci., 1163*, 410−413.

Harvey, L. A., & Propper, C. R. (1997). Effects of androgens on male sexual behavior and secondary sex characters in the explosively breeding spadefoot toad, *Scaphiopus couchii*. *Horm. Behav., 31*, 89−96.

Harvey, L. A., Propper, C. R., Woodley, S. K., & Moore, M. C. (1997). Reproductive endocrinology of the explosively breeding desert spadefoot toad, *Scaphiopus couchii*. *Gen. Comp. Endocrinol., 105*, 102−113.

Hasunuma, I., Sakai, T., Nakada, T., Toyoda, F., Namiki, H., & Kikuyama, S. (2007). Molecular cloning of three types of arginine vasotocin receptor in the newt, *Cynops pyrrhogaster. Gen. Comp. Endocrinol., 151*, 252−258.

Hasunuma, I., Toyoda, F., Yamamoto, K., Yamashita, M., & Kikuyama, S. (2005). Localization of prolactin receptor in the newt brain. *Cell. Tissue. Res., 320*, 477−485.

Hillery, C. (1983). Seasonality of two midbrain auditory responses in the treefrog. *Copeia, 1983*, 844−852.

Hollis, D. M., Chu, J., Walthers, E. A., Heppner, B. L., Searcy, B. T., & Moore, F. L. (2005). Neuroanatomical distribution of vasotocin and mesotocin in two urodele amphibians (*Plethodon shermani* and *Taricha granulosa*) based on *in situ* hybridization histochemistry. *Brain. Res., 1035*, 1−12.

Hollis, D. M., Coddington, E. J., & Moore, F. L. (2006). Neuroanatomical distribution of cannabinoid receptor gene expression in the brain of the rough-skinned newt, *Taricha granulosa*. *Brain. Behav. Evol., 67*, 135−149.

Houck, L. D. (1986). The evolution of salamander courtship pheromones. In D. Duvall, D. Muller-Schwarze, & R. M. Silverstein (Eds.), *"Chemical Signals in Vertebrates, Vol. IV: Ecology, Evolution, and Comparative Biology."* (pp. 173−190). New York: Plenum Press.

Houck, L. D., & Arnold, S. J. (2003). Courtship and mating. In D. M. Sever (Ed.), *"Reproductive Biology and Phylogeny of Urodela (Amphibia)"* (pp. 383−424). New Hampshire Science Publishers.

Houck, L. D., Arnold, S. J., & Thisted, R. A. (1985). A statistical study of mate choice: sexual selection in a plethodontid salamander (*Desmognathus ochrophaeus*). *Evolution, 39*, 370−386.

Houck, L. D., & Reagan, N. L. (1990). Male courtship pheromones increase female receptivity in a plethodontid salamander. *Anim. Behav., 39*, 729−734.

Houck, L. D., & Sever, D. M. (1994). Role of the skin in reproduction and behaviour. In H. Heatwole, & G. T. Barthalmus (Eds.), *"Amphibian Biology", Vol. 1* (pp. 351−381). Chipping Norton, New South Wales, Australia: Surry Beatty & Sons, The Integument.

Houck, L. D., Watts, R. A., Mead, L. M., Palmer, C. A., Arnold, A. P., Feldhoff, P. W., & Feldhoff, R. C. (2008). A candidate vertebrate pheromone, SPF, increases female receptivity in a salamander. In J. L. Hurst (Ed.), *"Chemical Signals in Vertebrates", Vol. 11* (pp. 213−221). New York: Springer.

Hull, E. M., & Dominguez, J. M. (2006). Getting his act together: roles of glutamate, nitric oxide, and dopamine in the medial preoptic area. *Brain. Res., 1126*, 66−75.

Ishii, S., & Itoh, M. (1992). Amplexus induces surge of luteinizing hormone in male toads, *Bufo japonicus*. *Gen. Comp. Endocrinol., 86*, 34−41.

Itoh, M., & Ishii, S. (1990). Changes in plasma levels of gonadotropins and sex steroids in the toad, *Bufo japonicus*, in association with behavior during the breeding season. *Gen. Comp. Endocrinol., 80*, 451−464.

Iwata, T., Kawahara, G., Yamamoto, K., Zhou, C. J., Nakajo, S., Shioda, S., & Kikuyama, S. (2000a). Effect of prolactin and androgen on the expression of the female-attracting pheromone silefrin in the abdominal gland of the newt, *Cynops ensicauda*. *Biol. Reprod., 63*, 1867−1872.

Iwata, T., Toyoda, F., Yamamoto, K., & Kikuyama, S. (2000b). Hormonal control of urodele reproductive behavior. *Comp. Biochem. Physiol. B. Biochem. Mol. Biol., 126*, 221−229.

Iwata, T., Umezawa, K., Toyoda, F., Takahashi, N., Matsukawa, H., Yamamoto, K., Miura, S., Hayashi, H., & Kikuyama, S. (1999). Molecular cloning of newt sex pheromone precursor cDNAs: evidence for the existence of species-specific forms of pheromones. *FEBS. Lett., 457*, 400−404.

Kalk, M. (1960). Climate and breeding in *Xenopus laevis*. *S. Afr. J. Sci., 11*, 271–276.

Kelley, D. B. (1980). Auditory and vocal nuclei in the frog brain concentrate sex hormones. *Science, 207*, 553–555.

Kelley, D. B. (1982). Female sex behaviors in the South African clawed frog, *Xenopus laevis*: gonadotropin-releasing, gonadotropic, and steroid hormones. *Horm. Behav., 16*, 158–174.

Kelley, D. B. (1996). Sexual differentiation in *Xenopus laevis*. In R. Tinsley, & H. Kobel (Eds.), *"The Biology of Xenopus"* (pp. 143–176). Oxford: Oxford University Press.

Kelley, D. B. (2004). Vocal communication in frogs. *Curr. Opin. Neurobiol., 14*, 751–757.

Kelley, D. B., & Pfaff, D. W. (1976). Hormone effects on male sex behavior in adult South African clawed frogs. *Xenopus laevis. Horm. Behav., 7*, 159–182.

Kelley, D. B., Lieberburg, I., McEwen, B. S., & Pfaff, D. W. (1978). Autoradiographic and biochemical studies of steroid hormone-concentrating cells in the brain of *Rana pipiens*. *Brain. Res., 140*, 287–305.

Kelley, D. B., Morrell, J. I., & Pfaff, D. W. (1975). Autoradiographic localization of hormone-concentrating cells in the brain of an amphibian, *Xenopus laevis*. I. Testosterone. *J. Comp. Neurol., 164*, 47–59.

Kikuyama, S., Nakano, R., & Yasumasu, I. (1975). Synergistic action of prolactin and androgen on the cloacal glands of the newt. *Comp. Biochem. Physiol. A., 51*, 823–826.

Kikuyama, S., Toyoda, F., Ohmiya, Y., Matsuda, K., Tanaka, S., & Hayashi, H. (1995). Sodefrin: a female-attracting peptide pheromone in newt cloacal glands. *Science, 267*, 1643–1645.

Kikuyama, S., Yamamoto, K., Iwata, T., & Toyoda, F. (2002). Peptide and protein pheromones in amphibians. *Comp. Biochem. Physiol. B. Biochem. Mol. Biol., 132*, 69–74.

Kikuyama, S., Yazawa, T., Abe, S., Yamamoto, K., Iwata, T., Hoshi, K., et al. (2000). Newt prolactin and its involvement in reproduction. *Can. J. Physiol. Pharmacol., 78*, 984–993.

Kime, N. M., Whitney, T. K., Davis, E. S., & Marler, C. A. (2007). Arginine vasotocin promotes calling behavior and call changes in male tungara frogs. *Brain. Behav. Evol., 69*, 254–265.

Klomberg, K. F., & Marler, C. A. (2000). The neuropeptide arginine vasotocin alters male call characteristics involved in social interactions in the grey treefrog, *Hyla versicolor. Anim. Behav., 59*, 807–812.

Kohno, S., Kamishima, Y., & Iguchi, T. (2003). Molecular cloning of an anuran V(2) type [Arg(8)] vasotocin receptor and mesotocin receptor: functional characterization and tissue expression in the Japanese tree frog (*Hyla japonica*). *Gen. Comp. Endocrinol., 132*, 485–498.

Koyama, T., Haraguchi, S., Vaudry, H., & Tsutsui, K. (2009). Diurnal changes in the synthesis of the neurosteroid 7alpha-hydroxypregnenolone stimulating locomotor activity in newts. *Ann. N. Y. Acad. Sci., 1163*, 444–447.

Kreitzer, A. C., & Regehr, W. G. (2002). Retrograde signaling by endocannabinoids. *Curr. Opin. Neurobiol., 12*, 324–330.

Laberge, F., Muhlenbrock-Lenter, S., Grunwald, W., & Roth, G. (2006). Evolution of the amygdala: new insights from studies in amphibians. *Brain. Behav. Evol., 67*, 177–187.

Landys, M. M., Ramenofsky, M., & Wingfield, J. C. (2006). Actions of glucocorticoids at a seasonal baseline as compared to stress-related levels in the regulation of periodic life processes. *Gen. Comp. Endocrinol., 148*, 132–149.

Larcher, A., Delarue, C., Idres, S., & Vaudry, H. (1992). Interactions between vasotocin and other corticotropic factors on the frog adrenal gland. *J. Steroid. Biochem. Mol. Biol., 41*, 795–798.

Largen, W., & Woodley, S. K. (2008). Cutaneous tail glands, noxious skin secretions, and scent marking in a terrestrial salamander (*Plethodon shermani*). *Herpetologica, 64*, 270–280.

Leary, C. J., Garcia, A. M., & Knapp, R. (2006a). Elevated corticosterone levels elicit non-calling mating tactics in male toads independently of changes in circulating androgens. *Horm. Behav., 49*, 425–432.

Leary, C. J., Garcia, A. M., & Knapp, R. (2006b). Stress hormone is implicated in satellite-caller associations and sexual selection in the Great Plains toad. *Am. Nat., 168*, 431–440.

Leary, C. J., Garcia, A. M., & Knapp, R. (2008a). Density-dependent mating tactic expression is linked to stress hormone in Woodhouse's toad. *Behav. Ecol., 19*, 1103–1110.

Leary, C. J., Garcia, A. M., Knapp, R., & Hawkins, D. L. (2008b). Relationships among steroid hormone levels, vocal effort and body condition in an explosive-breeding toad. *Anim. Behav., 76*, 175–185.

Leary, C. J., Jessop, T. S., Garcia, A. M., & Knapp, R. (2004). Steroid hormone profiles and relative body condition of calling and satellite toads: implications for proximate regulation of behavior in anurans. *Behav. Ecol., 15*, 313–320.

Lewis, C. M., & Rose, J. D. (2003). Rapid corticosterone-induced impairment of amplectic clasping occurs in the spinal cord of roughskin newts (*Taricha granulosa*). *Horm. Behav., 43*, 93–98.

Licht, P. (1982). Endocrine patterns in the reproductive cycle of turtles. *Herpetologica, 38*, 51–61.

Licht, P. (1984). In G. E. Lamming (Ed.), *"Marshall's Physiology of Reproduction". Reptiles, Vol. 1* (pp. 206–282). Edinburgh: Churchill Livingstone.

Lofts, B. (1972). The Sertoli cell. *Gen. Comp. Endocrinol. Supplement., 3*, 636–648.

Lofts, B. (1984). In G. E. Lamming (Ed.), *"Marshall's Physiology of Reproduction". Amphibians, Vol. 1* (pp. 127–205). Edinburgh: Churchill Livingstone.

Lowry, C. A., & Moore, F. L. (1991). Corticotropin-releasing factor (CRF) antagonist suppresses stress-induced locomotor activity in an amphibian. *Horm. Behav., 25*, 84–96.

Lowry, C. A., & Moore, F. L. (2006). Regulation of behavioral responses by corticotropin-releasing factor. *Gen. Comp. Endocrinol., 146*, 19–27.

Lowry, C. A., Richardson, C. F., Zoeller, T. R., Miller, L. J., Muske, L. E., & Moore, F. L. (1997). Neuroanatomical distribution of vasotocin in a urodele amphibian (*Taricha granulosa*) revealed by immunohistochemical and *in situ* hybridization techniques. *J. Comp. Neurol., 385*, 43–70.

Lynch, K. S., & Wilczynski, W. (2005). Gonadal steroids vary with reproductive stage in a tropically breeding female anuran. *Gen. Comp. Endocrinol., 143*, 51–56.

Lynch, K. S., & Wilczynski, W. (2008). Reproductive hormones modify reception of species-typical communication signals in a female anuran. *Brain. Behav. Evol., 71*, 143–150.

Lynch, K. S., Crews, D., Ryan, M. J., & Wilczynski, W. (2006). Hormonal state influences aspects of female mate choice in the Tungara Frog (*Physalaemus pustulosus*). *Horm. Behav., 49*, 450–457.

Malacarne, G., & Giacoma, C. (1980). Effects of lesions to the rostral preoptic area on courtship behaviour in the male crested newt *Triturus cristatus carnifex* (Laur.). *Monitore. Zool. Ital., 14*, 9−17.

Marler, C. A., Boyd, S. K., & Wilczynski, W. (1999). Forebrain arginine vasotocin correlates of alternative mating strategies in cricket frogs. *Horm. Behav., 36*, 53−61.

Marler, C. A., Chu, J., & Wilczynski, W. (1995). Arginine vasotocin injection increases probability of calling in cricket frogs, but causes call changes characteristic of less aggressive males. *Horm. Behav., 29*, 554−570.

Marler, C. A., & Ryan, M. J. (1996). Energetic constraints and steroid hormone correlates of male calling behavior in the tungara frog. *J. Zool. Lond., 240*, 397−409.

Matsumoto, A., Arai, Y., Kouki, T., & Kikuyama, S. (1995). Comparison of Mauthner cell size in sexually developed and undeveloped male red-bellied newts. *J. Neurobiol., 28*, 126−132.

Matsunaga, M., Ukena, K., Baulieu, E. E., & Tsutsui, K. (2004). 7alpha-Hydroxypregnenolone acts as a neuronal activator to stimulate locomotor activity of breeding newts by means of the dopaminergic system. *Proc. Natl. Acad. Sci. U.S.A., 101*, 17282−17287.

Mazzi, V., & Vellano, C. (1987). Prolactin and reproduction. In D. O. Norris, & R. E. Jones (Eds.), *"Hormones and Reproduction in Fishes, Amphibians, and Reptiles"* (pp. 87−115). New York: Plenum Press.

Meccariello, R., Franzoni, M. F., Chianese, R., Cottone, E., Scarpa, D., Donna, D., Cobellis, G., Guastalla, A., Pierantoni, R., & Fasano, S. (2008). Interplay between the endocannabinoid system and GnRH-I in the forebrain of the anuran amphibian *Rana esculenta*. *Endocrinology, 149*, 2149−2158.

Mendonça, M. T., Licht, P., Ryan, M. J., & Barnes, R. (1985). Changes in hormone levels in relation to breeding behavior in male bullfrogs (*Rana catesbeiana*) at the individual and population levels. *Gen. Comp. Endocrinol., 58*, 270−279.

Mesner, P. W., Mahmoud, I. Y., & Cyrus, R. V. (2005). Seasonal testosterone levels in Leydig and Sertoli cells of the snapping turtle (*Chelydra serpentina*) in natural populations. *J. Exp. Zool., 266*, 266−276.

Miranda, J. A., & Wilczynski, W. (2009). Female reproductive state influences auditory midbrain response. *J. Comp. Physiol. A, 195*, 341−349.

Moore, F. L. (1978). Differential effects of testosterone plus dihydrotestosterone on male courtship of castrated newts, *Taricha granulosa*. *Horm. Behav., 11*, 202−208.

Moore, F. L., & Evans, S. J. (1999). Steroid hormones use non-genomic mechanisms to control brain functions and behaviors: a review of the evidence. *Brain. Behav. Evol., 54*, 41−50.

Moore, I. T., & Jessop, T. S. (2003). Stress, reproduction, and adrenocortical modulation in amphibians and reptiles. *Horm. Behav., 43*, 39−47.

Moore, F. L., & Miller, L. J. (1983). Arginine vasotocin induces sexual behavior of newts by acting on cells in the brain. *Peptides, 4*, 97−102.

Moore, F. L., & Miller, L. J. (1984). Stress-induced inhibition of sexual behavior: corticosterone inhibits courtship behavior of a male amphibian. *Horm. Behav., 18*, 400−410.

Moore, F. L., & Muller, C. H. (1977). Androgens and male mating behavior in rough-skinned newts, *Taricha granulosa*. *Horm. Behav., 9*, 309−320.

Moore, F. L., & Rose, J. D. (2002). In D. W. Pfaff, A. P. Arnold, A. Etgen, S. E. Fahrbach, & R. T. Rubin (Eds.), *"Hormones, Brain and Behavior". Sensorimotor processing model: how vasotocin and corticosterone interact and control reproductive behaviors in an amphibian, Vol. 2* (pp. 515−544). London: Academic Press, Elsevier Science.

Moore, F. L., & Zoeller, R. T. (1979a). Endocrine control of amphibian sexual behavior: evidence for a neuorhormone-androgen interaction. *Horm. Behav., 13*, 207−213.

Moore, F. L., & Zoeller, R. T. (1979b). Endocrine control of amphibian sexual behavior: evidence for a neurohormone-androgen interaction. *Horm. Behav., 13*, 207−213.

Moore, F. L., Boyd, S. K., & Kelley, D. B. (2005). Historical perspective: Hormonal regulation of behaviors in amphibians. *Horm. Behav., 48*, 373−383.

Moore, F. L., Lowry, C. A., & Rose, J. D. (1994). Steroid-neuropeptide interactions that control reproductive behaviors in an amphibian. *Psychoneuroendocrinology, 19*, 581−592.

Moore, F. L., Richardson, C., & Lowry, C. A. (2000). Sexual dimorphism in numbers of vasotocin-immunoreactive neurons in brain areas associated with reproductive behaviors in the roughskin newt. *Gen. Comp. Endocrinol., 117*, 281−298.

Moore, F. L., Roberts, J., & Bevers, J. (1984). Corticotropin-releasing factor (CRF) stimulates locomotor activity in intact and hypophysectomized newts (Amphibia). *J. Exp. Zool., 231*, 331−333.

Moore, F. L., Seide, R. L., Specker, J. L., & Swanson, L. (1978). Effects of prolactin and methallibure on second metamorphosis and plasma androgens in male newts, *Taricha granulosa*. *Comp. Biochem. Phys. A. Phys., 61*, 419−422.

Moore, F. L., Wood, R. E., & Boyd, S. K. (1992). Sex steroids and vasotocin interact in a female amphibian (*Taricha granulosa*) to elicit female-like egg-laying behavior or male-like courtship. *Horm. Behav., 26*, 156−166.

Narins, P. M., & Capranica, R. R. (1976). Sexual differences in the auditory system of the tree frog *Eleutherodactylus coqui*. *Science, 192*, 378−380.

Nelson, R. J. (2005). *"An Introduction to Behavioral Endocrinology."* New York: Sinauer Associates.

Norris, D. O. (2007). *"Vertebrate Endocrinology."* Burlington: Elsevier Academic Press.

Norris, D. O., Austin, H. B., & Hijazi, A. S. (1989). Induction of cloacal and dermal skin glands of tiger salamander larvae, (*Ambystoma tigrinum*): effects of testosterone and prolactin. *Gen. Comp. Endocrinol., 74*, 194−204.

Orchinik, M., Gasser, P. J., & Breuner, C. (2002). In D. W. Pfaff, A. P. Arnold, A. Etgen, S. E. Fahrbach, & R. T. Rubin (Eds.), *"Hormones, Brain and Behavior". Rapid corticosteroid actions on behavior, Vol. 3* (pp. 567−600). London: Academic Press, Elsevier Science.

Orchinik, M., Licht, P., & Crews, D. (1988). Plasma steroid concentrations change in response to sexual behavior in *Bufo marinus*. *Horm. Behav., 22*, 338−350.

Orchinik, M., Matthews, L., & Gasser, P. J. (2000). Distinct specificity for corticosteroid binding sites in amphibian cytosol, neuronal membranes, and plasma. *Gen. Comp. Endocrinol., 118*, 284−301.

Orchinik, M., Murray, T. F., & Moore, F. L. (1991). A corticosteroid receptor in neuronal membranes. *Science, 252*, 1848−1851.

Palka, Y. S., & Gorbman, A. (1973). Pituitary and testicular influenced sexual behavior in male frogs,. *Rana pipiens. Gen. Comp. Endocrinol., 21*, 148−151.

Park, D., & Propper, C. R. (2001). Repellent function of male pheromones in the red-spotted newt. *J. Exp. Zool., 289*, 404−408.

Pearl, C. A., Cervantes, M., Chan, M., Ho, U., Shoji, R., & Thomas, E. O. (2000). Evidence for a mate-attracting chemosignal in the dwarf African clawed frog *Hymenochirus*. *Horm. Behav., 38*, 67−74.

Potter, K. A., Bose, T., & Yamaguchi, A. (2005). Androgen-induced vocal transformation in adult female African clawed frogs. *J. Neurophysiol., 94*, 415−428.

Propper, C. R. (1991). Courtship in the rough-skinned newt *Taricha granulosa*. *Anim. Behav., 41*, 547−554.

Propper, C. R., & Dixon, T. B. (1997). Differential effects of arginine vasotocin and gonadotropin-releasing hormone on sexual behaviors in an anuran amphibian. *Horm. Behav., 32*, 99−104.

Propper, C. R., & Moore, F. L. (1991). Effects of courtship on brain gonadotropin hormone-releasing hormone and plasma steroid concentrations in a female amphibian (*Taricha granulosa*). *Gen. Comp. Endocrinol., 81*, 304−312.

Rollmann, S. M., Houck, L. D., & Feldhoff, R. C. (1999). Proteinaceous pheromone affecting female receptivity in a terrestrial salamander. *Science, 285*, 1907−1909.

Romero, L. M. (2002). Seasonal changes in plasma glucocorticoid concentrations in free-living vertebrates. *Gen. Comp. Endocrinol., 128*, 1−24.

Romero, L. M., & Reed, J. M. (2005). Collecting baseline corticosterone samples in the field: is under 3 min good enough? *Comp. Biochem. Physiol. A., 140*, 73−79.

Rose, J. D. (2000). Corticosteroid actions from neuronal membrane to behavior: neurophysiological mechanisms underlying rapid behavioral effects of corticosterone. *Biochem. Cell. Biol., 78*, 307−315.

Rose, J. D., & Moore, F. L. (2002). Behavioral neuroendocrinology of vasotocin and vasopressin and the sensorimotor processing hypothesis. *Front. Neuroendocrinol., 23*, 317−341.

Rose, J. D., Kinnaird, J. R., & Moore, F. L. (1995). Neurophysiological effects of vasotocin and corticosterone on medullary neurons: implications for hormonal control of amphibian courtship behavior. *Neuroendocrinology, 62*, 406−417.

Rose, J. D., Marrs, G. S., & Moore, F. L. (1998). Rapid, corticosterone-induced disruption of medullary sensorimotor integration related to suppression of amplectic clasping in behaving roughskin newts (*Taricha granulosa*). *Horm. Behav., 34*, 268−282.

Rugh, R. (1935). Ovulation in the frog. I. Pituitary relations in induced ovulation. *J. Exp. Zool., 71*, 149.

Rugh, R. (1962). *"Experimental embryology techniques and procedures."* Minnesota: Burgess, Minneapolis.

Ryan, M. J. (1985). *"The Tungara Frog: a study in sexual selection and communication."*. Chicago: University of Chicago Press.

San Mauro, D., Vences, M., Alcobendas, M., Zardoya, R., & Meyer, A. (2005). Initial diversification of living amphibians predated the breakup of Pangaea. *Am. Nat., 165*, 590−599.

Sanderson, N. S., Le, B., Zhou, Z., & Crews, D. (2008). Preoptic neuronal nitric oxide synthase induction by testosterone is consistent with a role in gating male copulatory behavior. *Eur. J. Neurosci., 27*, 183−190.

Sapolsky, R. M. (2002). Endocrinology of the stress response. In J. B. Becker, S. M. Breedlove, D. Crews, & M. M. McCarthy (Eds.), *Behavioral Endocrinology* (pp. 409−450). Cambridge, MA: MIT Press.

Schmidt, R. S. (1985). Prostaglandin-induced mating call phonotaxis in female American toad: facilitation by progesterone and arginine vasotocin. *J. Comp. Physiol. A., 156*, 823−829.

Schmidt, A., & Roth, G. (1990). Central olfactory and vomeronasal pathways in salamanders. *J. Hirnforsch., 31*, 543−553.

Schubert, S. N., Houck, L. D., Feldhoff, P. W., Feldhoff, R. C., & Woodley, S. K. (2006). Effects of androgens on behavioral and vomeronasal responses to chemosensory cues in male terrestrial salamanders (*Plethodon shermani*). *Horm. Behav., 50*, 469−476.

Schubert, S. N., Houck, L. D., Feldhoff, P. W., Feldhoff, R. C., & Woodley, S. K. (2008). The effects of sex on chemosensory communication in a terrestrial salamander (*Plethodon shermani*). *Horm. Behav., 54*, 270−277.

Schubert, S. N., Wack, C. L., Houck, L. D., Feldhoff, P. W., Feldhoff, R. C., & Woodley, S. K. (2009). Exposure to pheromones increases plasma corticosterone concentrations in a terrestrial salamander. *Gen. Comp. Endocrinol., 161*, 270−275.

Semsar, K., Klomberg, K. F., & Marler, C. (1998). Arginine vasotocin increases calling-site acquisition by nonresident male grey treefrogs. *Anim. Behav., 56*, 983−987.

Sever, D. M. (2002). Female sperm storage in amphibians. *J. Exp. Zool., 292*, 165−179.

Soderstrom, K., Leid, M., Moore, F. L., & Murray, T. F. (2000). Behavioral, pharmacological, and molecular characterization of an amphibian cannabinoid receptor. *J. Neurochem., 75*, 413−423.

Specker, J. L., & Moore, F. L. (1980). Annual cycle of plasma androgens and testicular composition in the rough-skinned newt, *Taricha granulosa*. *Gen. Comp. Endocrinol., 42*, 297−303.

Stebbins, R. C., & Cohen, N. W. (1995). *"A natural history of amphibians."*. Princeton, NJ: Princeton University Press.

Sullivan, B. K., Ryan, M. J., & Verrell, P. A. (1995). Female choice and mating system structure. In H. Heatwole, & B. K. Sullivan (Eds.), *"Amphibian Biology", Vol. 2* (pp. 470−517). Chipping Norton, Australia, New South Wales, Australia: Surrey Beatty & Sons.

Taigen, T. L., & Wells, K. D. (1985). Energetics of vocalization by an anuran amphibian (*Hyla versicolor*). *J. Comp. Physiol. B., 155*, 163−170.

Tanaka, S., & Takikawa, H. (1983). Seasonal changes in plasma testosterone and 5 alpha dihydrotestosterone levels in the adult male newt, *Cynops pyrrhogaster pyrrhogaster. Endocrinol. Japan, 30*, 1−6.

Taylor, J. A., & Boyd, S. K. (1991). Thyrotropin-releasing hormone facilitates display of reproductive behavior and locomotor behavior in an amphibian. *Horm. Behav., 25*, 128−136.

Ten Eyck, G. R. (2005). Arginine vasotocin activates advertisement calling and movement in the territorial Puerto Rican frog, *Eleutherodactylus coqui*. *Horm. Behav., 47*, 223−229.

Thompson, R. R., & Moore, F. L. (2000). Vasotocin stimulates appetitive responses to the visual and pheromonal stimuli used by male roughskin newts during courtship. *Horm. Behav., 38*, 75−85.

Thompson, R. R., & Moore, F. L. (2003). The effects of sex steroids and vasotocin on behavioral responses to visual and olfactory sexual stimuli in ovariectomized female roughskin newts. *Horm. Behav., 44*, 311−318.

Thompson, R. R., Dickinson, P. S., Rose, J. D., Dakin, K. A., Civiello, G. M., Segerdahl, A., et al. (2008). Pheromones enhance

somatosensory processing in newt brains through a vasotocin-dependent mechanism. *Proc. Biol. Sci., 275*, 1685−1693.

Tito, M. B., Hoover, M. A., Mingo, A. M., & Boyd, S. K. (1999). Vasotocin maintains multiple call types in the gray treefrog, *Hyla versicolor. Horm. Behav., 36*, 166−175.

Tobias, M. L., Viswanathan, S. S., & Kelley, D. B. (1998). Rapping, a female receptive call, initiates male-female duets in the South African clawed frog. *Proc. Natl. Acad. Sci. U.S.A., 95*, 1870−1875.

Toyoda, F., & Kikuyama, S. (2000). Hormonal influence on the olfactory response to a female-attracting pheromone, sodefrin, in the newt, *Cynops pyrrhogaster. Comp. Biochem. Phys. B., 126*, 239−245.

Toyoda, F., Hasunuma, I., Yamamoto, K., Yamashita, M., & Kikuyama, S. (2005). Prolactin acts centrally to enhance newt courtship behavior. *Gen. Comp. Endocrinol., 141*, 172−177.

Toyoda, F., Ito, M., Tanaka, S., & Kikuyama, S. (1993). Hormonal induction of male courtship behavior in the Japanese newt, *Cynops pyrrhogaste*r. *Horm. Behav., 27*, 511−522.

Toyoda, F., Matsuda, K., Yamamoto, K., & Kikuyama, S. (1996). Involvement of endogenous prolactin in the expression of courtship behavior in the newt, *Cynops pyrrhogaster. Gen. Comp. Endocrinol., 102*, 191−196.

Toyoda, F., Tanaka, S., Matsuda, K., & Kikuyama, S. (1994). Hormonal control of response to and secretion of sex attractants in Japanese newts. *Physiol. Behav., 55*, 569−576.

Toyoda, F., Yamamoto, K., Ito, Y., Tanaka, S., Yamashita, M., & Kikuyama, S. (2003). Involvement of arginine vasotocin in reproductive events in the male newt *Cynops pyrrhogaster. Horm. Behav., 44*, 346−353.

Toyoda, F., Yamamoto, K., Iwata, T., Hasunuma, I., Cardinali, M., Mosconi, G., Polzonetti-Magni, A. M., & Kikuyama, S. (2004). Peptide pheromones in newts. *Peptides, 25*, 1531−1536.

Trainor, B. C., Rouse, K. L., & Marler, C. A. (2003). Arginine vasotocin interacts with the social environment to regulate advertisement calling in the gray treefrog (*Hyla versicolor*). *Brain. Behav. Evol., 61*, 165−171.

Tso, E., & Lofts, B. (1977). Seasonal changes in the newt, *Trituroides hongkonggensis*, Testis. I. A histological and histochemical study. *Acta. Zool., 58*, 1−8.

Wabnitz, P. A., Bowie, J. H., Tyler, M. J., Wallace, J. C., & Smith, B. P. (1999). Aquatic sex pheromone from a male tree frog. *Nature, 401*, 444−445.

Wabnitz, P. A., Bowie, J. H., Tyler, M. J., Wallace, J. C., & Smith, B. P. (2000). Differences in the skin peptides of the male and female Australian tree frog *Litoria splendida*. *Eur. J. Biochem., 267*, 269−275.

Wada, M., & Gorbman, A. (1977). Relation of mode of administration of testosterone to evocation of male sexual behavior in frogs. *Horm. Behav., 8*, 310−319.

Watson, J. T., & Kelley, D. B. (1992). Testicular masculinization of vocal behavior in juvenile female *Xenopus laevis* reveals sensitive periods for song duration, rate, and frequency spectra. *J. Comp. Physiol. [A], 171*, 343−350.

Wells, K. D. (1977). The social behaviour of anuran amphibians. *Anim. Behav., 25*, 666−693.

Wells, K. D. (2007). *"The ecology and behavior of amphibians."* Chicago: The University of Chicago Press.

Wetzel, D. M., & Kelley, D. B. (1983). Androgen and gonadotropin effects on male mate calls in South African Clawed Frogs, *Xenopus laevis*. *Horm. Behav., 17*, 388−404.

Wilczynsk, W., & Chu, J. (2001). Acoustic communication, endocrine control, and the neurochemical systems of the brain. In M. J. Ryan (Ed.), *Anuran communication* (pp. 23−35). Washington: Smithsonian Institution Press.

Wilczynski, W., & Endepols, H. (2007). Central auditory pathways in anuran amphibians: the anatomical basis of hearing and sound communication. In P. M. Narins, A. S. Feng, R. R. Fay, & A. N. Popper (Eds.), *"Hearing and sound communication in amphibians"* (pp. 221−249). New York: Springer.

Wilczynski, W., Lynch, K. S., & O'Bryant, E. L. (2005). Current research in amphibians: studies integrating endocrinology, behavior, and neurobiology. *Horm. Behav., 48*, 440−450.

Wilczynski, W., Yang, E. J., & Simmons, D. (2003). Sex differences and hormone influences on tyrosine hydroxylase immunoreactive cells in the leopard frog. *J. Neurobiol., 56*, 54−65.

Will, U. (1991). Amphibian Mauthner cells. *Brain. Behav. Evol., 37*, 317−332.

Wingfield, J. C., & Silverin, B. (1986). Effects of corticosterone on territorial behavior of free-living male song sparrows *Melospiza melodia*. *Horm. Behav., 20*, 405−417.

Wingfield, J., Hahn, T. P., Levin, R. N., & Honey, P. (1992). Environmental predictability and control of gonadal cycles in birds. *J. Exp. Zool., 261*, 214−231.

Wirsig-Wiechmann, C. R., Houck, L. D., Feldhoff, P. W., & Feldhoff, R. C. (2002). Pheromonal activation of vomeronasal neurons in plethodontid salamanders. *Brain. Res., 952*, 335−344.

Woodley, S. K. (1994). Plasma androgen levels, spermatogenesis, and secondary sexual characteristics in two species of plethodontid salamanders with dissociated reproductive patterns. *Gen. Comp. Endocrinol., 96*, 206−214.

Woodley, S. K. (2007). Sex steroid hormones and sexual dimorphism of chemosensory structures in a terrestrial salamander (*Plethodon shermani*). *Brain. Res., 1138*, 95−103.

Woolley, S. C., Sakata, J. T., & Crews, D. (2004). Evolutionary insights into the regulation of courtship behavior in male amphibians and reptiles. *Physiol. Behav., 83*, 347−360.

Yamaguchi, A., & Kelley, D. B. (2003). In A. M. Simmons, A. N. Popper, & R. R. Fay (Eds.), *"Handbook of Auditory Research". Hormonal mechanisms in acoustic communication, Vol. 16* (pp. 275−323). New York: Springer Verlag, Acoustic Communication.

Yamamoto, K., Toyoda, F., Tanaka, S., Hayashi, H., & Kikuyama, S. (1996). Radioimmunoassay of a newt sex pheromone, sodefrin, and the influence of hormones on its level in the abdominal gland. *Gen. Comp. Endocrinol., 104*, 356−363.

Yang, E. J., Nasipak, B. T., & Kelley, D. B. (2007). Direct action of gonadotropin in brain integrates behavioral and reproductive functions. *Proc. Natl. Acad. Sci. U.S.A., 104*, 2477−2482.

Yao, M., Hu, F., & Denver, R. J. (2008). Distribution and corticosteroid regulation of glucocorticoid receptor in the brain of *Xenopus laevis*. *J. Comp. Neurol., 508*, 967−982.

Yazawa, T., Yamamoto, K., Kikuyama, S., & Abe, S. I. (1999). Elevation of plasma prolactin concentrations by low temperature is the cause of spermatogonial cell death in the newt, *Cynops pyrrhogaster. Gen. Comp. Endocrinol., 113*, 302−311.

Zerani, M., & Gobbetti, A. (1993). Corticosterone during the annual reproductive cycle and in sexual behavior in the crested newt, *Triturus cristatus*. *Horm. Behav., 27*, 29−37.

Zerani, M., & Gobbetti, A. (1996). NO sexual behaviour in newts. *Nature, 382*, 31.

Zerani, M., Vellano, C., Amabili, F., Carnevali, O., Andreoletti, G. E., & Polzonetti-Magni, A. (1991). Sex steroid profile and plasma vitellogenin during the annual reproductive cycle of the crested newt (*Triturus carnifex* Laur.). *Gen. Comp. Endocrinol., 82*, 337−344.

Zoeller, R. T., & Moore, F. L. (1986). Correlation between immunoreactive vasotocin in optic tectum and seasonal changes in reproductive behaviors of male rough-skinned newts. *Horm. Behav., 20*, 148−154.

Zoeller, R. T., & Moore, F. L. (1988). Brain arginine vasotocin concentrations related to sexual behaviors and hydromineral balance in an amphibian. *Horm. Behav., 22*, 66−75.

Zornik, E., & Yamaguchi, A. (2008). Sexually differentiated central pattern generators in *Xenopus laevis*. *Trends. Neurosci., 31*, 296−302.

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Chapter 9

Hormones and Reproductive Cycles in Anuran Amphibians

Rakesh K. Rastogi,* Claudia Pinelli,† Gianluca Polese,* Biagio D'Aniello,* and Gabriella Chieffi-Baccari†

* *University of Naples Federico II, Napoli, Italy,* † *Second University of Naples, Caserta, Italy*

SUMMARY

Anuran amphibians display a variety of reproductive modes including, in one genus, a non-placental viviparous species. In frogs and toads, reproduction is hormonally controlled and, in most species, environmentally mediated. The reproductive cycle includes several components: oogenesis and spermatogenesis, including cell maturation and vitellogenesis; courtship and mating; spermiation and oviposition; and the timing of these events in terms of seasonality or its absence. These topics are briefly summarized. In many species, breeding is synchronized within the population, whereas in others it is not. Some species of anuran have a predictable breeding season, whereas others are more flexible and some breed opportunistically in response to the unpredictable humidity availability. Temperature is the principal environmental cue used to time and tune the pre-breeding stages of the reproductive cycle, allowing anurans to adapt their physiology in advance of predictable environmental changes. Endocrine and neuroendocrine plasticity allows non-thermal cues (photoperiod, food availability) to modulate timing to enable individuals to reproduce. Circumstantial evidence indicates that GnRH from the hypothalamus exerts its regulatory functions on the pituitary gonadotropins via blood circulation. The gonadotropins are responsible for the seasonal and/or annual developmental changes in gametogenesis and in the production of sex steroids that drive reproductive organ function, culminating in reproduction. There are many areas of uncertainty, and future integrated studies are required.

1. INTRODUCTION

The hormonally and environmentally monitored reproductive cycle, male or female, is part of one overwhelming impulse: to continue to be.

The anuran fauna is extremely complex in terms of biogeography, ecology, and life history patterns. Reproduction in frogs and toads is controlled by a suite of endogenous and environmental factors, which appear to interact in a complex synergistic fashion. Depending on the species, various seasonally variable environmental factors, including temperature, photoperiod, and food availability, may be important in regulating the different stages of gametogenesis (proliferation, growth, and differentiation) and breeding. Because amphibians are poikilotherms (cold-blooded), the most important regulator of anuran reproduction is environmental temperature. Photoperiod appears to play a role in some species living in temperate areas, but only if temperature is 'permissive' or adequate to sustain reproductive activity. Another important environmental trigger for reproduction in many species, particularly those inhabiting desertic or semidesertic geographical areas, is seasonal rainfall, which creates high humidity and a favorable spawning habitat.

Like most vertebrates, reproductive function in anuran amphibians depends on interactions between the hypothalamus, adenohypophysis, and gonads that are mediated by neural pathways operating across a classic endocrine circuitry. Within this classic system, the synthesis and release of hormones involves complex stimulatory and inhibitory pathways and diverse receptor-mediated actions of these hormones on their target tissues. Reproductive function is also mediated by important autocrine, paracrine, and juxtacrine mechanisms acting at a local level. Gonadotropic hormones are essential for promoting gonadal growth, gamete formation, and their release at the appropriate times. Some instances of ovarian and/or testicular inactivity are associated with an apparent lack of gonadotropin stimulation, and local regulators of gonadal function are probably involved. It is not well known whether nutritional, genetic, and other influences act at the brain-pituitary level to change gonadotropin secretion; however, changes in sensitivity to gonadotropins at the gonadal level may be of great physiological significance; researchers have investigated the phenomenon of post-reproductive gonadal refractoriness in some species of vertebrate, including anurans.

A detailed picture of the endocrine mediation of gonadal activity as well as of the secondary sexual characteristics in the male and of the oviduct structure and function in the female is available for only a few species. As a consequence, generalizations are usually extended to many species in which only a part of the picture has been explored. The sex steroids are synthesized most notably in the gonads and function as hormones to control or influence every aspect of reproduction.

There is information available on the reproductive cycles of a large number of frogs and toads, but hormonal data are available for only a few species. Following the reviews of pertinent data in the 1970s (Lofts, 1974) and 1980s (Lofts, 1984), almost two decades elapsed before another attempt was made to put together the available literature on amphibian reproduction (Rastogi et al., 2005). For editorial reasons this last review was updated to the works published until 2001. Since then some progress has been made as to the molecular mechanisms involved in the regulation of reproductive cycles. Some new species have been added to the repertoire, and greater insight has been gained into the tumultuous panorama of the hormonal and environmental modulation of anuran reproduction. This paper reviews information on reproductive cycles and their endocrine and exocrine control and constraints. For citations prior to the 1980s, with a few exceptions, the reader is referred to a recent review (Rastogi et al., 2005).

2. REPRODUCTIVE STRATEGIES

Amphibians exhibit an amazing variety of reproductive strategies related most probably to the complex and often unpredictable egg/larval environment. Anuran amphibians have a wide diversity of reproductive modes, but external aquatic fertilization without parental care is the ancestral and most widespread strategy, particularly evident in frogs and toads inhabiting temperate regions. Indeed, the known temperate anurans are all oviparous and all breed in water, lay their eggs there, and have free-living larvae. Although most of these species have usually monogamic mating, polyandrous mating, in which several males attempt to mate simultaneously with a female, is rare in temperate species (described later), but is not uncommon in tropical and subtropical species (Bagnara, Iela, Morrisett, & Rastogi, 1986; Byrne & Roberts, 1999).

2.1. Seasonality

Most frogs and toads reproduce with a discrete and predictable annual cycle of gametogenesis and breeding. The interval of time between two reproductive seasons in sequence roughly represents the length of reproductive cycle of a species. Most anurans, including all temperate and many tropical species, breed seasonally once a year. In many species, in fact, annual cyclic changes in the histological composition and weight of the ovary are consistent with the fact that they undergo a single breeding period each year. The female gonads of the seasonal spawners increase conspicuously in weight as ovarian follicles grow and are filled with yolk, followed by a sharp reduction in weight due to gamete release. Thus, the reproductive cycle of most annual breeding frogs and toads can be followed by calculating the female gonadosomatic index (GSI; gonad weight $\div$ body weight $\times$ 100). Each reproductive cycle within a species is nearly identical to all the others, but not quite.

2.2. Oviparity/Viviparity

Among frogs and toads, the majority are oviparous, some are ovoviviparous (lecithotrophic mode of viviparity: growing embryos and larvae feed exclusively upon the yolk contained in the egg, which is retained within the oviduct or in some 'ectopic site' such as the vocal sac or a specialized venue inside the stomach), and only one species yet known is truly viviparous (see Rastogi et al., 2005). *Nimbaphrynoides* (*Nectophrynoides*) *occidentalis*, (Western Nimba or Mount Nimba viviparous toad), has internal fertilization and provides maternal nutrition to the oviductally developing young by epithelial secretions (see Wake, 1980). It is now classified as an endangered species in the International Union for Conservation of Nature (IUCN) Red List 2004 and hence we might never obtain knowledge of its reproductive endocrinology. Strikingly, this same genus includes species such as *Nimbaphrynoides osgoodi*, which is oviparous with free-living larvae; *N. malcolmi*, with direct development; and *N. viviparous* and *N. torineri*, which are ovoviviparous (lecithotrophic live-bearing toads).

Oviparity in some anurans differs from oviparity in many other frogs and toads in that a few eggs of a large size are laid and taken care of by one of the parents. Indeed, in a terrestrial egg-laying frog (Townsend, Stewart, & Pough, 1984) and in an arboreal breeding frog (Chen, Yu, & Kam, 2007), parental attendance during embryonic development is performed exclusively by males.

2.3. Oviposition/Nesting Site

In contrast with temperate species, in tropical and semitropical regions, anurans show a tendency to lay eggs away from the aquatic environments that are richly populated by a high diversity of predators. Many species lay eggs on leaves above water (commonly called leaf frogs), some in foam nests, some in burrows at the edge of water, and some in tree holes or bromeliads where rain water creates miniature pools (Duellman & Trueb, 1986; Caldwell, 1992). Of these frog species, only for one species, the Mexican leaf

frog, *Pachymedusa* (*Agalychnis*) *dacnicolor*, do we have data as to the environmental and endocrine correlates of its reproductive biology (Bagnara et al., 1986; Bagnara & Rastogi, 1992). Recently, reproductive mode plasticity has been observed in a treefrog, *Dendropsophus ebraccatus*, which lays most of its egg masses aquatically and can also lay eggs terrestrially, on vegetation over water, all during a single night (Touchon & Warkentin, 2008).

2.4. Mating Systems

From a behavioral point of view, polyandry has recently been described in detail as a tool to insure against nest failure. This is a form of sequential polyandry. In the Australian toadlet, *Pseudophryne bibronii*, males construct soil depressions as terrestrial nests for egg-laying; the females of this species are highly promiscuous and each female of the population mates with multiple males and lays the eggs in different nests, each guarded by a male with which the female has mated (Byrne & Keogh, 2009). These authors made, for the first time, a genetic analysis of the parentage. Based on behavioral observations in the African leaf-folding frog, *Afrixalus delicatus* (Backwell & Passmore, 1990), the European water frog, *Rana esculenta* (Reyer, Frei, & Som, 1999), and the Asian fanged frog, *Rana* (*Limnonectes*) *kuhlii* (Tsuji & Lue, 2000), it has been suggested that sequential polyandry is likely to occur. In these species, however, genetic paternity analyses should be conducted to confirm the occurrence of sequential polyandry.

2.5. Clutch Size

Some species lay all their eggs at one time, whereas in some other species eggs can be laid in several clutches during the reproductive season with many individual females breeding several times during the season (Rastogi et al., 1983; Bagnara et al., 1986; Reyer et al., 1999). In some cases a reduced oviposition during one breeding season may be followed in the next breeding by an increased number of eggs being deposited (Rastogi et al., 1983; Bagnara et al., 1986; Tejedo, 1992). In such species, a positive correlation was observed between potential clutch size and female body weight. This implies that bigger females lay bigger egg masses. Food availability may also be an important factor in this context.

3. MALE REPRODUCTIVE CYCLE

3.1. Testicular Cycles

There is no generalized male reproductive cycle valid for all anurans studied so far. It is based specifically on gonadal activity (spermatogenesis, steroidogenesis, and spermiation) during the solar year or across seasons, and hence it is often called the annual or seasonal testicular cycle. Spermatogenesis is a dynamic, synchronized process, taking place in the seminiferous tubules of the testis, whereby spermatogonial stem cells proliferate and differentiate to produce mature haploid germ cells. This complex process is dependent upon pituitary gonadotropins and androgens produced by Leydig cells in the interstitium, as well as upon paracrine-acting factors produced by supporting Sertoli cells intimately associated with the spermatogenetic cysts (see Rastogi et al., 2005). Some anuran species may display a discontinuous type of spermatogenetic cycle during the year in which spermatogenesis is either impaired or completely interrupted during a part of the year when the harsh weather sets in, or sometimes even in summer (Figures 9.1(a) and 9.2). The testis may be characterized by the presence of germinal cysts with degenerating cells during the period of stasis; e.g., *Bufo japonicus* (Itoh, Inoue, & Ishii, 1990), *Leptodactylus chaquensis* (Cei et al., 1996), *P. dacnicolor* (Rastogi, Iela, Delrio, & Bagnara, 1986; Bagnara & Rastogi, 1992), *Rana dalmatina* (Guarino & Bellini, 1993), and *Rana italica* (Guarino et al., 1993). It is in the period of seasonal stasis that the testis becomes refractory to any of the stimulatory factors, hormonal or environmental; however, to our knowledge, only two anuran species so far investigated possess a discontinuous spermatogenetic cycle *sensu stricto*. They are the European red frog, *Rana temporaria* (van Oordt, 1960) and the large four-eyed frog from austral Argentina, *Pleurodema bufonia* (Cei, 1980). It has not been possible to stimulate spermatogenesis in these two species by any means during the seasonal testicular quiescence. Nevertheless, the beginning of active spermatogenesis in these species may vary in nature among localities with different seasonal profiles of the mean environmental temperature.

The continuous type of spermatogenetic cycle (Figure 9.1 (b)), instead, is characterized by the presence of different stages of spermatogenesis throughout the year, whether or not reproduction is seasonal (e.g., midwife toad, *Alytes obstetricans* (Crespo, 1982); tropical ranid, *Amolops larutensis* (Emerson & Hess, 1996); Creole frog, *Leptodactylus ocellatus* (Vivas, Nicora, Di Tada, & Ibanez, 1995); common India treefrog, *Polypedates maculatus* (Kanamadi & Jirankali, 1992); North American bullfrog, *Rana catesbeiana* (Yoneyama & Iwasawa, 1985); Chinese or Thai bullfrog, *Rana rugulosa* (Kao, Alexander, Yang, & Yu, 1993)). However, there may occur some degree of seasonal variation in the relative frequency of spermatogenetic cysts containing meiotic and/or spermiogenic stages. In such species, there is more or less a continuous supply of male gametes for much of the year.

A third type of spermatogenetic cycle, occurring in some anuran species, is defined as the potentially continuous type (Figure 9.1(c)). The European common green or

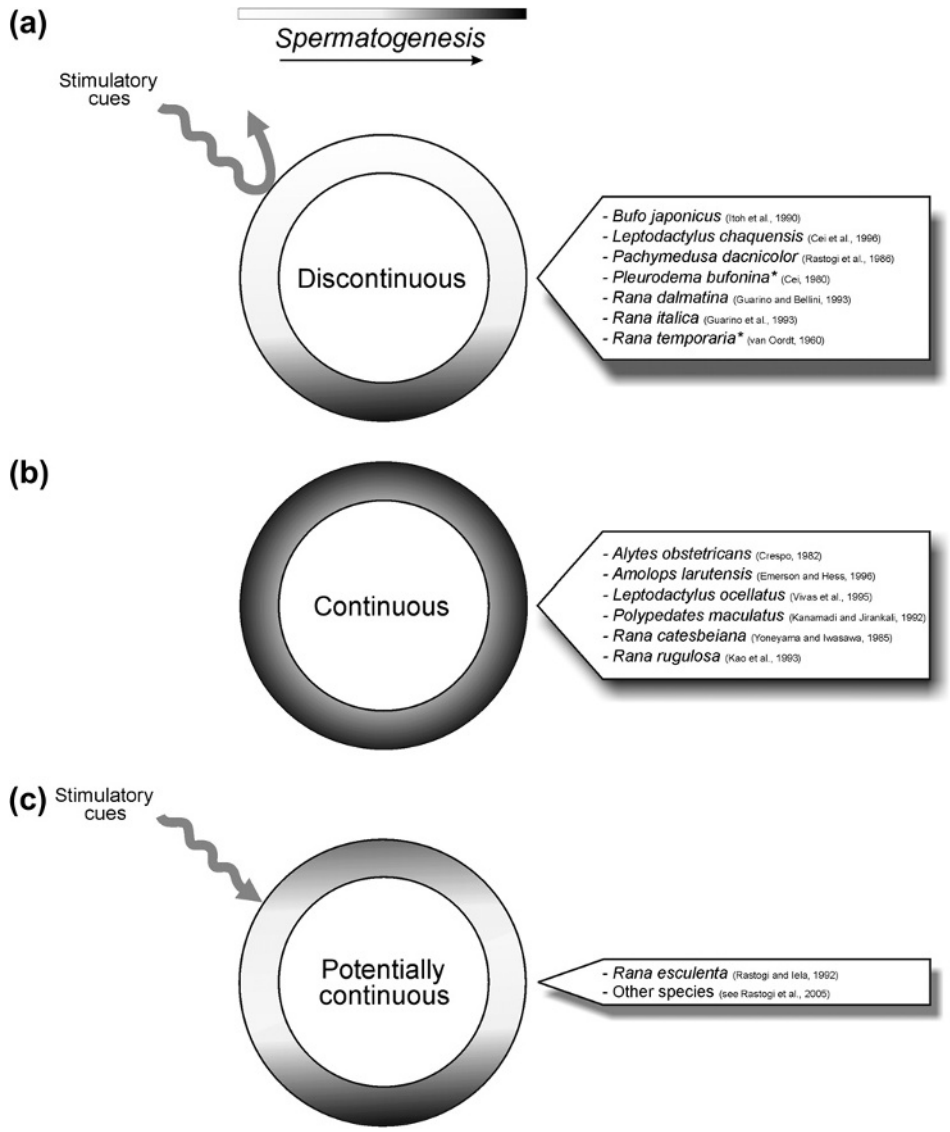

FIGURE 9.1 Generalized diagram of the three types of spermatogenetic cycle displayed in anuran species. In 'stimulatory cues' are included some endogenous factors such as hormones and environmental factors such as temperature, humidity, photoperiod, food availability, and rainfall. For each type of spermatogenetic cycle (A, B, C), examples are reported in the label on the right. * Discontinuous *sensu stricto*: spermatogenesis cannot be stimulated by any means during the seasonal quiescence.

edible frog, *R. esculenta*, is a well-known example (Rastogi, 1976; Rastogi & Iela, 1992). Males are strictly seasonal, with moderate to strong suppression of spermatogenesis during the cold months of the year when massive degeneration of meiotic cells, primary spermatocytes in particular, may occur. There are several other species of anuran, from different continents, in which this type of spermatogenetic cycle has been described (see Rastogi et al., 2005).

The differences between the discontinuous and the potentially continuous types of spermatogenetic cycles are based upon the total refractoriness, in the former, of the spermatogenetic tissue to stimulatory cues, both intrinsic and extrinsic, while in the latter the spermatogenetic tissue can be stimulated at any time in winter by appropriate thermal and hormonal input. A recent study on a professionally raised colony of *R. catesbeiana* in Brazil clarified that the males show a continuous type of spermatogenetic cycle and the testicular weight index does not change significantly during the year (Sasso-Cerri, De Faria, Freymüller, & Miraglia, 2004). In line with many earlier studies, a recent work on the seasonal changes in the testis and thumb pads of the male *Rana ridibunda* from the East Marmara region of Turkey confirmed the existence of a seasonal cycle (Kaptan & Murathanoğlu, 2008). *Pseudis limellum*, a hylid from Brazil, is a continuous breeder

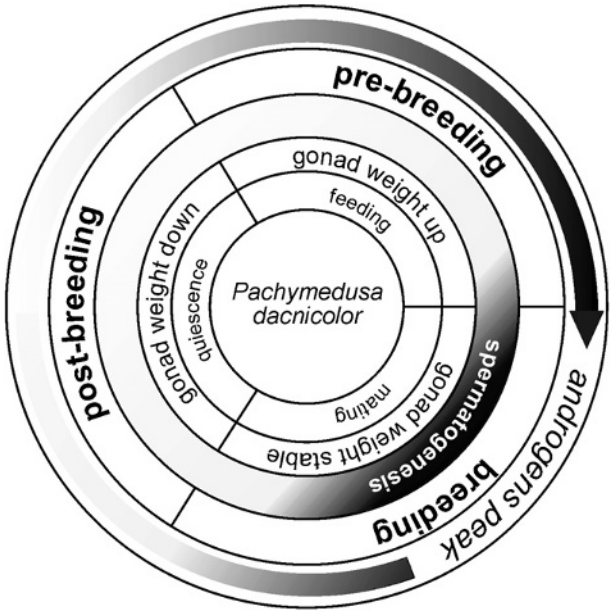

FIGURE 9.2 Example of discontinuous spermatogenetic cycle displayed by males of *Pachymedusa dacnicolor*. Black color indicates the highest hormones level/activity and white the lowest.

(Ferreira, Mehanna, & Prado, 2008). There are species in which the testicular and breeding cycles correspond (*P. dacnicolor* (Rastogi et al., 1986); Figure 9.2), species in which the two occur at different times of the year (*R. italica* (Guarino et al., 1993)), and species intermediate between these two types (*Rana perezi* (Delgado, Gutiérrez, & Alonso-Bedate, 1989)). In many species, the testis mass or the gonadosomatic index remains more or less uniform throughout the year (see Rastogi, Iela, Saxena, & Chieffi, 1976; Rastogi et al., 2005). In such species, although breeding is seasonal, different stages of spermatogenesis can be observed throughout the year (one exception is *P. maculatus* from South India, in which seasonal testicular mass changes are rather conspicuous (Kanamadi & Jirankali, 1992)). In species with a discontinuous type of testis cycle, testis weight change is a reliable index of the seasonal reproductive status, with the annual maximal testicular weight occurring at the onset of the breeding season and a drastic decline observed immediately after breeding, when all the seminiferous tubules are almost completely emptied (e.g., *P. dacnicolor* (Rastogi et al., 1986); Figure 9.2). After breeding, in species with a discontinuous cycle, there is a short refractory period and then testicular recrudescence begins and the spermatogenetic cycle resumes.

3.2. Hormones

Males with well-developed callosities or other secondary sexual characteristics usually have higher androgen concentrations than males with regressed callosities. Such males are ready for breeding. Most secondary sexual characteristics exhibit seasonal modifications and reach their maximal development during the breeding season; the seasonal trend of regression and recrudescence of secondary sexual characteristics is usually correlated with fluctuations in circulating levels of androgens (Di Fiore et al., 2005; Rastogi et al., 2005). Thus, the causal role of high androgen titers in eliciting and modulating male behavioral activity during the breeding season is widely accepted. The seasonal cycle of the circulating and testicular levels of sex steroids, mainly androgens, may be dissociated with the seasonal testis weight cycle or even the seasonal spermatogenetic cycle (e.g., *B. japonicus*, *R. italica*, *R. esculenta* (Itoh et al., 1990; Guarino et al., 1993; Rastogi et al., 2005)). Of these only *R. esculenta* has a potentially continuous type of spermatogenetic cycle. A close seasonal association between testis weight, spermatogenetic activity, and plasma concentration of androgens is found in frogs such as *P. dacnicolor* (Rastogi et al., 1986) with a discontinuous type of spermatogenetic cycle (Figure 9.2).

The blood titers of androgens vary greatly, not only during a seasonal reproductive cycle within the same species but also between different species. Published information on plasma levels of testosterone (T) in anurans presents an enormous range of values: from species such as *Bufo mauritanicus*, in which maximal levels are as high as 595 ng/ml in amplecting males (against a low in winter of 4.4 ng/ml), to species in which maximal levels at the peak of the seasonal cycle are a little less than 1 ng/ml (*Dicroglossus occipitalis*) (see Rastogi et al., 2005). The same holds true for other androgens studied, such as 5α-dihydrotestosterone (DHT), Δ^4-androstenedione (see Rastogi et al., 2005) or 11-ketotestosterone (Murphy et al., 2006). Some of this disparity may be due to species differences in plasma steroid-binding protein and some may be due to the method, place, or period of sampling. In addition, it is remarkable that during a seasonal cycle androgen titers may increase enormously, up to and in excess of 100 times the seasonal nadir value. Androgens are known to be involved in the regulation of the spermatogenetic process. A seasonal increase in circulating levels of androgens corresponds reasonably well to the period during which the males engage most intensely in reproductive behavior. An exception to this scheme is the male Bornean voiceless frog (*Rana blythii*), in which low androgen levels are maintained during the mating behavioral display (Emerson & Hess, 1996). In such species, it would be interesting to explore the spatial and seasonal variation of androgen receptors. Besides androgens, estrogens are also produced by the anuran testis, the highest plasma concentration of 17β-estradiol (E_2) ever recorded in an anuran being up to 4 ng/ml in *R. esculenta*. In a few species, plasma progesterone

(P_4) was also assayed and was found in very minute quantities. What specific role(s) estrogens or P_4 may have in the male reproductive cycle of anurans is far from clear. A preliminary *in-vitro* study has, nevertheless, indicated that E_2 promotes the proliferation of primary spermatogonia as well as of the interstitial mast cells, and at the same time tends to downregulate intratesticular androgen levels in the *R. esculenta* testis (Minucci, Di Matteo, Chieffi, Pierantoni, & Fasano, 1997).

The dependence of the gametogenic compartment of the testis on the secretory activity of the endocrine compartment has been explored experimentally in *R. esculenta*. The use of a cytotoxic compound, such as ethane dimethane sulfonate, leads to morphological and functional disorganization of the endocrine tissue accompanied by a decrease in testicular and plasma androgen levels, and this subsequently is reflected in damaged spermatogenetic tissue (Minucci, Fasano, Di Matteo, Chieffi-Baccari, & Pierantoni, 1990; Minucci, Di Matteo, Fasano, Chieffi-Baccari, & Pierantoni, 1994; Palmiero, Ferrara, De Rienzo, & Minucci, 2001). Following the suspension of cytotoxic treatment, the damaged tissues recover together with the circulating levels of androgens, showing that some local, intratesticular factor intervenes in the process of recovery. Several earlier attempts to experimentally demonstrate the involvement of thyroid hormones in gonadal activity have not provided convincing evidence of this relationship (Leatherland, 1987; Kühn et al., 1990). Nevertheless, our latest unpublished findings are worthy of notice: the presence of thyroid hormone receptors through the TRβ-A1 mRNA expression in the frog testis during winter months (unpublished observations).

4. FEMALE REPRODUCTIVE CYCLE

4.1. Ovarian Cycles

The female reproductive cycle, in all anurans whether oviparous, ovoviviparous, or viviparous, is characterized by a vitellogenic phase of ovarian growth (see Polzonetti-Magni, 1999; Rastogi & Iela, 1999; Rastogi et al., 2005). Prior to this there occurs recruitment of previtellogenic follicles. In general, the anuran ovarian cycle can be subdivided into the preparatory phase (also known as the prebreeding or prespawning phase, during which much of the vitellogenesis takes place) followed by the ovulatory or spawning phase, after which comes the postovulatory/postbreeding phase, which may be, in some species, refractory to hormonal stimulation (Figure 9.3). During the vitellogenic phase and under the influence of pituitary gonadotropins, ovarian follicles secrete E_2, which is required to stimulate the liver to synthesize and secrete yolk precursor protein, vitellogenin. This protein is sent into the blood circulation and is taken up at specific receptor sites by the growing follicle, where it is processed into yolk. This is followed, when the appropriate environmental conditions set in, by ovulation and oviposition. In most anurans studied so far, the female reproductive cycle has an annual duration; rarely, as in *Bufo bufo*, is it biennial (see Rastogi et al., 2005). In tropical and subtropical species of anuran, the recruitment of previtellogenic follicles for vitellogenesis, and vitellogenesis itself, may occur throughout the year. The ovary is characterized by the presence of multiple stages of follicular growth during much of the year. In temperate species this recruitment is synchronous, at least at the individual level, and may be potentially asynchronous at the population level. Vitellogenesis may also take place during a surprisingly short period of time before breeding (e.g., *P. dacnicolor* (Iela, Rastogi, Delrio, & Bagnara, 1986)). In explosive breeders, such as the spadefoot toad (*Scaphiopus couchii*), usually there is a short period of postovulatory quiescence followed by a period of vitellogenesis, which terminates before overwintering so that the female is ready to oviposit as soon as favorable environmental conditions (rainfall) occur. Thus, in species like the explosive breeders there is synchrony of vitellogenic oocytes, whereas in species with prolonged breeding there is asynchrony in the recruitment of previtellogenic oocytes with correlated changes occurring in the secretion of regulatory hormones, such as pituitary gonadotropins and gonadal steroids. There is a major convergence of opinion that the absence of vitellogenic oocytes within the ovary is a prerequisite for the recruitment of early oocytes under the monitoring of pituitary gonadotropins. There may be variations in the period of oogonial proliferation, which may occur around the time of breeding, soon after breeding, or throughout the year. Female reproductive cycles are also characterized by conspicuous changes in ovarian weight during the year owing to the production of eggs rich in yolk. In some species, e.g., *B. japonicus*, the peak ovarian weight has been calculated to constitute up to 20% of the total body weight (Itoh et al., 1990).

Frogs and toads are the most fecund among amphibians, laying a few hundred to a few thousand small yolked eggs, ca. 1 or 2 mm in diameter.

4.2. Hormones

The presence of large vitellogenic follicles within the ovary is always associated with higher circulating levels of sex hormones, particularly of E_2. In seasonally breeding oviparous anurans, maximal plasma levels of P_4 were recorded during the prebreeding (preovulatory) period, gradually declining during breeding; e.g., *P. dacnicolor* (Iela et al., 1986), *D. occipitalis* (Kühn, Gevaerts, Jacobs, & Vandorpe, 1987), *B. japonicus* (Itoh et al., 1990), *R. italica* (Guarino et al., 1993), *R. esculenta* (Paolucci & Di Fiore, 1994), and *Bufo arenarum* (Medina, Ramos, Crespo,

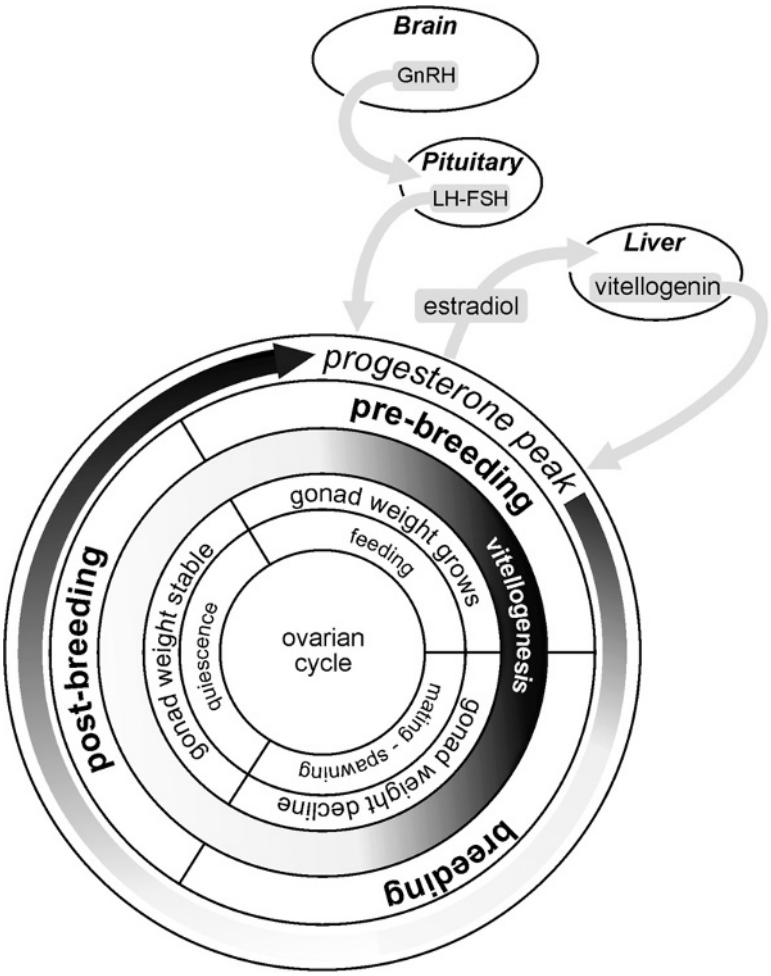

FIGURE 9.3 Simplified anuran ovarian cycle with involvement of the main hormonal components. Black color indicates the highest hormones level/activity and white the lowest. LH, luteinizing hormone; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone.

González-Calvar, & Fernández, 2004) (Figure 9.3). This preovulatory peak is obviously associated with the induction of nuclear maturation in full-grown oocytes for spawning. Similarly, whereas in all species investigated serum levels of androgens peaked during the preovulatory period together with E_2, only in *B. arenarum* did the later hormone peak soon after ovulation, which correlated with the onset of a new wave of follicular growth and vitellogenesis (Medina et al., 2004). More detailed accounts of the annual hormonal cycles for many species (female as well as male cycle) can be found in Rastogi et al. (2005); also see this source for earlier references.

In many tropical species, asynchronous oogenesis occurs in order to maintain readiness to breed at any time that favorable breeding conditions are created; i.e., periods of rainfall. In such species, e.g., *Physalaemus pustulosus*, the highest plasma levels of P_4 and estrogen (the authors do not report what estrogen they were measuring; presumably E_2) were found in amplexing females and the lowest levels were observed in post-mated females (Lynch & Wilczynsky, 2005). Androgens, however, peaked prior to amplexus and were extremely low in amplexed and post-mated females. In water frogs (*Rana lessonae*/*R. esculenta* complex), an interesting causal relationship has been described between a higher T level during the previous autumn and a greater magnitude of oviposition in the following breeding season (Reyer et al., 1999). Indeed, in many temperate species, circulating levels of T increase substantially in autumn, but whether this increase is always correlated with an increased ovulation in the following reproductive season remains a matter of investigation. But then, the hormonal and breeding cycle should be investigated for at least two or three years in a row. In the Mexican leaf frog, P_4, T, and E_2 were all peaking during amplexus, with P_4 being even higher in concentration in females with oviductal eggs (Iela et al., 1986). All three hormones had decreased drastically by 48 hours post-breeding. A very similar situation has been described for an explosive breeder, *S. couchii* (Harvey, Propper, Woodley, & Moor, 1997). Based on the known hormonal data and some

experimental designs, it is considered that E_2 and P_4 synergize to induce female receptive behavior and trigger molecular steps leading to ovulation. Certainly, there is need for further experiments in which the hormone levels are manipulated to subsequently monitor the process of amplexus and spawning. As in the male, in female anurans there is a conspicuous interspecific variation in plasma concentrations of E_2 and T. Of nearly a dozen species of anuran explored in this regard, the highest plasma concentration of E_2 was observed in breeding females of the Mexican leaf frog (Iela et al., 1986) at 17 ng/ml, and the lowest titer was recorded in *B. japonicus*, barely above 0.02 ng/ml (Itoh et al., 1990). Plasma P_4 reached values over 3 ng/ml in *R. esculenta* during breeding (Paolucci & Di Fiore, 1994). Interestingly, in females of several species, circulating T levels are higher than those of E_2. Progesterone is always present in quantities lower than those of E_2. Usually, the seasonal profile of plasma E_2 is correlated with ovarian growth and with the period of vitellogenesis.

Environmental information received by the sense organs may activate neuroendocrine pathways that control, through the synthesis of gonadotropin-releasing hormone (GnRH), the synthesis and release of pituitary gonadotropins (GTHs). The control of GTH secretion may involve complex interactions of several regulatory factors, including amino acid neurotransmitters, neuropeptides, and gonadal steroids. In female *R. esculenta*, the seasonal pattern of gonadal weight was found to be positively correlated with seasonal changes in the brain content of GnRHs (Rastogi, King, Di Fiore, D'Aniello, & Pinelli, 1997). Two GnRH forms are present in the anuran brain (see Rastogi, Meyer, Pinelli, Fiorentino, & D'Aniello, 1998). One, similar to the mammalian form, is located in the diencephalon, whence this decapeptide can be transferred to the pituitary via the hypothalamo–hypophysial portal vessels. This form is considered largely responsible for the regulation of pituitary GTH synthesis and secretion. Reproductive status-related variations in the brain content of this GnRH have been demonstrated in *R. esculenta* (Rastogi et al., 1997). There may be sexual differences in pituitary responsiveness to GnRH, and there may be correlations between pituitary responsiveness and the phase of the gonadal cycle in some species. As to the other form of GnRH, similar to chicken-II GnRH molecule, localized in the midbrain, its role is not yet clear. Pituitary gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH) also show conspicuous seasonal variations correlated with seasonal reproductive activity (see Rastogi et al., 2005). An interesting study on *Rana pipiens* has shown that pituitary gonadotropin secretion is indeed affected by gonadectomy and exogenous steroid hormone treatment, evidently through the hypothalamo–hypophysial–gonadal (HPG) feedback loop (Pavgi & Licht, 1989).

5. ENVIRONMENTAL CONTROL

Reproductive cycles in most frogs and toads are influenced by external environmental cues as well as their endogenous reproductive rhythms. The relative importance of each of these mechanisms in modulating and controlling reproductive cycles, both male and female, varies somewhat among anurans. Many studies on the control of seasonal or annual reproductive cycles in frogs and toads have investigated exogenous cues, whereas only in one anuran species has the endogenous circannual rhythm been examined, and only in the male (Rastogi et al., 1981). To demonstrate that a reproductive cycle is endogenously driven, animals must be maintained under constant photo–thermal and feeding conditions for a long period of time (at least a year or more). The study conducted with male *R. esculenta* convincingly demonstrated that an underlying endogenous rhythm is an important component of the seasonal testicular cycle. More species, and both sexes, must be investigated in this regard.

The timing of breeding in most temperate species is highly seasonal. Breeding activity may occur in early spring, early summer, when there is a summer storm, or even in late winter. The precise timing of reproduction in temperate, tropical, and subtropical species of frog and toad may vary annually and geographically, depending on external cues such as temperature, humidity, food, and photoperiod. Many aspects of environmental change during the year are roughly the same each year and thus predictable. Temperature increase in spring/summer and decrease in autumn/winter, outside the tropics, is predictable, as is the approximate time and duration of food availability. Physiological change in a process does not occur instantly. Hence, anurans may use reliable environmental cues to anticipate the onset of predictable seasonal change. However, whether absolute temperature or seasonal change in temperature acts directly to control reproductive cycles, or an environmental cue acts to synchronize the endogenous circannual rhythm of reproductive cycles, is yet to be clarified.

5.1. Temperature

Temperature is the main environmental variable that influences gonadal cycles and reproductive activity as a whole in most frogs and toads (see Paniagua, Fraile, & Sáez, 1990; Rastogi & Iela, 1992; Rastogi et al., 2005). In the male anuran, increasing temperatures may promote gonadal growth and spermatogenesis, and low temperatures tend to depress the gonadal activity. Irrespective of geographical location, the thermal optimum and the upper and lower thermal limits within which gametogenesis can proceed normally varies greatly from species to species.

This species-specific discrepancy of thermal sensitivity has been described in two sympatric species of the genus *Leptodactylus*, *viz. L. ocellatus* and *L. chaquensis*, living in South America in an area with mean monthly temperatures varying from around 16°C in July to around 28°C in January. While in the former species spermatogenesis occurs at a continuous rate, indicating that its thermal optimum ranges widely between 16°C and 28°C, in the latter species there occurs total stasis of spermatogenesis in summer (December to March) and the thermal optimum for active spermatogenesis lies between 20°C and 24°C (Cei, 1980). The thermal optimum for maintaining active gametogenesis in the common green frog (*R. esculenta*) in Southern Europe lies roughly between 10°C and 20°C (Rastogi et al., 1981). This range of temperature can maintain spermatogenesis even during winter months, whereas temperatures around 4°C or less, applied during summer, impair spermatogenesis, primarily causing degeneration of primary spermatocytes and inhibiting spermatogonial proliferation (Rastogi et al., 1981; Rastogi, Di Matteo, Minucci, di Meglio, & Iela, 1990). In this ranid, temperatures between 20°C and 24°C may stimulate testicular gametogenic activity at any time of the year. Higher temperatures have deleterious effects on the testis; at 28°C testis activity is impaired within a very short time. In the male of the Mexican leaf frog, the upper thermal limit for normal testicular activity is around 33−34°C (Rastogi et al., 1986). In a South American toad (*Bufo spinolosus*), spermatogenetic activity is decreased by low temperature (4°C) but remains normally active within the range of 18−37°C. In a Japanese hylid frog, exposure to a temperature of 30°C stimulated meiotic stages of spermatogenesis only if the animals were kept under a natural photoperiod (Toyoshima & Iwasawa, 1984). *R. esculenta* exhibit *in-vitro* and *in-vivo* thermal dependence of primary spermatogonial proliferation with or without stimulatory hormone priming (Minucci et al., 1986; Rastogi et al., 1990). In contrast to the cessation of primary spermatogonial proliferation in *R. esculenta* exposed to a temperature of 4°C, spermatogonial proliferation in *P. dacnicolor* stops when active spermatogenesis begins.

These facts may lead us to consider that the testis provides an inhibitory factor that maintains primary spermatogonia under mitotic arrest. Indeed, in many species it has been suggested that the sensitivity of primary germ cells to stimulatory hormonal factors varies with the season, which naturally implies the occurrence of seasonal thermal variation. Although it has been repeatedly recorded that very low and very high temperatures may, in some temperate species, induce degeneration of some stages of male gametogenesis, we have no clue as to the molecular mechanisms underlying differences in gonadal thermal sensitivity, either between species or between different gametogenic cell types within the same species.

Very little information is available on the relationship between environmental temperature and the female reproductive cycle. There is evidence in some anurans, such as *B. bufo*, *R. esculenta*, *Rana tigrina*, and *P. maculatus*, that the environmental temperature can affect the growth of ovarian follicles (Rastogi et al., 1983; Pancharatna & Saidapur, 1990; Kanamadi & Jirankali, 1992; Saidapur & Hoque, 1995; Rastogi et al., 2005). As in the male, the thermal range favorable for ovarian growth and vitellogenesis varies from species to species. In general, exposure to low temperatures during the phase of ovarian recrudescence induces follicular atresia or it simply stops vitellogenesis.

At present, we can simply say that the thermal *preferendum*, upper and lower limits within which the gonads manifest normal activity, varies greatly between species. It can be proposed, naturally with due caution, that anurans rely entirely on temperature to control the time of gonadal maturation and regression and that that is why these events occur roughly at the same time each year. The exact time of breeding can differ between years, and it can differ between different habitats at the same latitude (e.g., sea-level and montane populations of *R. esculenta* or *R. italica*). Some non-thermal cues (behavior, energy reserves, photoperiod, rainfall) can invariably intervene in the modulation of thermal control of gonadal growth and regression, thus evidencing the underlying physiological plasticity of the reproductive system.

5.2. Rainfall and Humidity

Rainfall and, as a consequence, the building up of high humidity, is the main causative factor that triggers breeding activity in many species (see Duellman & Trueb, 1976; Bagnara & Rastogi, 1992; Bagnara et al., 1986). In the wet tropics, breeding may occur throughout the year, but only in correspondence with the predictable periods of rainfall that induce females to lay eggs. In these tropical species, rainfall itself may act only as a short-term cue to trigger breeding. The physiological mechanisms underlying such rapid responses are unclear. In habitats with predictable rainfall, animals have regressed gonads outside the breeding season whereas, in habitats with unpredictable humidity, the reproductive system is fairly mature year-round so that it can respond quickly to the sudden and unpredictable rainfall. Many species inhabiting arid habitats breed following rain, the timing of which is often unpredictable. The relative ambient humidity may also affect spermatogenesis and steroidogenesis in the viviparous anuran *N. occidentalis* (Gavaud, 1975). By placing the males of *P. dacnicolor* in an environmental chamber with 80−90% humidity at a temperature of 30°C, spermatogenesis was greatly stimulated and aggressive behavior increased in all males as compared to those kept at the same

temperature but at an ambient humidity of 35–45%; in high-humidity males, the circulating levels of androgens were comparatively higher than in the low-humidity males (Rastogi & colleagues, unpublished). However, photoperiod and some biological cues such as food availability and social interactions may become important.

5.3. Photoperiod

In some frog species kept in total darkness for months in a row, spermatogenetic activity is not impaired, while in others a certain photoperiodic timetable becomes important for the initiation and continuation of some steps of the gonadal cycle. Semidesertic explosive breeders, such as the spadefoot toad or the Colorado River toad, preferably breed in the night hours during summer storms; plasma levels of androgens and E_2 were very low soon after egg-laying (Rastogi, personal observations, 1984). So far, not one amphibian species has been demonstrated to be photoperiodic. It seems that the absence of light, total blinding, or even exposure to red light has stimulatory effects on reproduction (see Joshi & Udaykumar, 2000). None-theless, in some species of frog, a positive response of the reproductive system to a favorable thermal input depends upon the presence of a 'permissive' photoperiod (Rastogi et al., 1978; Rastogi et al., 2005). Numerous documents have discussed the results obtained in pinealectomized or blinded animals in order to understand whether the pineal and/or the lateral eyes may act as neuroendocrine transducers of photic information, thereby linking the reproductive activity to prevailing environmental photoperiod (Alonso-Gomez, Tejera, Alonso-Berdate, & Delgado, 1990; Chanda & Biswas, 1984; Chanda & Biswas, 1992; Udaykumar & Joshi, 1996). The pineal gland produces melatonin and several investigations have been undertaken to analyze the seasonal cyclic nature of melatonin secretion in some anurans and its effects on the reproductive system. The results have been non-uniform and sometimes contradictory, and have not furnished sufficient support concerning whether and how the pineal and/or lateral eyes are related to photoperiod mediation of thermal response of reproductive activity (Hoque, Saidapur, & Pancharatna, 1993; Serino, d'Istria, & Monteleone, 1993; de Atenor, de Romero, Brauckmann, & Pisanò, & Legname et al., 1994; d'Istria, Monteleone, Serino, & Chieffi, 1994; Udaykumar & Joshi, 1997; d'Istria et al., 2001). Exogenous melatonin can inhibit GnRH-induced T secretion *in vitro* and leads to the disappearance of frog relaxin expression in Leydig cells (d'Istria et al., 2004). Earlier it was also shown that exogenous melatonin counteracts the stimulatory effects of blinding or red light exposure on ovarian activity in the skipper frog, *Rana cyanophlyctis* (Joshi & Udaykumar, 2000). The neuroanatomical pathways from eyes and pineal leading to the hypothalamus and specifically the preoptic area in the brain might lead us to think that these organs may be involved in translating photoperiod signals into hormonal signals through the central nervous system. However, there is no experimental data to support this. Thus, whether photic cues play an important role in the regulation of reproductive cycles in anurans is not yet unequivocally demonstrated.

5.4. Nutrition

Adequate food supply may be an important external biological factor. Frogs and toads usually store their energetic supplies in the abdominal fat bodies, and the total mass of these bodies may be used as a reliable indicator of the nutritional condition of an individual. Fat bodies store several classes of lipid and protein and can synthesize steroid hormones *in vitro* (Chieffi, Rastogi, Milone, & Iela, 1980). In temperate species, fat bodies become very fatty by the end of summer and thus are ready to supply the necessary energy during winter hibernation, when in many species gametogenesis may continue (though at a slower rate). In some cases, surgical removal of the fat bodies in females leads to ovarian regression due to atresia of vitellogenic follicles, markedly delays the onset of vitellogenesis, or impairs the recruitment of non-vitellogenic oocytes for vitellogenesis (Pierantoni et al., 1983; Pramoda & Saidapur, 1984; Prasadmurthy & Saidapur, 1987). In males, fat body removal may cause degeneration of primary spermatocytes and subsequent testicular atrophy (see Rastogi et al., 2005). Seasonal variation in mean mass of the abdominal fat bodies has been investigated in many species of anuran and one feature common to all species and both sexes is that the seasonal minimum fat body mass occurs during and soon after the breeding season. In females, seasonal variation in mean mass of abdominal fat bodies is generally inversely related to ovarian mass (Rastogi et al., 1983; Prasadmurthy & Saidapur, 1987). There is indirect evidence to indicate that fat bodies may be involved in the transfer of vitellogenin to the ovary (Varriale, Di Matteo, Minucci, Pierantoni, & Chieffi, 1988). Stored lipids may be utilized only in part for reproduction. They provide energy mainly for winter dormancy, at least in temperate species. Species inhabiting wet tropical and subtropical regions either contain developed fat bodies throughout the year or they show only slight annual changes in weight. Altogether, it appears that in both sexes fat bodies are needed to support normal gonadal activity and that in females they are essential for the transfer of basic material to the ovary for vitellogenesis. In other words, they are used as an immediate source of nutrients for gonadal activity, influencing size and number of eggs (Long, 1987; Girish & Saidapur, 2000).

6. BIOREGULATORY MECHANISMS (MOLECULAR MACHINERY)

The presence of many compounds belonging to a variety of chemical regulatory families has been described in the anuran gonads. Their presence in the testis might lead to the contention that they are somehow involved in intratesticular autocrine/paracrine mechanisms. For example, the frog testis expresses relaxin mRNA; frog relaxin has been identified as belonging to the relaxin/insulin gene family (De Rienzo, Aniello, Branno, & Minucci, 2001). It is specifically expressed in Leydig cells and its transcript shows changes during the reproductive cycle in close relationship with the seasonally changing titer of testicular androgen levels (De Rienzo, Aniello, Branno, Izzo, & Minucci, 2006). Perhaps T acts directly in controlling frog relaxin expression. Melatonin interferes with Leydig cell activity and probably inhibits GnRH-induced T secretion *in vitro* and induces the simultaneous total absence of frog relaxin transcript (d'Istria et al., 2004). Circumstantial evidence on the presence of endocannabinoids (lipophilic retrograde transmitters acting locally) in the central nervous system (CNS) and in the testicular extract of *R. esculenta* may indicate their role in bioregulatory mechanisms correlated with reproductive activity (Meccariello et al., 2006). Recently, on a neuroanatomical basis, it has also been suggested that there might be an interplay between the endocannabinoid system and brain GnRH neuronal activity related to seasonal reproduction (Meccariello et al., 2008). These authors also outlined that pituitary hormone-induced increase of estrogens in the frog testis stimulates the peritubular myoid cells, expressing an immediate early gene Fra-1 (extracellular-signal-regulated kinase), and suggested that estrogens play an important role in the process of spermiation (Cobellis et al., 2008). Regarding other intratesticular molecular regulatory mechanisms, in the *R. esculenta* testis, mRNA expression of some proto-oncogenes related to nuclear transcription factors or to the protein kinase family (such as c-fos, c-Jun, c-myc, and c-mos) has been observed in different stages of spermatogenetic cells as well as in somatic cell types (Chieffi, Minucci, Cobellis, Fasano, & Pierantoni, 1995; Chieffi, Colucci-D'Amato, Staibano, Franco, & Tramontano, 2000; Cobellis, Meccariello, Fienga, Pierantoni, & Fasano, 2002). High levels of c-fos corresponded to the E_2 peak, whereas low levels were observed when plasma androgens were at a peak; modulation of estrogen-induced proliferation of primary spermatogonia was suggested. Indeed, E_2 may be the main mitogenic factor within the frog testis. It has recently been shown to induce the activation of serine/threorine kinase Akt-1 phosphorylation, possibly via estrogen receptor-β (ERβ) to mediate the mitotic proliferation of germ cells (Stabile, Russo, & Chieffi, 2006). Further experimentation should clarify these relationships to spermatogenesis and/or steroidogenesis. More recently, evidence has been given of the presence of prothymosin alpha protein in the *R. esculenta* testis and it was suggested that it may play a role in frog spermatogenesis, particularly during meiosis (Aniello et al., 2002). Further experimental proof was obtained to show that this acidic protein transcript is strongly expressed in the frog testis during the year, peaking in a period when the testis shows the highest spermatogenetic activity (late summer/early autumn) and localizing abundantly in spermatocytes (Ferrara et al., 2008). It was suggested that it plays a role in spermatocyte differentiation and further maturation.

In the female, the role of prolactin and other physiological inducers in oocyte maturation (germinal vesicle breakdown) and ovulation are discussed in other chapters of this volume. Although far from being exhaustive and clear, data are accumulating on the presence of cell cycle regulators and their potential role in the meiotic maturation of oocytes. This type of research is using the African clawed frog, *X. laevis*, as the experimental model (e.g., Frank-Vaillant, Haccard, Ozon, & Jessus, 2001; Furuno, Kawasaki, & Sagata, 2003; Andersen et al., 2003). Evidence has accumulated showing that cAMP regulates the meiotic arrest of amphibian oocytes at the G2/M transition (see Ferrell, 1999). Further indirect evidence from *B. arenarum* indicates that the prostaglandin $PGF_{2\alpha}$ may be involved in mediating pituitary gonadotropin-progesterone-induced ovulation (Ramos, Cisint, Crespo, Medina, & Fernández, 2008). Notwithstanding some apparent flaws in the conclusions drawn, several *in-vivo* and *in-vitro* investigations in *R. esculenta* and *Xenopus laevis* point out that prostaglandins are involved in male and female reproduction (see Zhao & Kung, 1994; Gobbetti & Zerani, 1995). Endogenous D-aspartic acid (D-Asp), acting probably as a messenger molecule, has been found in the frog testis and ovary. It may be involved in the modulation of T synthesis and secretion in the gonads as well as T-dependent thumb pad growth and spermatogonial proliferation (Di Fiore, Assisi, Botte, & D'Aniello, 1998; Raucci et al., 2004). D-aspartic acid may be acting as a precursor for its methylated form, N-methyl-D-aspartic acid (NMDA) synthesis, which, in turn, through specific NMDA receptors, may be directly involved in the regulation of hormone synthesis and secretion and thus indirectly in the control of spermatogonial proliferation and thumb pad growth. Further research is needed to clarify the precise modulatory role of D-Asp in frog reproduction. The demonstrated presence of immunoreactive opioid peptides in the brain, pituitary, and gonads of *R. esculenta* during the reproductive cycle, has been taken to indicate that such peptides may be involved in the modulation of the HPG axis as well as play a local modulatory role in the gonads, both male and female (Facchinetti et al., 1992; 1993). More experimental evidence is required to clarify this issue. Whether all these

compounds, and presumably others to come in the proximal future, have an integrated role in gonadal regulation is yet to be defined.

7. CONCLUDING REMARKS

Within the class Amphibia, anurans have played a prominent role in exploring external environment−HPG interactions. Owing to their diverse reproductive strategies, frogs and toads are ideal model systems in which to investigate and clarify the role of hormones (steroid hormones; brain and pituitary hormones) in the maintenance and modulation of seasonal reproductive activity. Fundamental information on the physiological regulation of reproduction in anurans indicates that, as in other vertebrates, the brain hormone GnRH, pituitary GTHs, and gonadal sex steroids (androgens and estrogens plus P_4 in the female) constitute the basic set of chemical instructions necessary to promote gonadal growth and regulate the reproductive cycle. There are some gaps in our knowledge, however, and there are discrepancies in the literature concerning some seasonal events of the reproductive activity in a few well-known species.

In general, anuran reproductive cycles are tied to environmental factors such as temperature, rainfall/humidity, and, sometimes, photoperiod. Energetic intake (food availability) may also become an important factor in successful reproduction. Our knowledge of the neurochemical signals and neural pathways in frogs and toads by which environmental information influences neuroendocrine machinery, controlling the reproductive cycle, is still rudimentary. Much more needs to be done. For example, we do not know through which brain nucleus the thermal and/or photoperiodic information is relayed, nor which neural pathway is used to relay information on humidity, which is the prime factor for inducing females to deposit their eggs. While much has been done with regard to the role thermal cues play in the modulation of reproductive activity, the photoperiodic control of the anuran reproductive cycle is not yet well established. We do not know what role, if any, the pineal complex or melatonin (which is secreted in frogs and toads) play in the complex picture of anuran reproductive biology. It is to be noted that only two species of anuran stand out as the most widely used models among amphibians for research on morphological, physiological, biochemical, and molecular aspects of reproduction: *R. esculenta* and *X. laevis*.

In wet/dry tropics, temperatures are high and stable year-round but monsoonal rainfall is usually highly seasonal and may vary both annually and spatially. Many features of the reproductive biology of anurans of such regions are certainly adaptations to deal with such unpredictable variation in precipitation. Such species may show extreme annual variation in reproductive rates, linked to stochastic variation in wet-season rainfall. In most tropical oviparous anurans, both males and females exhibit year-round gametogenesis, with subsequent courtship (if any), amplexus, and oviposition during the breeding season or whenever the environmental conditions are appropriate for breeding.

While it is clear that chemical messengers (hormones) trigger initiation and progression of gametogenesis and release of gametes, we do not yet have clear evidence of how non-hormonal factors such as temperature and/or humidity act. In other words, we have circumstantial evidence that a pituitary GTH will induce ovulation or spermiation under a certain temperature range but we do not know the molecular steps that lead to this. We do not have a clear picture of the paracrine interactions between the spermatogenetic and interstitial compartments of the testis, especially in the context of seasonal testicular activity in most species of frog and toad. Similarly, we do not have a clear picture of the paracrine interactions of the endocrine and gametogenic compartments of the ovary related to the seasonal reproductive cycle in the female. Putative roles of molecules such as interleukins (see Jantra et al., 2007) and other possible regulators of reproduction are at the very initial level of investigation.

The events of reproduction in many frog and toad species are interesting and offer subjects for investigations that may have broad implications in several areas of biology, ranging from behavior to environmental endocrinology of reproduction to cell biology. The ready availability of all stages of development and the ease of their maintenance make them good candidates for future studies. Indeed, it becomes critically important to integrate endocrinological, genetic, and ecological approaches in order to understand the cyclic mechanisms of reproduction. Such a consideration is highlighted by one simple example: the manifestations of extreme sequential polyandry in some egg-laying frog species. Other key questions could concern: (1) how individual variations are considered within a pool of individuals; (2) what the 'baseline' or 'pretreatment' values are and how they fit within a natural population; and (3) how much variation is accounted for behavioral plasticity or the mode of determination of physiological measures; and so on.

Finally, a deeper knowledge of amphibian reproductive biology and life history in concert with knowledge of the environmental characteristics will undoubtedly aid in conservation efforts against the amphibian population decline worldwide (see Loyola et al., 2008).

ABBREVIATIONS

CNS	Central nervous system
D-Asp	D-aspartic acid
DHT	5α-dihydrotestosterone

E_2	17β-estradiol
ERβ	Estrogen receptor-β
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin releasing hormone
GSI	Gonadosomatic index
GTH	Gonadotropic hormone
HPG	Hypothalamo−hypophysial−gonadal
LH	Luteinizing hormone
NMDA	N-methyl-D-aspartic acid
P_4	Progesterone
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
T	Testosterone

REFERENCES

Alonso-Gomez, A. L., Tejera, M., Alonso-Berdate, M., & Delgado, M. J. (1990). Response of pinealectomy and blinding in vitellogenic female frog (*Rana perezi*) subjected to high temperature in autumn. *Can. J. Physiol. Pharamacol., 68*, 94−98.

Andersen, C. B., Sakaue, H., Nedachi, T., Kovacina, K. S., Clayberger, C., Conti, M., & Roth, R. A. (2003). Protein kinase B/Akt is essential for the insulin- but not progesterone-stimulated resumption of meiosis in *Xenopus* oocytes. *Biochem. J., 369*, 227−238.

Aniello, F., Branno, M., De Rienzo, G., Ferrara, D., Palmiero, C., & Minucci, S. (2002). First evidence of prothymosin alpha in a non-mammalian vertebrate and its involvement in the spermatogenesis of the frog *Rana esculenta*. *Mech. Dev., 110*, 213−217.

Backwell, P. R. Y., & Passmore, N. I. (1990). Polyandry in the leaf-folding frog, *Afrixalus delicatus*. *Herpetologica, 46*, 7−10.

Bagnara, J. T., & Rastogi, R. K. (1992). Reproduction in the Mexican leaf frog, *Pachymedusa dacnicolor*. In W. C. Hamlett (Ed.), *"Reproductive Biology of South American Vertebrates"* (pp. 98−111). New York: Springer-Verlag.

Bagnara, J. T., Iela, L., Morrisett, F., & Rastogi, R. K. (1986). Reproduction in the Mexican leaf frog (*Pachymedusa dacnicolor*). I. Behavioral and morphological aspects. *Museum Nat. History Univ. Kansas, 121*, 1−31.

Byrne, P. G., & Keogh, J. S. (2009). Extreme sequential polyandry insures against nest failure in a frog. *Proc. R. Soc. Lond. B., 276*, 115−120.

Byrne, P. G., & Roberts, J. D. (1999). Simultaneous mating with multiple males reduces fertilization success in the myobatrachid frog *Crinia georgiana*. *Proc. R. Soc. London B., 266*, 717−721.

Caldwell, J. P. (1992). Diversity of reproductive modes in anurans: facultative nest construction in gladiator frogs. In W. C. Hamlett (Ed.), *"Reproductive Biology of South American Vertebrates"* (pp. 85−97). New York: Springer-Verlag.

Cei, J. M. (1980). Amphibians of Argentina. *Monit. Zool. Ital. (N.S.) Monogr., 2*, 1−609.

Cei, J. M., Ibanez, N., Alvarez, B. B., Carnevali, O., Mosconi, G., & Polzonetti-Magni, A. (1996). Divergent male androgen patterns in two sympatric species of *Leptodactylus* from subtropical South America. *Amphibia-Reptilia, 17*, 1−16.

Chanda, S., & Biswas, N. M. (1984). Influence of the pineal gland on spermatogenesis of the toad (*Bufo melanosticus*). *Horm. Res., 19*, 200−204.

Chanda, S., & Biswas, N. M. (1992). Effect of melatonin on the testis of pinealectomized and sham-operated toads during breeding season. *Comp. Physiol. Ecol., 17*, 1−5.

Chen, Y. H., Yu, H. T., & Kam, Y. C. (2007). The ecology of male egg attendance in an arboreal breeding frog, *Chirixalus eiffingeri* (Anura: Rhacophoridae), from Taiwan. *Zoolog. Sci., 24*, 434−440.

Chieffi, G., Rastogi, R. K., Milone, M., & Iela, L. (1980). Amphibian reproduction: reproductive physiology in the male *Rana esculenta*. *Boll. Zool., 47*, 63−70.

Chieffi, P., Minucci, S., Cobellis, G., Fasano, S., & Pierantoni, R. (1995). Changes on proto-oncogene activity in the testis of the frog, *Rana esculenta*, during the annual reproductive cycle. *Gen. Comp. Endocrinol., 99*, 127−136.

Chieffi, P., Colucci-D'Amato, G. L., Staibano, S., Franco, R., & Tramontano, D. (2000). Estradiol-induced mitogen-activated protein kinase (extracellular signal-regulated kinase 1 and 2) activity in the frog (*Rana esculenta*) testis. *J. Endocrinol., 167*, 77−84.

Cobellis, G., Cacciola, G., Chioccarelli, T., Izzo, G., Meccariello, R., Pierantoni, R., et al. (2008). Estrogen regulation of the male reproductive tract in the frog, *Rana esculenta*: a role in Fra-1 activation in peritubular myoid cells and in sperm release. *Gen. Comp. Endocrinol., 155*, 838−846.

Cobellis, G., Meccariello, R., Fienga, G., Pierantoni, R., & Fasano, S. (2002). Cytoplasmic and nuclear Fos protein forms regulate resumption of spermatogenesis in the frog, *Rana esculenta*. *Endocrinology, 143*, 163−170.

Crespo, E. G. (1982). Contribuicao para o conhecimento da biologia das especies ibericas de Alytes, *A. obstetricans boscai*, Lataste 1879 e *A. cisternasii*, Boscá 1879 (Amphibia, Discoglossidae): morfologia dos adultos e dos girinos. *Arquivos. do. Museu. Bocage. (C), 1*, 255−316.

Delgado, M. J., Gutiérrez, P., & Alonso-Bedate, M. (1989). Seasonal cycles in testicular activity in the frog, *Rana perezi*. *Gen. Comp. Endocrinol., 73*, 1−11.

d'Istria, M., Fuschino, A., Minucci, S., Palmiero, C., Rastogi, R. K., & Serino, I. (2001). Melatonin effects on the testicular activity in the green frog, *Rana esculenta*. In H. J. Th. Goos, R. K. Rastogi, H. Vaudry, & R. Pierantoni (Eds.), *"Perspectives in Comparative Endocrinology: Unity and Diversity"* (pp. 1089−1094). Bologna, Italy: Monduzzi Editore.

d'Istria, M., Monteleone, P., Serino, I., & Chieffi, G. (1994). Seasonal variations in the daily rhythm of melatonin and NAT activity in the Harderian gland, retina, pineal gland, and serum of the green frog, *Rana esculenta*. *Gen. Comp. Endocrinol., 96*, 6−11.

d'Istria, M., Serino, I., Izzo, G., Ferrara, D., De Rienzo, G., & Minucci, S. (2004). Effects of melatonin treatment on Leydig cell activity in the testis of the frog *Rana esculenta*. *Zygote, 12*, 293−299.

de Atenor, M. S. B., de Romero, I. R., Brauckmann, E., Pisanò, A., & Legname, A. H. (1994). Effects of the pineal gland and melatonin on the metabolism of oocytes *in vitro* and on ovulation in *Bufo arenarum*. *J. Exp. Zool., 268*, 436−441.

De Rienzo, G., Aniello, F., Branno, & Minucci, S. (2001). Isolation and characterization of a novel member of the relaxin/insulin family from the testis of the frog *Rana esculenta*. *Endocrinology, 142*, 3231−3238.

De Rienzo, G., Aniello, F., Branno, M., Izzo, G., & Minucci, S. (2006). The expression of frog relaxin mRNA (fRLX), in the testis of *Rana esculenta*, is influenced by testosterone. *J. Exp. Biol., 209*, 3806−3811.

Di Fiore, M. M., Assisi, L., Botte, V., & D'Aniello, A. (1998). D-Aspartic acid is implicated in the control of testosterone production by the vertebrate gonad. Studies on the female green frog *Rana esculenta*. *J. Endocrinol., 157*, 199−207.

Di Fiore, M. M., Chieffi-Baccari, G., Rastogi, R. K., di Meglio, M., Pinelli, C., & Iela, L. (2005). Hormonal regulation of secondary sexual characters. In H. Heatwole (Ed.), *"Amphibian Biology", Vol. 6*. (Endocrinology) (pp. 2229−2249). Chipping Norton NSW Australia: Surrey Beatty & Sons.

Duellman, W. E., & Trueb, L. (1986). *Biology of Amphibia*. New York: McGraw-Hill.

Emerson, S. B., & Hess, D. L. (1996). The role of androgens in opportunistic breeding, tropical frogs. *Gen. Comp. Endocrinol., 103*, 220−230.

Facchinetti, F., Genazzani, A. R., Pestarino, M., Vallarino, M., Pierantoni, R., Fasano, S., D'Antonio, M., Carnevali, O., Mosconi, G., & Polzonetti-Magni, A. (1992). Ovarian opioids and the reproductive cycle of the frog *Rana esculenta*. *Life. Sci., 50*, 1389−1398.

Facchinetti, F., Genazzani, A. R., Vallarino, M., Pestarino, M., Polzonetti-Magni, A., Carnevali, O., Ciarcia, G., Fasano, S., D'Antonio, M., & Pierantoni, R. (1993). Opioids and testicular activity in the frog, *Rana esculenta*. *J. Endocrinol., 137*, 49−57.

Ferrara, D., Izzo, G., Liguori, L., d'Istria, M., Aniello, F., & Minucci, S. (2008). Evidence for involvement of prothymosin alpha in the spermatogenesis of the frog *Rana esculenta*. *J. Exp. Zool., 309A*, 12−19.

Ferreira, A., Mehanna, M., & Prado, C. P. (2008). Morphologic and morphometric analysis of testis of *Pseudis limellum* (Cope, 1862) (Anura, Hylidae) during the reproductive cycle in the Pantanal, Brazil. *Biocell, 32*, 185−194.

Ferrell, J. E., Jr. (1999). *Xenopus* oocyte maturation: new lessons from a good egg. *BioEssays, 21*, 833−842.

Frank-Vaillant, M., Haccard, O., Ozon, R., & Jessus, C. (2001). Interplay between Cdc2 kinase and the c-Mos/MAPK pathway between metaphase I and metaphase II in *Xenopus* oocytes. *Dev. Biol., 231*, 279−288.

Furuno, N., Kawasaki, A., & Sagata, N. (2003). Expression of cell-cycle regulators during *Xenopus* oogenesis. *Gene. Expr. Patterns, 3*, 165−168.

Gavaud, J. (1975). Étude expérimentale du role des facteurs externs sur la spermatogenèse et la stéroideogenèse des males de *Nectophrynoides occidentalis* Angel. *J. Physiol. Paris., 70*, 549−559.

Girish, S., & Saidapur, S. K. (2000). Interrelationship between food availability, fat body, and ovarian cycles in the frog, *Rana tigrina*, with a discussion on the role of fat body in anuran reproduction. *J. Exp. Zool., 286*, 487−493.

Gobbetti, A., & Zerani, M. (1995). Amphibian oocyte: a model of a possible regulatory mechanism for prostaglandin E2 and prostaglandin F2α synthesis. *J. Endocrinol., 145*, 235−241.

Guarino, F. M., & Bellini, L. (1993). Reproductive activity and plasma concentrations in the male of *Rana dalmatina*. *Boll. Zool., 60*, 281−286.

Guarino, F. M., Di Fiore, M. M., Caputo, V., Angelini, F., Iela, L., & Rastogi, R. K. (1993). Seasonal analysis of the reproductive cycle in two wild populations of *Rana italica* Dubois 1985. *Anim. Biol., 2*, 23−42.

Harvey, L. A., Propper, C. R., Woodley, S. K., & Moore, M. C. (1997). Reproductive endocrinology of the explosively breeding desert spadefoot toad, *Scaphiopus couchii*. *Gen. Comp. Endocrinol., 105*, 102−113.

Hoque, B., Saidapur, S. K., & Pancharatna, M. (1993). Melatonin suppresses compensatory hypertrophy and follicular development in the unilaterally ovariectomized bullfrog *Rana tigrina*: a detailed analysis of follicular kinetics. *Zool. Anz., 230*, 85−93.

Iela, L., Rastogi, R. K., Delrio, G., & Bagnara, J. T. (1986). Reproduction in the Mexican leaf frog, *Pachymedusa dacnicolor*. III. The female. *Gen. Comp. Endocrinol., 63*, 381−392.

Itoh, M., Inoue, M., & Ishii, S. (1990). Annual cycle of pituitary and plasma gonadotropins and plasma sex steroids in a wild population of the toad, *Bufo japonicus*. *Gen. Comp. Endocrinol., 78*, 242−253.

Jantra, S., Bigliardi, E., Brizzi, R., Ietta, F., Bechi, N., & Paulesu, L. (2007). Interleukin 1 in oviductal tissues of viviparous, oviparous, and ovuliparous species of amphibians. *Biol. Reprod., 76*, 1009−1015.

Joshi, B. N., & Udaykumar, K. (2000). Melatonin counteracts the stimulatory effects of blinding or exposure to red light on reproduction in the skipper frog *Rana cyanophlyctis*. *Gen. Comp. Endocrinol., 118*, 90−95.

Kanamadi, R. D., & Jirankali, C. S. (1992). Testicular activity in *Polypedates maculatus* (Rhacophoridae): seasonal changes in spermatogenesis and fat bodies. *J. Herpetol., 26*, 329−335.

Kao, Y. H., Alexander, P. S., Yang, V. V. C., & Yu, J. Y. (1993). Annual patterns of testicular development and activity in the Chinese bullfrog (*Rana rugulosa* Wiegmann). *Zool. Sci., 10*, 337−351.

Kaptan, E., & Murathanoğlu, O. (2008). Annual morphological cycles of testis and thumb pad of the male frog (*Rana ridibunda*). *Anat. Rec., 291*, 1106−1114.

Kühn, E., Gevaerts, H., Jacobs, G., & Vandorpe, G. (1987). Reproductive cycle, thyroxine, and corticosterone in females of the giant swamp frog *Dicroglossus occipitalis* at the equator. *Gen. Comp. Endocrinol., 66*, 137−144.

Kühn, E., Jacobs, G., & Vandorpe, G. (1990). Thyroid function and a possible thyroidal−gonadal interaction in reproduction and in circannual rhythmicity. In W. Hanke (Ed.), *"Biology and Physiology of Amphibians", Vol. 38* (pp. 3−27). Stuttgart and New York: Gustav Fisher Verlag.

Leatherland, J. F. (1987). Thyroid hormones and reproduction. In D. O. Norris, & E. Jones (Eds.), *"Hormones and Reproduction in Fishes, Amphibians, and Reptiles"* (pp. 411−425). New York and London: Plenum Press.

Lofts, B. (1974). Reproduction. In B. Lofts (Ed.), *"Physiology of the Amphibia", Vol. 2* (pp. 107−218). New York: Academic Press.

Lofts, B. (1984). Amphibians. In G. E. Lemming (Ed.), *"Marshall's Physiology of Reproduction: Reproductive Cycles of Vertebrates", Vol. 1* (pp. 127−205). London: Churchill Livingstone.

Long, D. R. (1987). Reproductive and lipid patterns of a semiarid-adapted anuran, *Bufo cognatus*. *Texas J. Sci., 39*, 3−13.

Loyola, R. D., Becker, C. G., Kubota, U., Haddad, C. F. B., Fonseca, C. R., & Lewinsohn, T. M. (2008). Hung out to dry: choice of priority ecoregions for conserving threatened neotropical anurans depends on life-history traits. *PLoS ONE, 3*, 2120.

Lynch, K. S., & Wilczynski, W. (2005). Gonadal steroids vary with reproductive stage in a tropically breeding female anuran. *Gen. Comp. Endocrinol., 143*, 51−56.

Meccariello, R., Chianese, R., Cacciola, G., Cobellis, G., Pierantoni, R., & Fasano, S. (2006). Type-1 cannabinoid receptor expression in the frog, *Rana esculenta*, tissues: a possible involvement in the regulation of testicular activity. *Mol. Reprod. Dev., 73*, 551−558.

Meccariello, R., Franzoni, M. F., Chianese, R., Cottone, E., Scarpa, D., Donna, D., Cobellis, G., Guastalla, A., Pierantoni, R., & Fasano, S. (2008). Interplay between the endocannabinoid system and GnRH-I in the forebrain of the anuran amphibian *Rana esculenta*. *Endocrinology, 149*, 2149−2158.

Medina, M. F., Ramos, I., Crespo, C. A., González-Calvar, S., & Fernández, S. N. (2004). Changes in serum sex steroid levels throughout the reproductive cycle of *Bufo arenarum* females. *Gen. Comp. Endocrinol., 136*, 143−151.

Minucci, S., Fasano, S., Di Matteo, L., Chieffi-Baccari, G., & Pierantoni, R. (1990). Morphological and hormonal changes in the frog, *Rana esculenta*, testis after administration of ethane dimethane sulfonate. *Gen. Comp. Endocrinol., 79*, 335−345.

Minucci, S., Di Matteo, L., Fasano, S., Chieffi-Baccari, G., & Pierantoni, R. (1994). Regeneration of the testicular interstitial compartment after ethane dimethane sulfonate treatment in the hypophysectomized frog *Rana esculenta*: independence of pituitary control. *Gen. Comp. Endocrinol., 95*, 84−91.

Minucci, S., Di Matteo, L., Chieffi, P., Pierantoni, R., & Fasano, S. (1997). 17β-estradiol effects on mast cell number and spermatogonial mitotic index in the testis of the frog, *Rana esculenta*. *J. Exp. Zool., 278*, 93−100.

Minucci, S., Di Matteo, L., Pierantoni, R., Varriale, B., Rastogi, R. K., & Chieffi, G. (1986). *In vivo* and *in vitro* stimulatory effect of a gonadotropin-releasing hormone analog (HOE 766) on spermatogonial multiplication in the frog, *Rana esculenta*. *Endocrinology, 119*, 731−736.

Murphy, M. B., Hecker, M., Coady, K. K., Tompsett, A. R., Higley, E. B., Jones, P. D., Du Preez, L. H., Solomon, K. R., Carr, J. A., Smith, E. E., Kendall, R. J., Van Der Kraak, G., & Giesy, J. P. (2006). Plasma steroid hormone concentrations, aromatase activities and GSI in ranid frogs collected from agricultural and non-agricultural sites in Michigan (USA). *Aquat. Toxicol., 77*, 153−166.

Palmiero, C., Ferrara, D., De Rienzo, G., & Minucci, S. (2001). Ethane 1,2-dimethane sulphonate is a useful tool to study testicular activity of the frog *Rana esculenta*. In H. J. Th. Goos, R. K. Rastogi, H. Vaudry, & R. Pierantoni (Eds.), *"Perspectives in Comparative Endocrinology: Unity and Diversity"* (pp. 1171−1179). Bologna, Italy: Monduzzi Editore.

Pancharatna, M., & Saidapur, S. K. (1990). Role of temperature in regulation of ovarian cycle in bullfrog, *Rana tigrina*. *Indian. J. Exp. Biol., 28*, 806−811.

Paniagua, R., Fraile, B., & Sáez, F. J. (1990). Effects of photoperiod and temperature on testicular function in amphibians. *Histol. Histopathol., 5*, 365−378.

Paolucci, M., & Di Fiore, M. M. (1994). Sex steroid binding proteins in the plasma of the green frog, *Rana esculenta*: changes during the reproductive cycle and dependence on pituitary gland and gonads. *Gen. Comp. Endocrinol., 96*, 401−411.

Pavgi, S., & Licht, P. (1989). Effect of gonadectomy and steroids on pituitary gonadotropin secretion in a frog, *Rana pipiens*. *Biol. Reprod., 40*, 40−48.

Pierantoni, R., Varriale, B., Simeoli, C., Di Matteo, L., Milone, M., Rastogi, R. K., & Chieffi, G. (1983). Fat body and autumn recrudescence of the ovary in *Rana esculenta*. *Comp. Biochem. Physiol., 76A*, 31−35.

Polzonetti-Magni, A. M. (1999). Amphibian ovarian cycles. In E. Knobil, & J. D. Neill (Eds.), *"Encyclopedia of Reproduction", Vol. 2* (pp. 154−160). San Diego: Academic Press.

Pramoda, S., & Saidapur, S. K. (1984). Effects of excision of abdominal fat bodies on vitellogenic growth of oocytes in the frog *Rana tigrina*. *Boll. Zool., 51*, 329−333.

Prasadmurthy, Y. S., & Saidapur, S. K. (1987). Role of fat bodies in oocyte growth and recruitment in the frog *Rana cyanophlictis* (Schn.). *J. Exp. Zool., 243*, 153−162.

Ramos, I., Cisint, S. B., Crespo, C. A., Medina, M. F., & Fernández, S. N. (2008). Modulators of *Bufo arenarum* ovulation. *Zygote, 16*, 65−72.

Rastogi, R. K. (1976). Seasonal cycle in anuran (Amphibia) testis: the endocrine and environmental controls. *Boll. Zool., 43*, 151−172.

Rastogi, R. K., & Iela, L. (1992). Spermatogenesis in amphibia: dynamics and regulation. In R. Dallai (Ed.), *"Sex Origin and Evolution"*. UZI Monograph 6 (pp. 231−249). Bologna, Italy: Mucchi Ediotore.

Rastogi, R. K., & Iela, L. (1999). Female reproductive system, amphibians. In E. Knobil, & J. D. Neill (Eds.), *"Encyclopedia of Reproduction", Vol. 2* (pp. 183−190). San Diego: Academic Press.

Rastogi, R. K., Iela, L., Delrio, G., di Meglio, M., Russo, A., & Chieffi, G. (1978). Environmental influence on testicular activity in the green frog, *Rana esculenta*. *J. Exp. Zool., 206*, 49−64.

Rastogi, R. K., Tammaro, L., di Meglio, M., Iela, L., Di Matteo, L., & Chieffi, G. (1981). Circannual testicular rhythm in the green frog, *Rana esculenta*. *Boll. Zool., 48*, 97−105.

Rastogi, R. K., Izzo-Vitiello, I., di Meglio, M., Di Matteo, L., Francese, R., Di Costanzo, M. G., et al. (1983). Ovarian activity and reproduction in the frog, *Rana esculenta*. *J. Zool. Lond., 200*, 233−247.

Rastogi, R. K., Iela, L., Delrio, G., & Bagnara, J. T. (1986). Reproduction in the Mexican leaf frog, *Pachymedusa dacnicolor*. II. The male. *Gen. Comp. Endocrinol., 62*, 23−35.

Rastogi, R. K., Di Matteo, L., Minucci, S., di Meglio, M., & Iela, L. (1990). Regulation of primary spermatogonial proliferation in the frog (*Rana esculenta*): an experimental analysis. *J. Zool. Lond., 220*, 201−211.

Rastogi, R. K., King, J. A., Di Fiore, M. M., D'Aniello, B., & Pinelli, C. (1997). Sex and reproductive status related brain content of mammalian and chicken-II GnRHs in *Rana esculenta*. *J. Neuroendocrinol., 9*, 519−522.

Rastogi, R. K., Meyer, D. L., Pinelli, C., Fiorentino, M., & D'Aniello, B. (1998). Comparative analysis of GnRH neuronal systems in the amphibian brain. *Gen. Comp. Endocrinol., 112*, 330−345.

Rastogi, R. K., Iela, L., di Meglio, M., Di Fiore, M. M., D'Aniello, B., Pinelli, C., et al. (2005). Hormonal regulation of reproductive cycles in amphibians. In H. Heatwole (Ed.), *"Amphibian Biology", Vol. 6*. (Endocrinology) (pp. 2045−2177). Chipping Norton, NSW: Surrey Beatty & Sons.

Rastogi, R. K., Iela, L., Saxena, P. K., & Chieffi, G. (1976). The control of spermatogenesis in the green frog, *Rana esculenta* L. *J. Exp. Zool., 196*, 151−166.

Raucci, F., Assisi, L., D'Aniello, S., Spinelli, P., Botte, V., & Di Fiore, M. M. (2004). Testicular endocrine activity is upregulated by D-Aspartic acid in the green frog, *Rana esculenta*. *J. Endocrinol., 182*, 365−376.

Reyer, H. U., Frei, G., & Som, C. (1999). Cryptic female choice: frogs reduce clutch size when amplexed by undesired males. *Proc. Biol. Sci., 266*, 2101−2107.

Saidapur, S. K., & Hoque, B. (1995). Effect of photoperiod and temperature on ovarian cycle of the frog *Rana tigrina* (Daud). *J. Biosci., 20*, 445−452.

Sasso-Cerri, E., De Faria, F. P., Freymüller, E., & Miraglia, S. M. (2004). Testicular morphological changes durino the seasonal reproductive cycle in the bullfrog *Rana catesbeiana*. *J. Exp. Zool., 301A*, 249−260.

Serino, I., d'Istria, M., & Monteleone, P. (1993). A comparative study of melatonin production in the retina, pineal gland and Harderian gland of *Bufo viridis* and *Rana esculenta*. *Comp. Biochem. Physiol., 106C*, 189−193.

Stabile, V., Russo, M., & Chieffi, P. (2006). 17β-estradiol induces Akt-1 through estrogen receptor-β in the frog (*Rana esculenta*) male germ cells. *Reproduction, 132*, 477−484.

Tejedo, M. (1992). Effects of body size and timing of reproduction on reproductive success in female natterjack toads *Bufo calamita*. *J. Zool. Lond., 228*, 545−555.

Touchon, J. C., & Warkentin, K. M. (2008). Reproductive mode plasticity: aquatic and terrestrial oviposition in a treefrog. *Proc. Natl. Acad. Sci. U.S.A., 105*, 7495−7499.

Townsend, D. S., Stewart, M. M., & Pough, F. H. (1984). Male parental care and its adaptive significance in a neotropical frog. *Anim. Behav., 32*, 421−431.

Toyoshima, S., & Iwasawa, H. (1984). Changes in the effect of light-cycles on spermatogenetic activity by temperature in late autumn frogs of *Hyla japonica*. *Sci. Rep. Niigata Univ. Ser. D., 21*, 9−13.

Tsuji, H., & Lue, K. (2000). The reproductive ecology of female *Rana* (*Limnonectes*) *kuhlii*, a fanged frog of Taiwan, with particular emphasis on multiple clutches. *Herpetologica., 56*, 153−165.

Udaykumar, K., & Joshi, B. N. (1996). Ovarian follicular kinetics in response to enucleation and parietal shielding in *Rana cyanophlyctis*. *Gen. Comp. Endocrinol., 103*, 244−248.

Udaykumar, K., & Joshi, B. N. (1997). Effect of exposure to continuous light and melatonin on ovarian follicular kinetics in the skipper frog, *Rana cyanophlyctis*. *Biol. Signals, 6*, 62−66.

Van Oordt, P. G. W. J. (1960). The influence of internal and external factors in the regulation of the spermatogenic cycle in amphibian. *Symp. Zool. Soc. Lond., 2*, 29−52.

Varriale, B., Di Matteo, L., Minucci, S., Pierantoni, R., & Chieffi, G. (1988). Fat body involvement in vitellogenin fate in the green frog, *Rana esculenta*. *Comp. Biochem., 91A*, 175−178.

Vivas, A. B., Nicora, O., Di Tada, I. E., & Ibanez, N. (1995). Reproductive features of *Leptodactylus ocellatus* (Amphibia: Leptodactylidae) in embalse de Rio Tersero (Cordoba, Argentina). *Physis., 50B*, 13−18.

Wake, M. H. (1980). The reproductive biology of *Nectophrynoides malcolmi* (Amphibia: Bufonidae) with comments on the evolution of reproductive modes in the genus *Nectophrynoides*. *Copeia, 2*, 193−209.

Yoneyama, H., & Iwasawa, H. (1985). Annual changes in the testis and accessory sex organs of the bullfrog *Rana catesbeiana*. *Zool. Sci., 2*, 229−237.

Zhao, J., & Kung, H. F. (1994). Modulation of progesterone-induced *Xenopus* oocyte maturation by prostaglandin E1 and E2. *Cell. Mol. Biol. Res., 40*, 63−68.

Chapter 10

Hormones and Reproductive Patterns in Urodele and Gymnophionid Amphibians

David O. Norris
University of Colorado at Boulder, Boulder, CO, USA

SUMMARY
Urodele and caecilian amphibians exhibit four basic life-history patterns: biphasic (terrestrial and aquatic phases), paedomorphic (aquatic phase only), direct development (terrestrial phase only), and viviparity (aquatic and/or terrestrial phases). Reproductive cycles of representative patterns are described together with the hormonal factors involved. Most knowledge of the hormonal regulation of reproduction in urodeles is based on studies of males, and much more work needs to be done with females. Caecilian reproductive endocrinology is poorly known even where the life cycles have been described. Although a few amphibian species are expanding their ranges, many species are declining due to the threats of habitat destruction, pollution, and global warming, all of which together highlights the need for better understanding of the reproductive biology of all amphibians balanced with the preservation of the diversity of amphibian species, which are such integral parts of so many ecosystems.

1. INTRODUCTION

Urodela (Caudata, salamanders, and newts) and the Gymnophiona (Apoda, caecilians) are members of the vertebrate subclass Lissamphibia (class Amphibia). Reproduction in these groups (as in the Anura, frogs and toads; see Chapter 9, this volume) is timed by environmental factors operating through the central nervous system and the hypothalamus–pituitary–gonadal (HPG) axis. These environmental factors include physical factors (e.g., temperature, rainfall, photoperiod), ecological factors (e.g., food availability, suitable breeding or nesting sites), and behavioral and chemical cues from conspecifics. Activation of the HPG axis results in gametogenesis, mating, and production of offspring. Other hormones including prolactin (PRL), thyroid hormones (THs), and adrenal hormones (glucocorticoids) may be involved, as well as numerous cytokines (e.g., paracrines) and pheromones. Although most studies show that reproductive patterns of seasonally breeding amphibians are annual, biennial patterns shown in some species may occur more commonly than generally assumed (see Barker Jorgensen, 1992).

In the following sections, life-history scenarios are coupled with what is known about endocrine regulation of these events in a variety of taxa. The names of taxa used in this chapter are based on Larson, Weisrock, and Kozak (2003) and Wilkinson and Nussbaum (2006). Many details of reproduction in these groups can be found in several comprehensive works including Taylor and Guttman (1977), Norris and Jones (1987), Barker Jorgensen (1992), Polzonetti-Magni (1999), Jamieson (2003), Sever (2003), Heatwole (2005), and Exbrayat (2006a).

2. LIFE-HISTORY PATTERNS IN URODELE AMPHIBIANS

Salamanders and newts comprise the taxon Urodela and exhibit the greatest life-history variations of any vertebrate taxon of equivalent rank (see Bruce, 2003). There are just over 500 extant species of urodele in contrast to almost 5000 extant species of anuran and fewer than 200 extant species of caecilian. Of these groups, salamanders and newts anatomically more closely resemble ancestral amphibians as well as the presumed progenitors of the reptiles, and they occupy a range of habitats that overlap with both fishes and reptiles. Some species are totally aquatic (resembling ancestral sarcopterygian fishes); many are aquatic as larvae and, following metamorphosis, become terrestrial or semi-terrestrial as adults (suggestive of the earliest vertebrate invaders of land); and still others are totally terrestrial (as is the case for modern reptiles).

The name 'urodele' is derived from the Greek *uro* meaning 'tail,' as they are the only amphibians with distinct tails as adults. An alternative name for this group, Caudata, is also derived from a word for 'tail.' Additionally, urodele amphibians are characterized by considerable longevity, with the majority of species exceeding 10 years of life in

the natural environment and with numerous species living 20−30 years (see review by Bruce, 2003).

Urodeles exhibit four basic reproductive patterns with variations that reflect their selection of breeding and non-breeding habitats as well as modifications in their life-history patterns: (1) the biphasic cycle characteristic of amphibians; (2) a paedomorphic, entirely aquatic cycle; (3) a terrestrial or land cycle with direct development from egg to metamorphosed juvenile upon hatching; and (4) a truly viviparous cycle (see Figure 10.1). Viviparity in salamanders is detailed in Chapter 6, this volume, and will not be discussed here. This way of categorizing reproductive cycles differs from that used by Houck and Woodley (1995), who focused on breeding times irrespective of habitat choice.

Additionally, there are numerous unisexual populations of all-female salamanders in the genus *Ambystoma* that are found in the northeastern USA. Studies reviewed by Bogart (2003) reveal that these 'species' are not parthenogenetic as originally supposed but depend on mating with males of a bisexual species in order to have their eggs undergo development. This complicated and unique reproductive mode recently has been named kleptogenesis (see Bogart, Bi, Fu, Noble, & Niedzwiecki, 2007).

2.1. Biphasic Life Cycle

The most primitive reproductive pattern in urodeles is the biphasic life cycle, which also characterizes most anuran amphibians (see Chapter 9, this volume). This biphasic pattern is characteristic of newts and many salamanders. Adults exhibiting this pattern lay eggs in water, where they hatch into free-living larvae. Unlike anuran tadpoles, the young urodeles emerge from the egg as aquatic larvae with external gills and already have four limbs emerged. Further, unlike the case for the anuran tadpole, only the tail fin of the urodele larva regresses at metamorphosis and the bulk of the tail is retained. Whereas most anuran larvae are herbivorous, salamander and newt larvae are largely carnivorous.

Weeks, months, or in some cases years later, a biphasic urodele larva will undergo a complex metamorphosis, involving gross morphological and physiological changes, to become a more terrestrial juvenile. After a second period of growth, the terrestrial juvenile undergoes sexual maturation and seeks out water to reproduce. The resumption of an aquatic existence for breeding may be accompanied by some minor body changes. In some species, the metamorphosed individuals may continue to reside in the aquatic habitat and spend little or no time on land, eventually becoming sexually mature.

2.2. Paedomorphic Life Cycle

The second reproductive pattern involves heterochrony; i.e., evolutionary changes in rate and/or timing of developmental events. In this case, aquatic larvae become

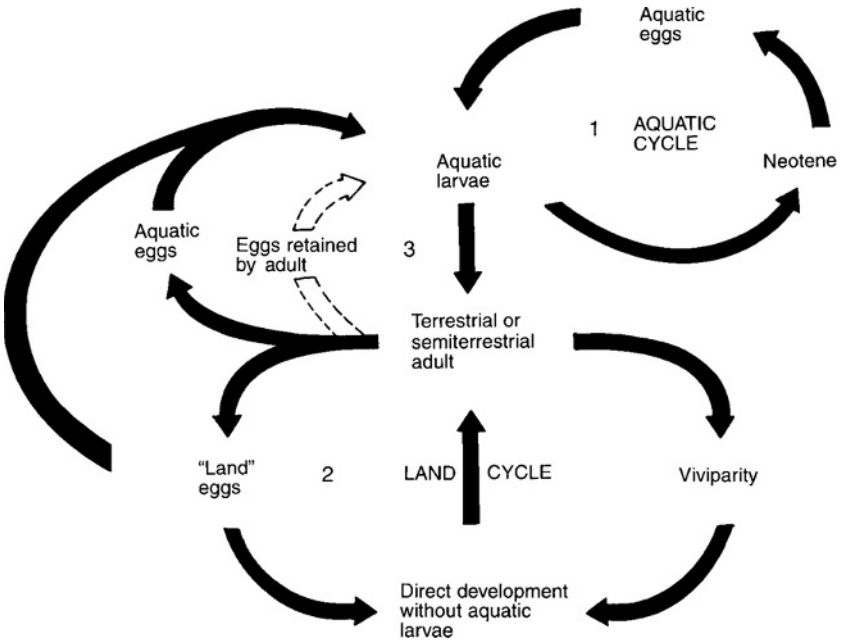

FIGURE 10.1 Amphibian life history patterns exhibited by anurans, urodeles, and caecilians. Animals in the aquatic cycle (1) may remain as paedomorphic larvae (Neotenes). The land cycle (2) may involve viviparity or direct development within an egg oviposited on land. Some individuals may retain the eggs within or on their bodies, where they complete metamorphosis prior to release, whereas others may deposit the larvae in water, where they complete their development. Most species exhibit a biphasic pattern (3) with a terrestrial or semi-terrestrial adult phase, returning to water to breed. Some animals with 'land eggs' may have a free-living larval form. *Reprinted with permission from Norris, D.O. (2007) 'Vertebrate Endocrinology,' Academic Press, San Diego.*

sexually mature prior to or without ever undergoing metamorphosis, a process termed paedomorphosis. Such animals are referred to as paedomorphs and often show little or no metamorphic change from the larval morphology. Some species exhibit obligate paedomorphosis whereas others exhibit facultative paedomorphosis where individuals within a population or certain entire populations of a species achieve sexual maturation without prior metamorphosis. In some species, the mature paedomorph may later undergo metamorphosis.

Certain species can be described as partial paedomorphs in that they exhibit some metamorphic changes characteristic of the biphasic life cycle (also called dissociative paedomorphosis), such as all species within the Cryptobranchidae and Amphiumidae as well as some plethodontid salamanders. In a few cases, animals remain virtually larval in body form or exhibit complete paedomorphosis; e.g., the Sirenidae (*Pseudobranchus*, *Siren*) and the closely related Proteidae (*Necturus*) as well as individuals or populations of some of the facultative species (e.g., *Ambystoma* spp.) The process of paedomorphosis may involve changes in the rate or timing of somatic tissue development or of reproductive development. In the case of retarded somatic development, the process has been referred to as neoteny and the paedomorph as a neotene. Progenesis refers to accelerated reproductive maturation with no change in the rate or timing of somatic development. One may infer that a complete paedomorphic species exhibits neoteny or progenesis only by inference from its phylogenetic position with respect to non-paedomorphic species. However, in the case of facultative paedomorphs, one can compare the timing of sexual maturation in the paedomorphic population with that of the metamorphosing population. Such comparisons reveal, as expected, that progenesis and neoteny are not mutually exclusively processes and that it often is difficult to characterize a given facultative species as neotenic or progenetic. Hence, all paedomorphic species will simply be termed paedomorphic in this review and the reader wishing to examine these topics more closely is referred to discussions by Gould (1977), Alberch, Gould, Oster, and Wake (1979), Reilly, Wiley, and Meinhardt (1997), Gould (2000), and Bruce (2003).

2.3. Direct Development in Fully Terrestrial Species without a Larval Stage

The third pattern of urodele reproduction involves an early acceleration of somatic development such that metamorphosis occurs during development within the egg and hence prior to hatching. Eggs are not deposited in ponds, as characterizes most amphibians, but are retained on land. Adults may spend considerable time tending the eggs or may simply deposit them in a relatively protected place and abandon them. This often is accompanied by a reduction in the number of eggs but with a concomitant increase in the size of the eggs. At hatching, the hatchling appears as a miniature version of the adult and there is no free-swimming larval form. In other words, metamorphic changes take place during development within the confines of the egg. This allows these salamanders to lay their eggs on land, where they can develop free from aquatic predators. The larger terrestrial egg size ensures sufficient stored energy to carry the young through the metamorphic process without the need for a free-living and feeding aquatic larval period. Thus, a fully transformed terrestrial juvenile emerges from the egg at hatching. Sexual maturation takes place after a period of growth to the adult body size, but does not appear to be accelerated as in some paedomorphic species (see Bruce, 2003). This pattern is termed direct development and is characteristic of many plethodontid salamanders, although it occurs elsewhere in the urodeles, too. Although these animals have been studied from an ecological and evolutionary perspective, little is known of their attendant endocrinology.

2.4. Viviparity

Viviparity among urodeles is limited to the European species of *Salamandra*. Greven (2003) proposed the term larviparity to refer to species that give birth to larvae, such as *Salamandra salamandra*, and pleuriparity for birth of completely metamorphosed offspring such as is the case for *Salamandra atra* (see Chapter 7, this volume). Little is known about the endocrinology underlying viviparity in these species, however.

3. ECOLOGICAL FACTORS AFFECTING THE TIMING OF REPRODUCTION

Regardless of the pattern of reproduction employed, urodele reproductive cycles can be further characterized by the timing of major reproductive events. Temperate species and many tropical species exhibit seasonal cycles determined primarily by temperature (temperate species) and the cycling of wet and dry seasons (tropical species). A few species have been described that appear to have aseasonal or continuous reproductive cycles. Reproduction may occur annually, biennially, or irregularly. Reproductive cycles may be associated or dissociated between the sexes in a single species (see Crews, 1984; Houck & Woodley, 1995). In some urodele species, peak steroid secretion is dissociated from mating, which may occur weeks or months later. For example, in the tiger salamander, *Ambystoma tigrinum*, spermatogenesis and peak androgen secretion in males occur during autumn (September–October) whereas mating occurs in the spring (March–May); i.e., these males exhibit a dissociated cycle. In

females of this species, oogenesis, estrogen secretion, and mating are all associated. In associated species spermiation, ovulation, and peak steroid secretion occur at the same time, after spermatogenesis and oogenesis are complete. It is also important to note that mating in some associated species with internal fertilization may occur at one time of year but oviposition may take place at a much later time when males may even be absent (e.g., in the mudpuppy *Necturus maculosus* (Harris, 1961)).

3.1. Roles for Hormones in Reproduction of Urodeles

Hypothalamic regulation of gonadotropin (GTH) secretion via gonadotropin-releasing hormone (GnRH), the roles of GTHs on gonadal secretions, and the actions of gonadal steroids and other hormones on reproductive physiology and behavior have been studied more extensively in anuran amphibians, but data on urodeles indicate that many similarities exist. The endocrine system of amphibians and its regulation have been reviewed recently, and only a brief summary is provided here (see Fernandez & Ramos, 2003; Kikuyama, Tanaka, & Moore, 2003; Norris & Lopez, 2005; Rastogi et al., 2005; Norris, 2007; see also Chapter 9, this volume).

Kikuyama et al. (2003) recently reviewed hypothalamic regulation of GTH secretion in urodeles and concluded that the urodele GnRH system operates in a similar manner to that of anurans, as demonstrated mostly in studies of *Xenopus laevis* and *Rana catesbeiana* and a few other species (Fernandez & Ramos, 2003; Rastogi et al., 2005; see also Chapters 2 and 9, this volume). Perhaps the most striking hypothalamic deviation from the anuran system is the observation that both mammalian GnRH (mGnRH) neurons and chicken II GnRH (cIIGnRH) neurons terminate in the median eminence of urodeles, as compared to anurans, where cIIGnRG neurons are confined to the midbrain. In response to GnRH stimulation, amphibian pituitaries produce two GTHs, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), that initiate gametogenesis and steroidogenesis by the gonads (details of spermatogenesis and oogenesis appear in Chapters 3 and 4, this volume, respectively).

3.2. Hormonal Regulation in Male Urodeles

Luteinizing hormone stimulates the synthesis of androgens by the gonads and FSH is responsible for their conversion to estrogens by stimulating aromatase production. Testosterone (T) and 5α-dihydrotestosterone (DHT) are the principle androgens secreted by urodele testes (Specker & Moore, 1980; Tanaka & Takikawa, 1983; Garnier, 1985a; Norris, Norman, Pancak, & Duvall, 1985; Woodley, 1994). The presence of 11-ketotestosterone (11-KT), an androgen characteristic of teleostean fishes (see Volume 1, Chapter 3), as well as other minor androgens also has been reported (Bolaffi, Lance, Callard, Walsh, & Idler, 1979). Androgens are responsible for stimulating the seasonal growth of the vas deferens, hypertrophy of the epithelium of the vas deferens, and development of cloacal glands (Norris, Duvall, Greendale, & Gern, 1977; Norris, 1987). Additionally, androgens apparently play a stimulatory role in the mating behavior of most species (Deviche & Moore, 1988; Iwata, Toyoda, Yamamoto, & Kikuyama, 2000b; Kikuyama, Hasunuma, Toyoda, Haraguchi, & Tsutsui, 2009). Androgens also stimulate development of nuptial pads on the limbs, which are used for clasping the female during courtship (Garnier, 1985a; Houck & Arnold, 2003). Contraction of the vas deferens necessary to transport sperm can be induced by arginine vasotocin (AVT) but also by the neurotransmitters norepinephrine or acetylcholine (Zoeller, Lais, & Moore, 1983).

Follicle-stimulating hormone stimulates spermatogenesis (Abe & Ji, 1994) but the role of androgens in spermatogenesis is unclear. Although some studies (e.g., Moore, 1975) indicate an enhancing role, in other cases low androgens are associated with spermatogenesis and high androgens with cessation of spermatogenesis (e.g., Lazard, 1979; Specker & Moore, 1980). In newts and other Salamandridae, there are two seasonal, circulating androgen peaks (Specker & Moore, 1980; Tanaka & Takikawa, 1983; Garnier, 1985a; Woodley, 1994; Houck & Woodley, 1995), one in the fall associated with development of the vasa deferentia and a second peak in the spring during breeding. Other species, such as *Plethodon jordani* (Woodley, 1994) and the tiger salamander (Norris et al., 1985), exhibit only a single peak of androgen secretion in the late summer and autumn (see also Houck & Woodley, 1995). In these latter species, elevated androgen levels do not occur in the circulation during spring mating.

3.3. Hormonal Regulation in Female Urodeles

Ovarian development is under the control of pituitary FSH whereas ovulation is regulated by LH acting through stimulation of progesterone (P_4) production (Norris & Duvall, 1981). The major estrogen reported in amphibians is 17β-estradiol (E_2) but ovaries also produce estrone as well as T, DHT, and P_4 (Cayrol, Garnier, & Deparis, 1985; Garnier, 1985b).

Oviductal development is stimulated by E_2 as well as by androgens (Norris, Carr, Summers, & Featherston, 1997). 17β-estradiol also stimulates jelly production by the oviductal epithelium although maximal secretion is obtained with E_2 plus PRL. Jelly is applied to the eggs as they pass through the oviduct on their way to the cloaca and

ultimate oviposition. Progesterone secreted by the short-lived postovulatory corpora lutea apparently prepares the oviduct for responding to AVT released from the pars nervosa (Guillette, Norris, & Norman, 1985). Arginine vasotocin stimulates contractions in the P_4-primed oviduct, and these contractions are essential for the process of egg-laying.

3.4. Hormonal Control of Courtship and Mating in Urodeles

Courtship and mating patterns have been described in all the major urodele groups (see Salthe, 1967; Moore, Boyd, Chu, & Hyde, 2005) and recently reevaluated by Houck and Arnold (2003). The pattern employed is fundamentally different in the most primitive salamanders, which employ external fertilization, as compared to the majority, which employ internal fertilization. Courtship among salamanders and newts with internal fertilization generally involves the male clasping the female in some characteristic manner (amplexus), setting down a spermatophore on the substrate, and inducing the female to pick up the sperm cap from the top of the spermatophore with her cloaca. Pheromones produced by sexes appear to play important roles in courtship by both aquatic breeders (e.g., red-bellied newts, *Cynops pyrrhogaster*, and the axolotl, *Ambystoma mexicanum*) and terrestrial breeders (e.g.,, the red-legged salamander *Plethodon shermani*) although the hormonal regulation of pheromone production has not been studied extensively (Iwata et al., 2000a; 2000b; Park, McGuire, Majchrzak, Ziobro, & Eisthen, 2004; Houck, 2009; Kikuyama et al., 2009).

Migration of adult urodeles to the breeding site (so-called water drive) is stimulated by PRL from the pituitary gland (see Dent, 1975). Additionally, PRL increases tail height (e.g., Platt, 1976; Moore, Seide, Specker, & Swanson, 1978), especially in males who often use their tails in courtship (Iwata et al., 2000b; Houck & Arnold, 2003). In hypophysectomized or gonadectomized red-bellied newts (*C. pyrrhogaster*), PRL and androgens are necessary for full restoration of tail vibrating, a common courtship behavior among urodeles, and treatment of intact males with PRL antibody decreases this behavior (Iwata et al., 2000b). Tail vibrating is controlled by giant Mauthner neurons located in the hind brain. Mauthner neurons are larger in sexually active male *C. pyrrhogaster* than in reproductive females or sexually inactive males (Iwata et al., 2000b). Further, hypophysectomy reduces the size of Mauthner cells, but the size is restored following treatment with PRL and androgens (Suzuki & Kikuyama, 1987; Matsumoto et al., 1995).

Zerani, Amabili, and Gobbetti (1992) reported that E_2 plasma levels and brain aromatase activity peak at the beginning of courtship in the males of *Triturus carnifex*, suggesting a role for estrogens possibly derived in the brain from circulating T. However, in *C. pyrrhogaster*, E_2 was ineffective at inducing courtship behavior in gonadectomized males even in the presence of PRL, and E_2 actually prevented the PRL-induced increase in tail height in this species (Iwata et al., 2000b). Obviously, more work needs to be done to clarify possible roles of estrogens in male courtship behavior.

In the rough-skinned newt, *Taricha granulosa*, amplexus involves clasping of the back of a female from above. Males have special androgen-dependent pads on all legs that aid in clasping. This neural-based behavior is induced with AVT in the presence of either androgens (Moore & Miller, 1983; Rose, Kinnaird, & Moore, 1995) or estrogens (Moore, Wood, & Boyd, 1992). Stress or the application of the stress hormone corticosterone (CORT) to a reproductively active male blocks clasping and causes immediate release of the female by a clasping male (see Rose, Moore, & Orchinick, 1993).

4. FERTILIZATION

In marked contrast to anuran amphibians, fertilization is internal in all but the most primitive groups of urodeles (Cryptobranchidae, Hynobiidae, Sirenidae). Transfer of sperm from male to female usually is accomplished through the deposition on the substrate of a stalked spermatophore. In a few closely related species, transfer of sperm may occur through cloacal apposition, suggesting that this mode of internal fertilization evolved only once in the urodeles (Houck & Arnold, 2003).

A spermatophore consists of a gelatinous stalk with a cap containing sperm and numerous proteins. For species that mate in aquatic habitats, spermatophores may be deposited on pond or stream bottoms. In the case of terrestrial species, the spermatophore is deposited on land. Male urodeles use visual and chemical cues to encourage a female to maneuver her cloacal opening over the spermatophore and to remove the cap containing the sperm. Mating can be relatively simple or involve extremely complicated steps. Additionally, in the case of the newts in the genus *Taricha*, a male will actually grasp the female in an amplectic clasp similar to that described in anurans. However, in the case of *Taricha*, amplexus is a prelude to behaviors that involve transfer of the spermatophore, whereas most anurans employ external fertilization as the eggs are shed by the female. Once a female *T. granulosa* appears in a pond, many males attempt to clasp her, forming large mating balls of males wrapped around a single female, but ultimately one male persists and actually completes spermatophore transfer to the female. It appears that the female secretes chemicals that attract the males. A similar sequence of events is reported for *Taricha rivularis* (Davis & Twitty, 1964).

5. REPRESENTATIVE URODELE REPRODUCTIVE CYCLES

I have chosen to illustrate urodele reproductive cycles by discussing three specific cases: the Eastern newt, *Notophthalmus (Triturus, Diemictylus) viridescens*; the Pacific newts, *Taricha (Triturus)* spp; and the paedomorphic tiger salamander, *Ambystoma tigrinum*.

5.1. The Eastern Newt, *Notophthalmus viridescens*

The eastern newt consists of several subspecies that inhabit the USA east of the Mississippi River, from the Gulf of Mexico northward and extending into the adjacent southern parts of Canada. It includes the red-spotted newt, *N. v. viridescens*, which is the primary basis for the following account. The eastern newt exhibits a biphasic reproductive cycle, typically breeding in ponds in April and May (Adams, 1940). After a period of rapid growth, the resultant larvae undergo metamorphosis and begin to migrate from the breeding ponds in the late summer and fall during rainy periods (Healy, 1975). The metamorphosed juvenile animal appears as a special terrestrial form known as the red eft. These terrestrial efts undergo a period of growth followed by sexual maturation and a return to the water to breed. The stimulus for this 'water drive' behavior is attributed to pituitary secretions, and migration of the efts to the breeding pond is caused by PRL (Reineke & Chadwick, 1939; 1940; Chadwick, 1941). In addition to migrating to the breeding ponds, efts undergo what has been called a second metamorphosis to an aquatic phase adult form, which involves molting of the keratinized reddish skin and its replacement with an olive-colored, smooth, non-keratinized skin as well as an enlargement of the tail fin. This second metamorphosis involves interactions of PRL and THs (Grant & Grant, 1958). Although adults may remain in the ponds after breeding (Healy, 1975), they often undergo another molt and leave the pond with a more keratinized skin to return to a terrestrial existence until the next breeding season.

During sexual maturation or gonadal recrudescence, gonads and sex accessory structures develop, and there undoubtedly is an increase in circulating hormones although we can only infer the latter from studies in other species. Testis weights reach a maximum in August and then decline as spermiation occurs during September and October. Sperm are stored in the vas deferens until the following spring. Studies in other species support the contention that spermatogenesis is stimulated by the secretion of pituitary FSH (e.g., Abe & Ji, 1994). Androgen secretion probably is lowest during the onset of spermatogenesis, as reported in a number of other species (e.g., Specker & Moore, 1980; Garnier, 1985a; Norris et al., 1985) (see Figure 10.2). Evidence of post-spermatogenetic androgen secretion is found in the development of the cloacal complex of glands (smallest in August to maximal in April), development of nuptial pads on the limbs that are used in courtship (present from September through June), and enlargement of the vasa deferentia (September through April). This suggests that pituitary LH is being secreted at this time to support androgen synthesis.

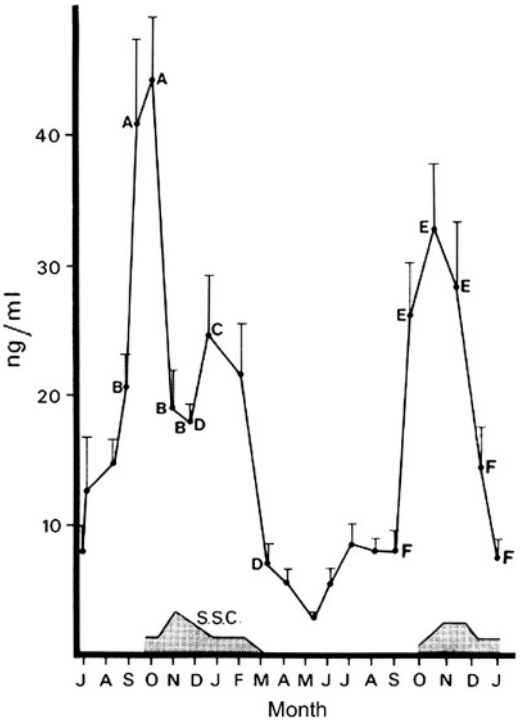

FIGURE 10.2 Seasonal changes in total androgens in captive male *Pleurodeles waltl*. Plasma androgens levels correspond to increased development of the thumb pads, a secondary sex characteristic (SSC). Total androgens are a mixture of testosterone, dihydrotestosterone, and androstenedione estimated from radioimmunoassay using a non-specific testosterone antibody. A significantly different from B ($p<0.001$), C from D ($p<0.01$), E from F ($p<0.0001$). *Reprinted with permission from Garnier, D.H. (1985a).*

Mating in the ponds may occur in the fall, winter, or spring, with oviposition usually occurring in the summer. In female efts, ovaries begin development in the summer but, because the process of oogenesis takes longer than spermatogenesis, females tend to appear at the ponds later than males in the fall and may not appear until spring for breeding. Some large yolked eggs are retained in the ovary after spawning and atretic follicles commonly are present until spring or summer. Development of the oviducts parallels the development of the ovaries, presumably a response to increased estrogen secretion. Sperm have been described in the spermatheca of female efts or aquatic females throughout the year, but most are seen during the fall and spring as a consequence of recent matings (Adams, 1940). The only study of seasonal plasma estrogen levels in

female urodeles was conducted in *Pleurodeles waltl*, (Cayrol et al., 1985; Garnier, 1985b). Ovarian weight was lowest for *P. waltl* in September following oviposition, began increasing in October, reaching the maximal level in December, and remained there until July, after which oviposition occurred. Plasma E_2 (Figure 10.3) increased from August through November with a second peak in February−March, then declined to its lowest level in May. Estrone levels were very low throughout the year with no significant fluctuations. Androgen levels were several-fold greater than E_2 (mostly due to T) and paralleled the increases seen in E_2. Although Garnier (1985b) reported the elevation of E_2 and T in February−March, this peak was not observed the following year (Cayrol et al., 1985). Unfortunately, no information on oviduct condition was reported for either study of *P. waltl*.

Mating behaviors do not include amplexus in *N. viridescens*, and this is true for most urodeles studied. Males apparently are attracted to females by pheromones (Park & Propper, 2001), and these may be secreted in response to PRL and E_2, as shown for the red-bellied newt, *C. pyrrhogaster* (Toyoda, Tanaka, Matsuda, & Kikuyama, 1994). Pheromones secreted by the males along with tail vibrating

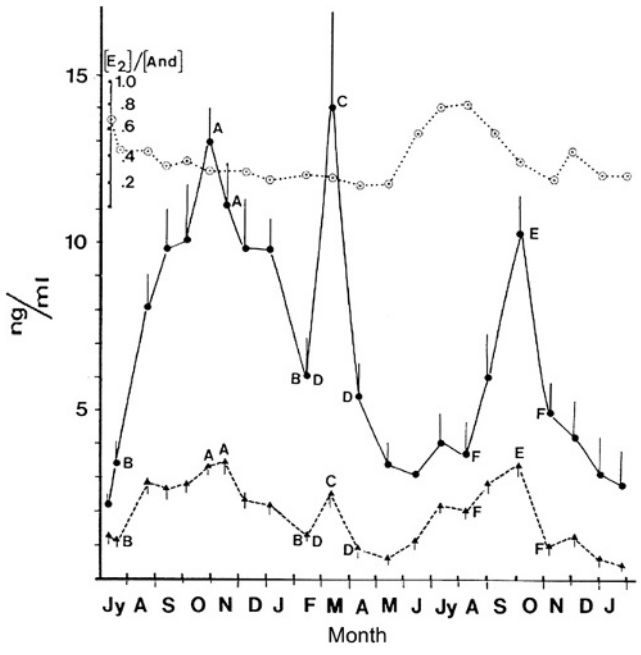

FIGURE 10.3 Seasonal changes in plasma steroids of female *Pleurodeles waltl*. Estradiol (E_2; solid triangles) and total androgens (And; solid circles) in captive females show hormonal peaks in the fall (September−October) as well as in the spring (February−March). Note that androgen levels are generally much greater than E_2 levels. Total androgens are a mixture of testosterone, dihydrotestosterone, and androstenedione estimated from radioimmunoassay using a non-specific testosterone antibody. The ratio of E_2 to total androgens is represented by the open circles. A significantly different from B ($p<0.01$), C from D ($p<0.01$), E from F ($p<0.005$). *Reprinted with permission from Garnier, D.H. (1985b).*

and blocking behaviors appear to be employed to elicit female responses in a number of newt species (see Moore et al., 2005). In *C. pyrrhogaster*, PRL and T are thought to be responsible for pheromone production (Iwata et al., 2000b; Kikuyama et al., 2009), although AVT may be involved in controlling release of the pheromone from the cloacal glands (Kikuyama et al., 2001). Testosterone levels generally are elevated in male newts during mating.

5.2. Pacific Newts, *Taricha* species

Studies of the Pacific newts in the genus *Taricha* include some hormonal data and provide considerable support for conclusions about endocrine events inferred for the eastern red-spotted newt, since these groups have similar life cycle patterns. According to Twitty (1941) and Miller and Robbins (1954), adult male California newts, *Taricha torosa*, begin migrating to ponds with the onset of rains in October about six to eight weeks before breeding ensues (December−February). Testes are already at maximal size when migration begins. A similar pattern is observed for the more northerly rough-skinned newt, *T. granulosa* (Specker & Moore, 1980). Sex accessory characteristics in male *T. torosa* begin to enlarge at the beginning of the migration/breeding season, including enlargement of various skin and cloacal glands as well as an increase in tail height and length. About four to six weeks later, spermiation occurs, followed by an increase in testicular interstitial tissue about two weeks prior to actual mating. Mating appears to involve elevated levels of both T and AVT in male *T. granulosa* (see Moore et al. 2005).

In female *T. torosa*, yolk deposition begins during the summer terrestrial estivation period and continues through migration, requiring a total of 5−6 months to achieve full oocyte development. Oviductal development begins about the time of migration, suggesting increased estrogen synthesis at that time. Following oviposition, the ovaries and oviducts regress over the next few weeks prior to resumption of a new cycle in July.

5.3. The Tiger Salamander, *Ambystoma tigrinum*

The tiger salamander is unique among salamanders in that it ranges throughout most of North America, from Canada to Mexico and from the Atlantic to the Pacific coast. In most regions, it exhibits the typical biphasic life cycle pattern that characterizes amphibians. However, it also undergoes a facultative paedomorphic life cycle, especially in the arid southwest of the USA, where it closely parallels the life cycle of the more southern Mexican axolotl, *A. mexicanum*, and overlaps with the axolotl in part of its range. In some southwestern locations, tiger salamanders are obligate paedomorphs similar to the axolotl.

Tiger salamanders breed in the late winter/early spring, depending on the climate. In environments where ponds tend to be ephemeral, *A. tigrinum* larvae may undergo metamorphosis during or at the end of their first summer and escape before the pond dries. Early field studies suggested that the stresses associated with pond drying, for example as a consequence of increased density of conspecifics (crowding), increased salinity, warmer temperatures, etc., were responsible for initiating metamorphosis. Long photoperiods are capable of activating the hypothalamus−pituitary−thyroid (HPT) axis and causing metamorphosis under crowded laboratory conditions (Norris et al., 1977), and could be the environmental trigger necessary to stimulate endocrine changes underlying metamorphosis. Frequently in arid environments where ponds are permanent, *A. tigrinum* larvae may become sexually mature and remain in the pond. Paedomorphosis has the advantage of providing a period of rapid growth and earlier sexual maturation in the pond, whereas metamorphosed individuals on land would have to contend with arid conditions and scarce food, and sexual maturity would be delayed. Thus, paedomorphs could mature sooner and reproduce sooner.

Some paedomorphs appear to retain the ability to undergo metamorphosis at a later time, should conditions change; i.e., during a long-term drought. Clearly, retention of these alternate strategies in the same population is beneficial for survival in an unpredictable environment. Additionally, there are populations that exist as a mixture of paedomorphs and individuals that undergo metamorphosis.

Prolactin appears to antagonize metamorphosis in larval amphibians (see Norris, 2007). Metamorphosis is accompanied by a decrease in PRL-based effects, as evidenced by a decrease in tail fin height as well as other changes associated with acquisition of a terrestrial habitat; e.g., loss of gills and skin changes (e.g., see Norman, 1985). Dopamine (DA) from the hypothalamus functions as a PRL release-inhibiting hormone (PRIH) in amphibians as it does in other vertebrates. Prolactin treatment is known to increase the height of the tail fin in larvae, and paedomorph pituitaries contain more PRL than do pituitaries of metamorphosed tiger salamanders (Norris, unpublished). Further, treatment of paedomorphic larvae with a DA antagonist causes tail fin regression (Platt, 1976).

Some interesting mechanisms have evolved among these salamanders occupying permanent ponds to prevent overpopulation. When larval tiger salamanders are confined in a limited space within ponds where food resources become limited, cannibalism may occur. Larger larvae readily consume smaller ones. In some cases, all first-year larvae may be eliminated by larger second-year larvae still present in the pond (Burger, 1950). Indeed, morphological adaptations for cannibalism have been described in some populations (Figure 10.4) (Armentrout, 1973, Rose & Armentrout, 1976). Paedomorphs also may impact reproductive success and prevent overcrowding through consumption of eggs. For example, *A. tigrinum* eggs have been found in the stomachs of adult females after they have completed spawning (Hamilton, 1949; Norris, 1989).

Prolactin also may be involved with the return of terrestrial adults of facultative populations to the breeding pond (i.e., water drive). Administration of PRL causes a preference by metamorphosed tiger salamanders to remain in the water whereas administration of THs induces a preference for a dryer substrate (Duvall & Norris, 1977; 1980; Moriya & Dent, 1986). Hence, in response to environmental stressors such as crowding or pond drying, metamorphosed individuals that experience elevated THs and decreased PRL leave the pond and assume a terrestrial existence.

Breeding of tiger salamanders occurs mainly from December to March with earlier dates in warmer climates (Twitty, 1941; Hassinger, Anderson, & Dalrymple, 1970; Anderson, Hassinger, & Dalrymple, 1971; Tanner, Fisher, & Willis, 1971; Armentrout, 1973; Semlitsch, 1983; Norris et al., 1985), although instances of oviposition in the fall have been described in some desert populations (Webb & Rouche, 1971). Mating and transfer of the spermatophore from the male to the female occur just prior to oviposition, as evidenced by the presence of sperm in the spermathecae of females (Figure 10.5). Gonadal recrudescence resumes soon after breeding, and spermatogenesis and oogenesis accelerate during the summer (July−September), when food is more abundant.

Male tiger salamanders exhibit a dissociated reproductive cycle in that peak androgen secretion occurs in the fall in association with spermatogenesis and storage of sperm in the vasa deferentia whereas mating takes place in the early spring (Figure 10.6). Spermatogenesis is

FIGURE 10.4 Morphological forms of *Ambystoma tigrinum*. From left to right, typical paedomorphic larva, cannibal paedomorphic larva, metamorphosed cannibal morph, metamorphosed non-cannibal morph. *Photograph courtesy of Hobart M. Smith.* See color palte section at the end of the book.

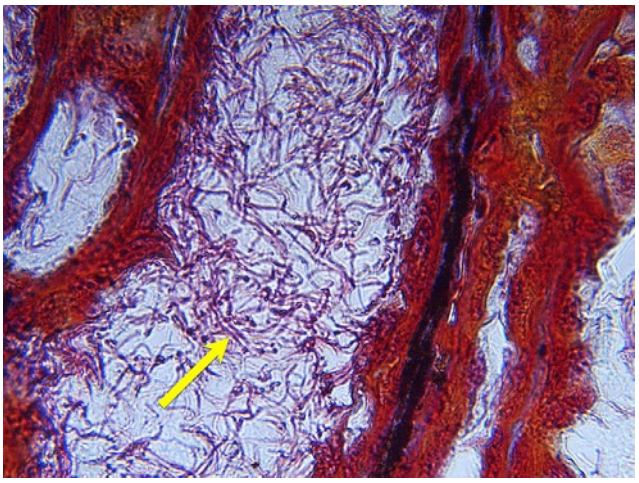

FIGURE 10.5 Presence of sperm in spermatheca of an adult female paedomorphic tiger salamander, *Ambystoma tigrinum*. Sperm (arrow) were observed in the lumina of spermathecal tubules only during the breeding season (late March to early May) and at no other time of year. See color plate section at the end of the book.

stimulated by mammalian FSH, and this is enhanced by PRL treatment (Table 10.1). Androgens appear to play an important role (Moore, 1975). Spermiation begins in September and the vasa deferentia are gorged with sperm by early November although sperm do not appear in the spermathecae of females until late March to early May. Androgen levels in males peak in October (Norris et al., 1985), as does development of the vasa deferentia, and androgens probably are responsible for the marked growth of the vasa deferentia and hypertrophy of their epithelia (Figure 10.7).

Differentiation of cloacal glands can be stimulated by treatment of larval male or female tiger salamanders with T, and this effect is enhanced by simultaneous treatment with PRL (Norris, Austin, & Hijazi, 1989). Cloacal glands exhibit increasing development in male paedomorphs, paralleling the increase in mean diameter of the vasa deferentia, with peak secretory activity evident during breeding (Norris, unpublished observations).

In paedomorphic females, oocytes grow rapidly during the summer months and achieve a large size by November, with additional growth occurring until breeding. Ovulation can be induced by GTHs *in vivo*

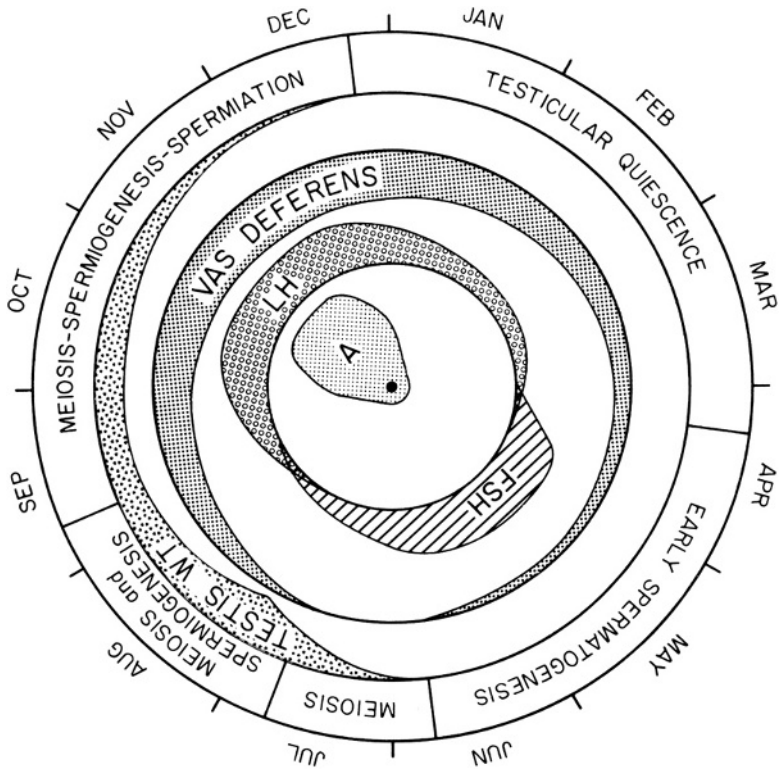

FIGURE 10.6 Seasonal reproductive events in paedomorphic male tiger salamanders, *Ambystoma tigrinum*. Timing of spermatogenesis events are depicted in the outermost circle. Variations in testicular weight and cross-sectional area of one vas deferens above the regressed conditions are shown in the next two inner circles. The central area represents the relative levels of testosterone plus dihydrotestosterone (A), which peak in the fall. Levels of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are hypothetical estimates based on events observed in the reproductive organs. *Modified with permission from Norris, D.O., Norman, M.F. Pancak M.K. and Duvall D. (1985).*

TABLE 10.1 Effects of mammalian follicle-stimulating hormone (FSH), prolactin (PRL), and thyroxine (T_4)[1] on testicular weight and spermatogenesis in immature tiger salamander larvae, *Ambystoma tigrinum*. *Data from Norris, D.O. and Norman, M.F., unpublished.*

Treatment (n)	Body Weight (g ± SEM)	Combined Testes Weight (mg ± SEM)[2]	Meiosis Present (% Animals)
Initial controls (5)	54 ± 3.7	210 ± 20[a]	nd
Saline (5)	56 ± 3.4	240 ± 50[a]	20
T_4 (6)	56 ± 3.4	280 ± 30	40
FSH (6)	56 ± 3.8	390 ± 50[b]	60
PRL (6)	56 ± 3.7	320 ± 40[b]	33
FSH + T_4 (6)	57 ± 2.9	380 ± 50[b]	20
FSH + PRL (6)	57 ± 3.2	440 ± 50[c]	83
T_4 + PRL (6)	57 ± 3.1	270 ± 40[a]	50
T_4 + PRL + FSH (6)	57 ± 2.9	380 ± 70[b]	100

[1] *TSH in a separate experiment had no effect on testicular weight, however.*
[2] *Values with different superscripts are significantly different ($p < 0.01$).*

or by LH or P_4 *in vitro*, and this effect is enhanced by PRL *in-vitro* (Norris & Duvall, 1981). Peak ovarian and oviductal weight follows the same pattern (Armentrout, 1973; Norris, unpublished) (Figure 10.8). Oviductal growth is stimulated in this species by estrogens as well as by androgens (Norris et al., 1997). Although seasonal data correlating circulating estrogens and androgens to oviductal weight and diameter are lacking for females of this species, oviductal weight and diameter as well as cloacal gland development in female *A. tigrinum* parallel increases in ovarian size, suggesting that increasing estrogen levels are correlated with these parameters, as shown for a few other species (e.g., Garnier, 1985b).

6. GYMNOPHIONA (CAECILIANS)

Although the reproductive biology of caecilians has been studied for many years, little is known about their reproductive endocrinology. There are only about 170 described species, about half of which are viviparous, and reproductive patterns of the remaining species vary from biphasic cycles to direct development (Wake, 2006). Because of their secretive lifestyles, their limited distributions, and the endangered nature of most species, neither the reproductive biology of field populations of caecilians nor detailed laboratory studies have been conducted as they have for anurans and urodeles.

In addition to the high incidence of viviparity in this group, there are some other unique reproductive features, especially in males. In the Gymnophiona, fertilization is always internal, and sperm transfer to the female is accomplished through the use by males of a copulatory organ called a phallodeum, associated with the cloacal complex of glands (Wake, 1977). Another interesting male reproductive structure is the Müllerian gland, which is possibly a functional homolog of the mammalian prostate gland, secreting chemicals similar to those associated with the reptilian epididymis and the mammalian ventral prostate and seminal vesicles (Akbarsha, Jancy, Smita, & Ommen, 2006). Müllerian ducts are retained in male caecilians as they sometimes are in certain anurans and urodeles. However, in caecilians, the posterior third of the Müllerian duct exhibits secretory activity during the breeding season and contributes these secretions to the seminal fluid.

Reproductive cycles (Wake, 1977) as well as the anatomy and histology of many reproductive organs (Exbrayat & Estabel, 2006) have been described for a number of species. Correlations exist between various reproductive parameters such as spermatogenesis, cloacal glands, oviductal development, and the abundance of pituitary gonadotropes, but data on the corresponding hormone levels are not available (see Exbrayat, 2006b). Nevertheless, the marked similarities between their anatomy and reproductive biology and that of other amphibian groups strongly suggest similar endocrine roles to those described in anurans and urodeles.

Gonadotropin-releasing hormone has been localized in the brains of viviparous *Typhlonectes natans* (Ebersole & Boyd, 2000), *Typhlonectes compressicaudus* (Rastogi, Meyer, Pinelli, Fiorentino, & d'Aniello, 1998), and oviparous *Ichthyophis beddomei* (Pinelli et al., 1997). Gonadotropic cells immunoreactive to mammalian LH and

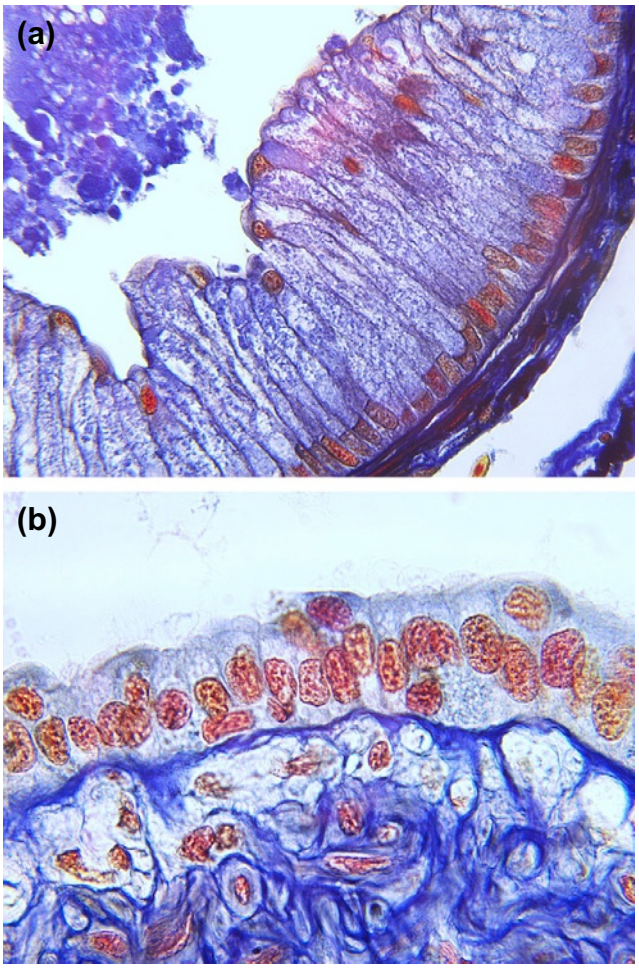

FIGURE 10.7 Vas deferens of paedomorphic tiger salamanders, *Ambystoma tigrinum*. (a) Vas deferens epithelium from a male captured in early November (40X). Secretion is visible in the upper left and at the luminal surface of the columnar epithelium. (b) Vas deferens from a male captured in early June. Note the minimal epithelial thickness and the thick connective tissue layer due to collapse of the vas deferens after evacuation of sperm. See color plate section at the end of the book.

lactotropes immunoreactive to mammalian PRL have been described in the pituitary of male *T. compressicaudus* (Doerr-Schott & Zuber-Vogeli, 1984), and these cells show seasonal increases in size with gonadal maturation and during breeding (which occurs during the rainy season), respectively. Further, the abundance of mRNA transcripts for PRL receptors in testes and Müllerian glands support a role for PRL in males during breeding (see Exbrayat, 2006b).

In females, gonadotropes and lactotropes reach maximal development at breeding and then decrease during gestation to reach minimal size by birth followed by recrudescence as the females prepare for the next breeding season (Exbrayat, 2006b). However, PRL receptor mRNA increases in the liver during pregnancy, suggesting another possible role for PRL during pregnancy (Exbrayat, 1988). In oviparous species, such as *I. beddomei*, oviducts exhibit a seasonal secretory cycle correlated with ovulation and oviposition, and the lumen is lined with ciliated epithelium during breeding. In viviparous species, the anterior portion of the oviducts exhibit changes similar to those described for oviparous species. However, this portion soon regresses after ovulation and probably has little influence on pregnancy. In contrast, the lower portion of the oviduct (the uterus) at first regresses only where it is in contact with developing embryos, although even these regions not associated with embryos progressively regress during pregnancy (see Exbrayat, 2006b). Ovarian steroidogenesis has not been studied extensively, but immunocytochemical methods have demonstrated the presence of 3β-hydroxysteroid dehydrogenase activity in previtellogenic and vitellogenic follicles as well as in the corpora lutea of *T. compressicaudus*, providing evidence of steroidogenic capability. However, no data are available on circulating steroids in any caecilian.

7. FUTURE DIRECTIONS

Endocrine research on model amphibian species, including urodeles, has provided fundamental discoveries about vertebrate endocrine systems, including the role of the pineal gland and melatonin as a mediator of photoperiod, the discovery of the olfactory origin of GnRH-secreting neurons and their subsequent migration into the brain, the discovery of a corticosteroid membrane receptor in the brain, and so on. The probable central position of urodeles on the main line of evolution in terrestrial vertebrates, the relative simplicity of urodele endocrine systems, and the adaptability of these animals to laboratory conditions make urodeles a favored group for basic research. Further, the critical ecological importance of amphibians to many ecosystems necessitates our knowing more about their reproductive biology. However, it is difficult to decide the proper direction for future research on amphibian reproductive endocrinology in light of the serious environmental and evolutionary situations facing modern amphibians.

From the standpoint of a comparative reproductive endocrinologist, it is clear that we need better studies among urodele species of circulating hormone levels correlated to specific events associated with hypothalamic, pituitary, and gonadal functioning as well as correlates with behavioral events. The lack of such correlations is especially evident in females. Certainly, there is much to be learned about the evolutionary history of reproductive events through the application of modern genomic approaches to endocrine functions in amphibians, which might provide additional insights on the aquatic to terrestrial transition of vertebrates as well as on fundamental aspects of reproductive endocrinology. Much remains to be learned about the interactions of nonapeptide hormones, prolactin, thyroid hormones, and glucocorticoids interacting with GTHs and gonadal steroid

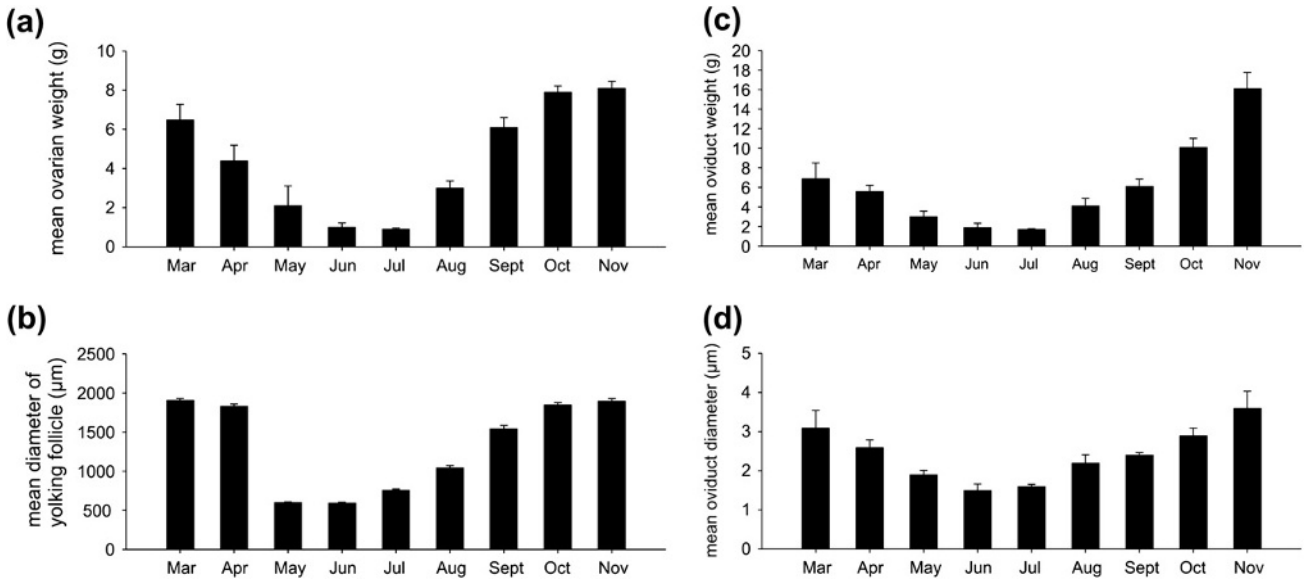

FIGURE 10.8 Seasonal variations in reproductive structures (± S.E.M.) of female paedomorphic tiger salamanders, *Ambystoma tigrinum*. Mean ovarian weight (a), mean oocyte diameter (b), mean oviduct weight (c), and mean oviduct diameter (d) exhibit significant changes throughout the reproductive seasonal cycle. These closely paralleled seasonal variations are likely to be positively correlated with unmeasured 17β-estradiol levels, as evidenced by correlations reported in anuran amphibians and other vertebrates. Figure prepared by Dr. Mathew Hale from unpublished data of D.O.Norris.

hormones as well as the involvement of a myriad of growth factors and other hormones. Urodeles also provide a fertile ground for the identification of pheromones and for the investigation of the roles of pheromones in vertebrate reproduction. Similarly, although numerous studies have examined the reproductive process in a few caecilians (see Exbrayat, 2006a), their reproductive endocrinology is practically an empty canvas waiting to be painted.

On the other hand, although a few invasive species are thriving, most species of modern amphibians are declining (Stuart et al., 2004) and globally are facing an unprecedented attack from human activities through destruction of habitat (e.g., draining of wetlands for home construction and/or agriculture; destruction of forests for lumber and fuel (see Stuart et al., 2004)); introduction of competing non-native species (e.g., the impact of introduced fish species on tadpole survival in Australia (see Gillespie & Hero, 1999)), diseases (e.g., chytrid fungus (Woodhams, Voyles, Lips, Carey, & Rollins-Smith, 2006), and parasitic trematodes (Stopper, Hecker, Franssen, & Sessions, 2002)); over-collection by scientists and non-scientists alike; and toxic chemical pollution (e.g., overtly toxic chemicals such as pesticides in aquatic systems (Mann & Bidwell, 1999; Fort et al., 2004) and estrogenic endocrine-disrupting chemicals (EDCs) (Kloas et al., 2009)). All of these factors can interact to accelerate decline; e.g., exposure to sublethal levels of a pesticide may increase the sensitivity of a population to invasion by parasitic trematodes (Kiesecker, 2002). However, in this author's opinion, the threat of EDCs in the environment may be the greatest threat of all to amphibians (and other vertebrates; see Diamanti-Kandarakis et al., 2009). Amphibians are especially vulnerable due to the nature of their life histories, the high permeability of their skin, and the widespread appearance of EDCs in surface waters that are capable of altering reproductive functions.

The current rate of extinction of amphibian species is estimated to be many times greater than historical rates based on the fossil record (McCallum, 2007). Further, recent climate changes and predictions of accelerated climate change due to global warming represent an additional threat that, through interaction with the above factors, could have catastrophic effects on amphibians. Clearly, researchers interested in reproductive endocrinology of wild populations of urodeles and caecilians must be cognizant of the importance of maintaining existing habitats, restoring damaged habitats, and increasing protection to enhance survival rates of wild amphibian populations. These goals must be balanced with the desire to understand their reproductive biology for both scientific value and conservation needs.

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ABBREVIATIONS

11-KT	11-Ketotestosterone
AVT	Arginine vasotocin
cIIGnRH	Chicken-II gonadotropin-releasing hormone
CORT	Corticosterone
DA	Dopamine
DHT	5α-dihydrotestosterone
E_2	17β-estradiol
EDC	Endocrine-disrupting chemical
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-releasing hormone
GTH	Gonadotropin
HPG	Hypothalamus−pituitary−gonad
HPT	Hypothalamus−pituitary−thyroid
LH	Luteinizing hormone
mGnRH	Mammalian gonadotropin-releasing hormone
P_4	Progesterone
PRIH	Prolactin release-inhibiting hormone
PRL	Prolactin
T	Testosterone
TH	Thyroid hormone

REFERENCES

Abe, S.-I., & Ji, Z.-S. (1994). Initiation and stimulation of spermatogenesis *in vitro* by mammalian follicle-stimulating hormone in the Japanese newt, *Cynops pyrrhogaster*. *Int. J. Dev. Biol., 38*, 201−208.

Adams, A. E. (1940). Sexual conditions in *Triturus viridescens*. III. The reproductive cycle of the adult aquatic form of both sexes. *Amer. J. Anat., 66*, 235−275.

Akbarsha, M. A., Jancy, J. M., Smita, M., & Ommen, O. V. (2006). Caecilian male Müllerian gland, with reference to *Uraeotyphlus narayani*. In J.-M. Exbrayat (Ed.), *"Reproductive Biology and Phylogeny of Gymnophiona (Caecilians)"* (pp. 157−182). Enfield NH: Science Publishers.

Alberch, P., Gould, S. J., Oster, G. F., & Wake, D. B. (1979). Size and shape in ontogeny and phylogeny. *Paleobiology, 5*, 296−317.

Anderson, J. D., Hassinger, D. D., & Dalrymple, G. H. (1971). Natural mortality of eggs and larvae of *Ambystoma t. tigrinum*. *Ecology, 52*, 1107−1112.

Armentrout, D. (1973). "Morphological, physiological, and behavioral polymorphism among populations of *Ambystoma tigrinum mavortium* residing in dissimilar habitats." *Ph.D. Dissertation*. Lubbock: Texas Tech University.

Barker Jorgensen, C. (1992). Growth and Reproduction. In M. E. Feder, & W. W. Burggren (Eds.), *"Environmental Physiology of the Amphibians"* (pp. 439−466). Chicago: University of Chicago Press.

Bogart, J. P. (2003). Genetics and systematics of hydrid species. In D. M. Sever (Ed.), *"Reproductive Biology and Phylogeny of Urodela"* (pp. 109−134). Enfield NH: Science Publishers, Inc.

Bogart, J. P., Bi, K., Fu, J., Noble, D. W. A., & Niedzwiecki, J. (2007). Unisexual salamanders (genus Ambystoma) present a new reproductive mode for eukaryotes. *Genome, 50*, 119−136.

Bolaffi, J. L., Lance, V., Callard, I. P., Walsh, J. M., & Idler, D. R. (1979). Identification of 11-ketotestosterone, 11β-hydrotestosterone, and testosterone in plasma of *Necturus maculosus* (Rafinesque). *Gen. Comp. Endocrinol., 38*, 127−131.

Bruce, R. C. (2003). Life histories. In D. M. Sever (Ed.), *"Reproductive Biology and Phylogeny of Urodela"* (pp. 477−526). Enfield NH: Science Publishers, Inc..

Burger, W. L. (1950). Novel aspects of the life history of two Ambystomas. *J. Tenn. Acad. Sci., 25*, 252−257.

Cayrol, C., Garnier, D. H., & Deparis, P. (1985). Comparative plasma levels of androgens and 17β-estradiol in the diploid and triploid newt, *Pleurodeles waltl*. *Gen. Comp. Endocrinol., 58*, 342−346.

Chadwick, C. S. (1941). Further observations on the water drive in *Triturus viridescens*. II. Induction of the water drive with the lactogenic hormone. *J. Exper. Zool., 86*, 175−187.

Crews, D. (1984). Gamete production, sex hormone secretion, and mating behavior uncoupled. *Horm. Behav., 18*, 22−28.

Davis, W. C., & Twitty, V. C. (1964). Courtship behavior and reproductive isolation in the species of *Taricha* (Amphibia, Caudata). *Copeia, 1964*, 601−610.

Dent, J. N. (1975). Integumentary effects of prolactin in the lower vertebrates. *Amer. Zool., 15*, 923−935.

Deviche, P., & Moore, F. L. (1988). Steroidal control of sexual behavior in the rough-skinned newt (*Taricha granulosa*): Effects of testosterone, estradiol, and dihydrotestosterone. *Horm. Behav., 22*, 26−34.

Diamanti-Kandarakis, E., Bourguignon, J.-P., Giudice, L. C., Hauser, R., Prins, G. S., Soto, A. M., Zoeller, R. T., & Gore, A. C. (2009). Endocrine-disrupting chemicals: an Endocrine Society scientific statement. *Endo. Revs., 30*, 293−342.

Doerr-Schott, J., & Zuber-Vogeli, M. (1984). Immunohistochemical study of the adenohypophysis of *Typhlonectes compressicaudus* (Amphibia: Gymnophiona). *Cell Tiss. Res., 235*, 211−214.

Duvall, D., & Norris, D. O. (1977). Prolactin and substrate stimulation of locomotor activity in adult tiger salamanders (*Ambystoma tigrinum*). *J. Exper. Zool., 200*, 103−106.

Duvall, D., & Norris, D. O. (1980). Stimulation of terrestrial substrate preferences and locomotor activity in newly transformed tiger salamanders (*Ambystoma tigrinum*) by exogenous and endogenous thyroxine. *Anim. Behav., 28*, 116.

Ebersole, T. J., & Boyd, S. (2000). Immunocytochemical localization of gonadotropin-releasing hormones in the brain of a viviparous caecilian amphibian, *Typhlonectes natans* (Amphibia: Gymnophiona). *Brain Behav. Evol., 55*, 14−25.

Exbrayat, J.-M. (1988). Variations ponderales des organs de reserve (corps adipeux et foie) chez *Typhlonectes compressicaudus*, Amphibien Apode vivipasre au cours des alternances saisonnaieres et des cycles de reproduction. *Ann. Sci. Nat. Zool., 13*, 45−53, (Cited by Exbrayat, 2006; Rastogi *et al.*, 2005).

Exbrayat, J.-M. (2006a). *"Reproductive Biology and Phylogeny of Gymnophiona (Caecilians)*. Enfield NH: Science Publishers.

Exbrayat, J.-M. (2006b). Endocrinology of reproduction in Gymnophiona. In J.-M. Exbrayat (Ed.), *"Reproductive Biology and Phylogeny of Gymnophiona (Caecilians)"* (pp. 183−229). Enfield NH: Science Publishers, Inc.

Exbrayat, J.-M., & Estabel, J. (2006). Anatomy with particular reference to the reproductive system. In J.-M. Exbrayat (Ed.), *"Reproductive Biology and Phylogeny of Gymnophiona (Caecilians)"* (pp. 79−155). Enfield NH: Science Publishers, Inc.

Fernandez, S. N., & Ramos, I. (2003). Endocrinology of Reproduction. In B. G. M. Jamieson (Ed.), *"Reproductive Biology and Phylogeny of Anura."* (pp. 73−117). Enfield NH: Science Publishers, Inc.

Fort, D. J., Guiney, P. D., Weeks, J. A., Thomas, J. H., Rogers, R. L., Noll, A. M., & Spaulding, C. D. (2004). Effect of methoxychlor on various life stages of *Xenopus laevis*. *Toxicol. Sci., 81*, 454−466.

Garnier, D. H. (1985a). Androgen and estrogen levels in the plasma of *Pleurodeles waltl* Michah., during the annual cycle. I. Male cycle. *Gen. Comp. Endocrinol., 58*, 376−385.

Garnier, D. H. (1985b). Androgen and estrogen levels in the plasma of *Pleurodeles waltl* Michah., during the annual cycle. II. Female cycle. *Gen. Comp. Endocrinol., 60*, 414−418.

Gillespie, G., & Hero, J.-M. (1999). Potential impacts of introduced fish and fish translocations on Australian amphibians. In A. Campbell (Ed.), *"Declines and Disappearances of Australian frogs"* (pp. 131−144). Canberra: Environment Australia.

Gould, S. J. (1977). *Ontogeny and Phylogeny.* Cambridge MA: Harvard University Press.

Gould, S. J. (2000). Of coiled oysters and big brains: how to rescue the terminology of heterochrony. *Evol. Devel., 2*, 241−248.

Grant, W. C., & Grant, J. A. (1958). Water drive studies on hypophysectomized efts of *Diemyctylus viridescens*. Part I. The role of the lactogenic hormone. *Biol. Bull., 114*, 1−9.

Greven, H. (2003). Larviparity and pleuriparity. In D. M. Sever (Ed.), *"Reproductive Biology and Phylogeny of Urodela"* (pp. 447−476). Enfield NH: Science Publishers, Inc.

Guillette, L. J., Norris, D. O., & Norman, M. F. (1985). Response of amphibian (*Ambystoma tigrinum*) oviduct to arginine vasotocin and acetylcholine *in vitro*: influence of steroid hormone pretreatment *in vivo*. *Comp. Biochem. Physiol., 80C*, 151−154.

Hamilton, R. S. (1949). "Natural history of the Rocky Mountain tiger salamander *Ambystoma tigrinum* and the occurrence of polydactylism in a local population." *M.A. Dissertation*. University of Colorado at Boulder.

Harris, J. P., Jr. (1961). The natural history of Necturus, IV. Reproduction. *Journal of the Graduate Research Center, 28*, 69−81.

Hassinger, D. D., Anderson, J. D., & Dalrymple, G. H. (1970). The early life history and ecology of *Ambystoma tigrinum* and *Ambystoma opacum* in New Jersey. *Amer. Midl. Nat., 84*, 474−495.

Healy, W. R. (1975). Breeding and postlarval migrations of the red-spotted newt, *Notophthalmus viridescens*, in Massachusetts. *Ecology, 56*, 673−680.

Heatwole, H. (2005). *"Amphibian Biology Vol. 6, Endocrinology."*. Chipping Norton NSW, Australia: Surrey Beatty & Sons.

Houck, L. D. (2009). Pheromone communication in amphibians and reptiles. *Ann. Rev. Physiol., 71*, 161−176.

Houck, L. D., & Arnold, S. J. (2003). Courtship and mating behavior. In D. M. Sever (Ed.), *"Reproductive Biology and Phylogeny of Urodela"* (pp. 383−424). Enfield NH: Science Publishers, Inc.

Houck, L. D., & Woodley, S. K. (1995). Field studies of steroid hormones and male reproductive behaviour in amphibians. In H. Heatwole, & B. K. Sullivan (Eds.), *"Amphibian Biology Vol. 2"* (pp. 677−703). Chipping Norton NSW, Australia: Surrey Beatty & Sons.

Iwata, T., Kawahara, G., Yamamoto, K., Zhou, C. J., Nakajo, S., Shioda, S., & Kikuyama, S. (2000a). Effect of prolactin and androgen on the expression of the female-attracting pheromone silefrin in the abdominal gland of the newt, *Cynops ensicauda*. *Biol. Reprod, 63*, 1867−1872.

Iwata, T., Toyoda, F., Yamamoto, K., & Kikuyama, S. (2000b). Hormonal control of urodele reproductive behavior. *Comp. Biochem. Physiol, 126B*, 221−229.

Jamieson, B.G.M. (2003). *Reproductive Biology and Phylogeny of Anura*. Enfield NH:Science Publishers, Inc.

Kiesecker, J. M. (2002). Synergism between trematode infection and pesticide exposure: a link to amphibian limb deformities in nature? *Proc. Natl. Acad. Sci. U.S., 99*, 9900−9904.

Kikuyama, S., Hasunuma, I., Toyoda, F., Haraguchi, S., & Tsutsui, K. (2009). Hormone-mediated reproductive behavior in the red-bellied newt. *Ann. N.Y. Acad. Sci., 1163*, 179−186.

Kikuyama, S., Tanaka, S., & Moore, F. L. (2003). Endocrinology of reproduction. In D. M. Sever (Ed.), *"Reproductive Biology and Phylogeny of Urodela"* (pp. 275−321). Enfield NH: Science Publishers, Inc.

Kikuyama, S., Toyoda, F., Iwata, T., Yamamoto, K., Tanaka, S., Matsuda, K., & Hayashi, H. (2001). Endocrine control of reproductive behavior of the newt, *Cynops pyrrhogaster.* In H. J. T. Goos, R. K. Rastogi, H. Vaudry, & R. Pierantoni (Eds.), *"Perspective in Comparative Endocrinology: Unity and Diversity"* (pp. 317−324). Italy: Monduzzi Editore, Bologna.

Kloas, W., Urbatzka, R., Opitz, R., Würtz, S., Behrends, T., Hermelink, B., et al. (2009). Endocrine disruption in aquatic vertebrates. *Ann. N.Y. Acad. Sci., 1163*, 187−200.

Larson, A., Weisrock, A., & Kozak, K. H. (2003). Phylogenetic systematics of salamanders (Amphibia: Urodela), a review. In D. M. Sever (Ed.), *"Reproductive Biology and Phylogeny of Urodela"* (pp. 31−108). Enfield NH: Science Publishers, Inc..

Lazard, L. (1979). Steroidogenesis in axolotl testis. Histochemistry of two major enzymes related to cell type, spermatogenesis, and substrate. *Gen. Comp. Endocrinol., 39*, 381−387.

Mann, R., & Bidwell, J. (1999). Toxicological issues for amphibians in Australia. In A. Campbell (Ed.), *"Declines and Disappearances of Australian frogs"* (pp. 185−201). Canberra: Environment Australia.

Matsumoto, A., Arai, Y., Kouki, T., & Kikuyama, S. (1995). Comparison of Mauthner cell size in sexually developed and undeveloped red-bellied newts. *J. Neurobiol., 28*, 126−132.

McCallum, M. (2007). Amphibian decline or extinction? Current declines dwarf background extinction rate. *J. Herpetol., 41*, 483−491.

Miller, M. R., & Robbins, M. E. (1954). The reproductive cycle in *Taricha torosa (Triturus torosus). J. Exp. Zool., 125*, 415−445.

Moore, F. L. (1975). Spermatogenesis in larval *Ambystoma tigrinum*: positive and negative interactions of FSH and testosterone. *Gen. Comp. Endocrinol., 26*, 525−533.

Moore, F. L., Boyd, S. K., Chu, J., & Hyde, G. (2005). Hormonal regulation of behaviour. In H. Heatwole (Ed.), *"Amphibian Biology Vol. 6, Endocrinology"* (pp. 2178−2207). Chipping Norton NSW, Australia: Surrey Beatty & Sons.

Moore, F. L., & Miller, L. J. (1983). Arginine vasotocin induces sexual behavior of newts by acting on cells in the brain. *Peptides, 4,*, 97−102

Moore, F. L., Seide, R. L., Specker, J. L., & Swanson, L. (1978). Effects of prolactin and methallibure on second metamorphosis in male newts, *Taricha granulosa*. *Comp. Biochem. Physiol., 61A*, 419−422.

Moore, F. L., Wood, R. E., & Boyd, S. K. (1992). Sex steroids and vasotocin interact in a female amphibian (*Taricha granulosa*) to elicit female-like egg-laying behavior or male-like courtship. *Horm. Behav., 26*, 156−166.

Moriya, T., & Dent, J. N. (1986). Hormonal interaction in the mechanism of migratory movement in the newt, *Notophthalmus viridescens*. *Zool. Sci., 3*, 669−676.

Norman, M. F. (1985). A practical method for staging metamorphosis in the tiger salamander *Ambystoma tigrinum*. *Anat. Rec., 211*, 102−109.

Norris, D. O. (1987). Regulation of male gonaducts and sex accessory structures. In D. O. (Norris, & R. E. Jones (Eds.), *"Hormones and Reproduction in Fishes, Amphibians and Reptiles"* (pp. 327−354). New York: Plenum Press.

Norris, D. O. (1989). Seasonal changes in diet of paedogenetic tiger salamanders (*Ambystoma tigrinum mavortium*). *J. Herpetol., 23*, 87−89.

Norris, D. O. (2007). *Vertebrate Endocrinology.* San Diego: Academic Press.

Norris, D. O., Austin, H. A., & Hijazi, A. S. (1989). Effects of testosterone and prolactin on cloacal and dermal skin glands of tiger salamander larvae, *Ambystoma tigrinum*. *Gen. Comp. Endocrinol., 73*, 194−204.

Norris, D. O., Carr., J. A., Summers, C. H., & Featherston, R. (1997). Interactions of androgens and estradiol on sex accessory ducts of larval tiger salamanders, *Ambystoma tigrinum*. *Gen. Comp. Endocrinol., 106*, 348−355.

Norris, D. O., & Duvall, D. (1981). Hormone-induced ovulation in *Ambystoma tigrinum*: influence of prolactin and thyroxine. *J. Exper. Zool., 216*, 175−180.

Norris, D. O., Duvall, D., Greendale, K., & Gern, W. A. (1977). Thyroid function in pre- and postspawning neotenic tiger salamanders (*Ambystoma tigrinum*). *Gen. Comp. Endocrinol., 33*, 512−517.

Norris, D. O., & Jones, R. E. (1987). *Hormones and Reproduction in Fishes, Amphibians and Reptiles.* New York: Plenum Press.

Norris, D. O., & Lopez, K. H. (2005). Anatomy of the amphibian endocrine system. In H. Heatwole (Ed.), *"Amphibian Biology Vol. 6, Endocrinology"* (pp. 2021−2044). Chipping Norton NSW, Australia: Surrey Beatty & Sons.

Norris, D. O., Norman, M. F., Pancak, M. K., & Duvall, D. (1985). Seasonal variations in spermatogenesis, testicular weights, vasa deferentia, and androgen levels in neotenic male tiger salamanders, *Ambystoma tigrinum*. *Gen. Comp. Endocrinol., 60*, 51−57.

Park, D., & Propper, C. R. (2001). Repellent function of male pheromones in the red-spotted newt. *J. Exp Zool., 289*, 404−408.

Park, D., McGuire, J. M., Majchrzak, A. L., Ziobro, J. M., & Eisthen, H. L. (2004). Discrimination of conspecific sex and reproductive condition using chemical cues in axolotls (*Ambystoma mexicanum*). *J. Comp. Physiol. A, 1190*, 415−427.

Pinelli, C., d'Aniello, B., Fiorentino, M., Bhat, G. K., Saidapur, S. K., & Rastogi, R. K. (1997). Distribution of gonadotropin-releasing hormone immunoreactivity in the brain of *Ichthyophis beddomei* (Amphibia: Gymnophiona). *J. Comp. Neurol., 384*, 283−292.

Platt, J. E. (1976). The effects of ergocornine on tail height, spontaneous and T4-induced metamorphosis and thyroidal uptake of radioiodine in neotenic *Ambystoma tigrinum*. *Gen. Comp. Endocrinol., 28*, 71−81.

Polzonetti-Magni, A. M. (1999). Amphibian Ovarian Cycles. In E. Knobil, & J. D. Neilll (Eds.), *"Encyclopedia of Reproduction, Vol. 1,"* (pp. 154−160). San Diego: Academic Press.

Rastogi, R. K., Iela, L., di Meglio, M., Di Fiore, M. M., D'Aniello, B., Pinelli, C., & Fiorentino, M. (2005). Hormonal regulation of reproductive cycles in amphibians. In H. Heatwole (Ed.), *"Amphibian Biology Vol. 6, Endocrinology"* (pp. 2045−2177). Chipping Norton NSW, Australia: Surrey Beatty & Sons.

Rastogi, R. K., Meyer, D. L., Pinelli, C., Fiorentino, M., & d'Aniello, B. (1998). Comparative analysis of GnRH neuronal systems in the amphibian brain. *Gen. Comp. Endocrinol., 112*, 330−345.

Reilly, S. M., Wiley, E. O., & Meinhardt, D. J. (1997). An integrative approach to heterochrony: the distincion between interspecific and intraspecific phenomena. *Biol. J. Linn. Soc., 60*, 119−143.

Reineke, E. E., & Chadwick, C. S. (1939). Inducing land stage *Triturus viridescens* to assume water habitat by pituitary implants. *Proc. Soc. Exp. Biol. Med, 40*, 691−693.

Reineke, E. E., & Chadwick, C. S. (1940). The origin of water drive in *Triturus viridescens*. I. Induction of the water drive in thyroidectomized and gonadectomized land phases by pituitary implantation. *J. Exp Zool., 83*, 223−233.

Rose, F. L., & Armentrout, D. (1976). Adaptive strategies of *Ambystoma tigrinum* Green inhabiting the llano estacado of west Texas. *J. Anim. Ecol., 45*, 713−729.

Rose, J. D., Kinnaird, J. R., & Moore, F. L. (1995). Neurophysiological effects of vasotocin and corticosterone on medullary neurons: Implications for hormonal control of amphibian courtship behavior. *Neuroendocrinology, 62*, 406−417.

Rose, J. D., Moore, F. L., & Orchinick, M. (1993). Rapid neurophysiological effects of corticosterone on medullary neurons: Relationship to stress-induced suppression of courtship clasping in an amphibian. *Neuroendocrinology, 57*, 815−824.

Salthe, S. N. (1967). Courtship patterns and the phylogeny of the urodeles. *Copeia, 1967*, 100−117.

Semlitsch, R. D. (1983). Structure and dynamics of two breeding populations of the eastern tiger salamander, *Ambystoma tigrinum*. *Copeia, 1983*, 608−616.

Sever, D. M. (2003). *Reproductive Biology and Phylogeny of Urodela,* Enfield NH: Science Publishers, Inc.

Specker, J. L., & Moore, F. L. (1980). Annual cycle of plasma androgens and testicular composition in the rough-skinned newt, *Taricha granul*osa. *Gen. Comp. Endocrinol., 42*, 297−303.

Stopper, G. F., Hecker, L., Franssen, R. A., & Sessions, S. K. (2002). How trematodes cause limb deformities in amphibians. *J. Exp. Zool. (Mol Dev Evol), 294*, 252−263.

Stuart, S. N., Chanson, J. S., Cox, N. A., Young, B. E., Rodrigues, A. S. L., Fischman, D. L., & Waller, R. W. (2004). Status and trends of amphibian declines and extinctions worldwide. *Science, 306*, 1783−1786.

Suzuki, Y., & Kikuyama, S. (1987). Effect of PRL and testosterone on the newt Mauthner neuron. *Zool. Sci., 4*, 1095.

Tanaka, S., & Takikawa, H. (1983). Seasonal changes in plasma testosterone and 5α-dihydrotestosterone levels in the adult male newt, *Cynops pyrrhogaster pyrrhogaster*. *Endocrinol. Japon, 30*, 1−6.

Tanner, W. W., Fisher, D. L., & Willis, T. J. (1971). Notes on the life history of *Ambystoma tigrinum nebulosum* Hallowell in Utah. *The Great Basin Naturalist, 31*, 213−222.

Taylor, D. H., & Guttman, S. I. (1977). *The Reproductive Biology of Amphibians*. NY: Plenum Publishing Press.

Toyoda, F., Tanaka, S., Matsuda, K., & Kikuyama, S. (1994). Hormonal control of response to and secretion of sex attractants in Japanese newts. *Physiol. Behav., 55*, 569−576.

Twitty, V. C. (1941). Data on the life history of *Ambystoma tigrinum californiense* Gray. *Copeia, 1941*, 1−3.

Wake, M. H. (1977). The reproductive biology of caecilians: an evolutionary perspective. In D. H. Taylor, & S. I. Guttman (Eds.), *"The Reproductive Biology of Amphibians."* (pp. 73−102). New York: Plenum Publishers.

Wake, M. H. (2006). A brief history of research on gymnophionan reproductive biology and development. In J.-M. Exbrayat (Ed.), *"Reproductive Biology and Phylogeny of Gymnophiona (Caecilians)"* (pp. 1−37). Enfield NH: Science Publishers, Inc.

Webb, R. G., & Roueche, W. L. (1971). Life history aspects of the tiger salamander (*Ambystoma tigrinum mavortium*) in the Chihuahuan Desert. *The Great Basin Naturalist, 31*, 193−212.

Wilkinson, M., & Nussbaum, R. A. (2006). Caecilian phylogeny and classification. In J.-M. Exbrayat (Ed.), *"Reproductive Biology and Phylogeny of Gymnophiona (Caecilians)"* (pp. 39−78). Enfield NH: Science Publishers, Inc.

Woodhams, D. C., Voyles, J., Lips, K. R., Carey, C., & Rollins-Smith, L. A. (2006). Predicted disease susceptibility in a Panamanian amphibian assemblage based on skin peptide defenses. *J. Wild. Dis., 42*, 207−218.

Woodley, S. K. (1994). Plasma androgen levels, spermatogenesis, and secondary sex characters in two species of plethodontid salamanders with dissociated reproductive patterns. *Gen. Comp. Endocrinol., 96*, 206−214.

Zerani, M., Amabili, F., & Gobbetti, A. (1992). Plasma testosterone and 17β-estradiol concentrations, and aromatase activity, during courtship in male. *Triturus carnifex. Horm. Behav., 26*, 56−61.

Zoeller, R. T., Lais, I. T., & Moore, F. L. (1983). Contractions of amphibian Wolffian duct in response to acetylcholine, norepinephrine, and arginine vasotocin. *J. Exper. Zool., 226*, 53−57.

Chapter 11

Endocrine Disruption of Reproduction in Amphibians

David O. Norris
University of Colorado at Boulder, Boulder, CO, USA

SUMMARY
Amphibians are important members of many aquatic and terrestrial communities and represent the transitional evolutionary stage between fishes and terrestrial amniote vertebrates. Because of the unique life-history patterns of many amphibians with aquatic larvae that become terrestrial adults, amphibians bridge the aquatic and terrestrial habitats during their lives and hence are subjected to both aquatic and terrestrial pollutants. These pollutants include endocrine-disrupting chemicals that can affect amphibian reproduction and survival. Many amphibian species are declining worldwide due to a number of causes, and the effects of endocrine-disrupting contaminants may be important contributing factors. Disruption of the amphibian thyroid system upon which metamorphosis depends may affect survival and the opportunity to reproduce. Disruption of sexual differentiation, gonadal maturation, and reproductive behavior likewise may be contributing to the worldwide decline of amphibians.

1. INTRODUCTION

In less than a century, humans have created thousands of unique chemicals that perhaps have never before been experienced by organisms on Earth. Many of these compounds have found their way into the environment either intentionally or by accident. Further, we have concentrated to an unprecedented degree many natural inorganic chemicals found in the Earth's crust, such as heavy metals from mining activities and organic chemicals normally produced by organisms, such as plant sterols (Vajda & Norris, 2006) and animal hormones concentrated in wastewater effluents from our cities (Kolpin et al., 2002). We previously assumed that these chemicals were innocuous if diluted sufficiently until studies first in alligators (e.g., Guillette et al., 1994; 1996) and later in fishes (see Mattiessen, 1998) revealed that levels previously considered to be extreme dilutions (parts per billion (ppb) or parts per trillion (ppt)) were sufficient to alter reproductive structures and behaviors.

Since these first observations, we have discovered numerous chemicals that can affect reproduction, development, metabolism, growth, neural function, endocrine functions, and so on. Early studies demonstrated that chemicals such as certain pesticides and industrial products could act by mimicking estrogenic hormones and altering estrogen-dependent physiological processes. This was referred to as 'endocrine disruption' and the offending chemicals were termed endocrine-disrupting chemicals or EDCs. It might be more accurate to call them endocrine-active chemicals (EACs) in that they may produce effects other than disruption. Because we are finding other chemicals that affect physiological systems and behaviors not always associated with hormones and because our awareness of them has been very recent, some prefer to address these interfering environmental chemicals as 'emerging contaminants' (ECs).

Although the term 'endocrine disruption' has been used only recently to describe the disturbance of endocrine systems by chemicals introduced or artificially concentrated in the environment by human activities, it has been studied in animals for several decades, especially since the appearance of Rachel Carson's *Silent Spring* in 1962. However, it was the observations of demasculinized alligators in Florida, effects of polychlorinated biphenyls (PCBs) in the North American Great Lakes area, disrupted reproduction in marine mammals, and the appearance of feminized fishes in rivers of the UK and Europe reported in the late 1980s and early 1990s that caused Theo Colborn to bring together scientists and other concerned individuals in 1991 at the Wingspread Conference Center in Racine, Wisconsin, with the goal of examining the topic. As a result of this conference and communication of the conclusions by the attending scientists to elected members of government, the US Congress instructed the US Environmental Protection Agency (USEPA) to develop a plan of action. With

input from many scientists across the USA, the USEPA drafted a committee in 1996 called EDSTAC, the Endocrine Disruptor Screening, Testing, and Advisory Committee, to develop a plan for investigating the magnitude and extent of endocrine disruption, including the establishment of a series of bioassays to assess systematically the impacts of the thousands of untested chemicals in the environment.

The Endocrine Disruptor Screening and Testing Committee issued their report in 1998 and proposed the establishment of an Endocrine Disruptor Screening Program (EDSP). Included in the battery of bioassays they proposed for use in screening for possible chemical effects was the Frog Embryo Teratogenesis Assay—*Xenopus* (FETAX), a 96-hour whole-embryo toxicity test employing mortality, malformation, and growth inhibition assays focusing on the developmental responses of an amphibian, the African clawed frog, *Xenopus laevis*. Although not a native amphibian, this species was chosen as a common and universal testing parameter for scientists because of the wealth of biochemical and genetic data known for *X. laevis* as well as the thorough understanding of the hormones involved in its metamorphosis process. Although the planning process was completed by 1998, the resultant EDSP has yet to be activated, seemingly due in part to the strong lobbying by the chemical and pharmaceutical industries as well as by politically based suppression of the USEPA (see extensive discussion of this issue by Schapiro, 2007).

Laboratory and field studies demonstrate that EDCs can produce significant effects on reproduction at extremely low doses (e.g., 4−6 ng/L in an aquatic system; see Kidd et al. (2007)). Numerous reports document the presence of EDCs at physiologically relevant concentrations in the environment (e.g., Kolpin et al., 2002) and there is a wealth of supporting laboratory studies demonstrating disruption at these levels in fishes, amphibians, reptiles, birds, and mammals (for detailed summaries see Guillette and Crain (2000); McLachlan, Guillette, Iguchi, and Toscano (2001); Norris and Carr (2006); Gore (2007); Diamanti-Kandarakes et al., 2009; Vandenberg, Maffini, Sonnenschein, Rubin, & Sotop (2009); Denver et al. (2010); see also in this series Volume 1 Chapter 13, Volume 3 Chapter 14, Volume 4 Chapter 9, and Volume 5 Chapter 14). Further, reproductive disorders documented in laboratory animals have been reported in newborn human males whose mothers exhibited evidence of high exposures of these same EDCs (Swan et al., 2005; Swan, 2008). Meanwhile, invoking the precautionary principle, the European Union began requiring products sold in Europe to be free from some of the chemicals of particular concern such as phthalates and bisphenol A (BPA), whereas the USEPA has failed to act on a single chemical (Schapiro, 2007; Diamanti-Kandarakes et al., 2009; Vandenberg et al., 2009), although a list of proposed chemical targets has been provided recently.

2. SOURCES OF ENDOCRINE-DISRUPTING CHEMICALS (EDCS)

Many manufactured chemicals and their metabolites present in the environment as a result of human activities can function as EDCs. This list of EDCs includes some pesticides (e.g., dichlorodiphenyltrichloroethane (DDT), carbaryl, methoxychlor); certain prescription (e.g., fluoxetine, ethinylestradiol (EE_2)) and non-prescription pharmaceuticals (e.g., ibuprofen); flame retardants (polybrominated biphenylethers (PBDEs)); herbicides (e.g., glyphosates, atrazine); industrial products and byproducts (polychlorinated biphenyls (PCBs), dioxins); and certain components of plastics, personal care products, latex paints, etc., including phthalates, such as diethyl phthalate (DEPH), BPA, and nonylphenol (NP). These chemicals may be applied directly to the environment or be released into the environment via wastewater effluents or biosolids from wastewater treatment plants (WWTPs).

Natural chemicals excreted by humans and domestic animals may function as EDCs. These chemicals may appear in surface waters either through leaching of animal wastes from livestock and poultry-rearing areas or by the addition of WWTP effluents to rivers and lakes. They also may occur in surface waters as a result of their direct application or indirectly from biosolids spread on agricultural fields. These EDCs appear at very low concentrations, e.g., µg/L (ppb) or ng/L (ppt), that are far below those concentrations shown to be overtly toxic and leading to disease or death of exposed animals in toxicological studies (typically, mg/L or g/L).

3. AMPHIBIANS AS TARGETS FOR ENDOCRINE-DISRUPTING CHEMICALS (EDCS) AND OTHER EMERGING CONTAMINANTS (ECS)

Amphibians occupy a unique position among vertebrates in that they represent the evolutionary transition from totally aquatic vertebrates to terrestrial vertebrates that occurred millions of years before the present. Further, frogs still best exemplify the molecular and morphological changes that were necessary for this transition during their life histories. Amphibian eggs usually are deposited in or very near water, where they hatch and develop into a tadpole larval form that is fish-like in its morphology, physiology, and behavior. Later, tadpoles develop legs as they undergo a distinct metamorphosis to a semi-terrestrial or terrestrial frog and thereby lose most of their fish-like features. This metamorphosis involves specific morphological and biochemical adaptations that are crucial to survival in the relatively arid, terrestrial environment (for details see Gilbert & Frieden, 1981; Balls & Bownes, 1985; Shi, 2000;

Norris, 2007). Most amphibians still require the aquatic environment for reproduction and some species remain in the water even after metamorphosis (see Chapters 7, 9, and 10 for details of amphibian reproductive cycles). Adults typically are semi-aquatic and never live far from water although some spend most of their lives in moist terrestrial habitats, returning to water primarily for breeding. Other species, such as paedomorphic urodeles, may forego metamorphosis and remain aquatic for their entire lives. In marked contrast, some species of frog, salamander, and gymnophionid have become completely terrestrial, exhibiting internal fertilization and in some cases bearing live young. Some terrestrial frogs have become adapted to desert communities by spending most of their lives underground, coming to the surface only during rainy periods to breed in temporary ponds in which the young develop rapidly into juveniles before seeking an underground existence themselves.

One anatomical feature that restricts most amphibians to moist habitats is their thin, highly permeable glandular skin. Mucus-secreting glands keep the skin soft and protect it to a limited degree. The additional presence of dermal poison glands in the skin makes some amphibians distasteful or even extremely toxic and may serve as a deterrent to predators. The moist and permeable skin often is important in respiration as well as in ionic and water balance of both aquatic and terrestrial forms. Because of their highly permeable skin, amphibians are especially sensitive to a variety of toxic chemicals in the environment that can enter their bodies and disrupt their normal physiology. In addition to absorbing ECs, amphibians also may concentrate some of these chemicals, allowing them to be passed up the food chain.

Amphibians occupy key positions in the food chains of freshwater and moist terrestrial habitats. Larval amphibians may be herbivores or carnivores. Larvae and adults may be both predators on insects, annelids, and other invertebrates as well as being prey themselves for fishes, snakes, and birds. Amphibians are often assessed as sentinel species that signal the health of an ecosystem. Because they are both aquatic and terrestrial at different unique phases of their life histories, amphibians are especially sensitive to degradation of either habitat. Because early development usually occurs in the aquatic habitat, amphibians are especially sensitive to agricultural, industrial, and household chemicals that inevitably find their way into the aquatic environment. Further, any chemical factors that alter the timing and success of metamorphosis and survival of the metamorphosed young can ultimately have effects on the number of breeding adults that in turn can affect the number of offspring produced and ultimately the reproductive success of the species.

It is well known that amphibian species are declining worldwide. These declines are associated with a great number of environmental factors including habitat destruction, bacterial and fungal infections, increased ultraviolet light exposure, parasite loads, global warming, and possibly interactions between these factors as they affect general stress levels and hence susceptibility to disease and parasites (see Linder, Krest, & Sparling, 2003). It is not inconceivable that exposures to toxic chemicals as well as EDCs are also playing a part (see Sparling, Linder, & Bishop, 2000).

Amphibians, then, are often exposed to EDCs or other ECs that potentially mimic or inhibit natural body chemicals responsible for normal control of their development, physiology, and behavior. We emphasize their actions on the processes of metamorphosis and reproduction in this review while recognizing they can have numerous other effects on development, physiology, and behavior through other mechanisms of action.

4. AMPHIBIAN HORMONES RELATED TO METAMORPHOSIS AND REPRODUCTION

Reproduction in amphibians, as in other vertebrates, is regulated through the hypothalamus−pituitary−gonad (HPG) axis (see Norris, 2007; see also Chapters 2, 3, 4, 5, 9, and 10, this volume). Briefly, gonadotropin-releasing hormone (GnRH) produced in the hypothalamus travels to the pituitary gland and controls secretion of two gonadotropins (GTHs): follicle-stimulating hormone (FSH) and luteinizing hormone (LH). These GTHs regulate gamete development and steroid hormone secretion by the testes and ovaries. The gonadal steroids include estrogens such as 17β-estradiol (E_2) and estrone (E_1); androgens such as testosterone (T), 5α-dihydrotestosterone (DHT), and 11-ketotestosterone (11-KT); and progesterone (P_4). These hormones influence a variety of sex accessory structures and can direct reproductive behaviors (see Chapter 8, this volume). Environmental factors (temperature, rainfall, food abundance, vocalization by conspecifics, etc.) operating through the HPG axis may normally initiate and/or modify reproductive events and alter reproductive success.

Metamorphosis is directed through the hypothalamus−pituitary−thyroid (HPT) axis and the hypothalamus−pituitary−adrenal (HPA) axis. The HPT axis involves production of corticotropin-releasing factor (CRF) in the hypothalamus, which stimulates release of thyrotropin (TSH) from the pituitary. Thyrotropin in turn stimulates production of two thyroid hormones, triiodothyronine (T_3) and thyroxine (T_4), that are responsible for directing metamorphosis. CRF, which also serves to activate the HPA axis, stimulates secretion of corticotropin (ACTH) from the pituitary that in turn stimulates corticosterone (CORT) secretion from the adrenocortical (interrenal) tissue. Finally, the pituitary hormone prolactin (PRL) is known

both to have inhibitory actions on metamorphosis in younger larvae and to actively participate in metamorphosis in older tadpoles (for reviews of the endocrine regulation of amphibian metamorphosis see Shi (2000), Tata (2005), Carr and Norris (2006a; 2006b), Norris (2007), and Denver (1997; 2008)).

Endocrine-disrupting chemicals may alter the synthesis and/or release of amphibian hormones from any level within the HPG, HPT, and HPA axes. They also may alter (1) transport of hormones to their target tissues; (2) actions of hormones on their target tissues, where they mimic or block the actions of the natural hormones; or (3) the rates at which hormones are metabolized or excreted (for reviews see Orchinik & Propper, 2006; Norris, 2007).

5. AMPHIBIANS AS MODELS FOR ENDOCRINE-DISRUPTING CHEMICAL (EDC) STUDIES

For all of the above reasons, amphibians represent logical subjects for monitoring the quality of the environment with respect to EDCs. Because of their unique ecological position, they can serve as monitors for both terrestrial and aquatic habitats. Consequently, numerous bioassays have been developed that involve whole animal models. The USEPA FETAX bioassay for monitoring the effects of toxic chemicals on early growth and development of amphibians is focused on general toxicity and does not directly address the problems of disruption of the HPT and HPG axes (e.g., see Hoke & Ankley, 2005). The main strength of the FETAX bioassay is that it involves the response of the entire organism, which can be more meaningful than *in-vitro* assays that simplify the analysis but at the same time ignore the natural complexity inherent in living systems. A more relevant test than FETAX is the reed frog (*Hyperolius argus*) skin coloration bioassay, which has proven to be an effective screen for estrogenic chemicals (Noriega & Hayes, 2000).

A radioreceptor assay in *X. laevis* has been described by Lutz and Kloas (1999) for screening potential estrogenic chemicals by comparing them to the principal estrogenic steroid of amphibians, E_2. Competitive binding studies showed that displacement of E_2 occurred with known estrogenic EDCs including DEPH, BPA, and NP: chemicals that commonly are found in wastewater effluents. These EDCs were better at displacing E_2 than several natural steroid hormones (i.e., CORT, T, and P_4). These authors also showed that E_2 displacement activity was present in three of five wastewater effluents they tested. Although this is an *in-vitro* assay, it is more relevant when assessing potentials of exogenous chemicals for amphibian disruption to employ an amphibian receptor than to rely on mammalian estrogen receptor (ER) assays such as the transfected yeast YES bioassay or the mammalian breast cancer cell bioassay.

17β-estradiol, T, and DHT are all effective at stimulating hypertrophy of undeveloped Müllerian ducts in immature female or male larvae of tiger salamanders, *Ambystoma tigrinum*, whereas T and DHT also stimulate hypertrophy of the Wolffian ducts, which is blocked by the presence of E_2 (Norris, Carr, Summers, & Featherston, 1997). This system also has been used as a bioassay to monitor the sensitivity of these reproductive tissues to potential EDCs such as DDT (Clark et al., 1998) and the potent dioxin 2,3,7,8-tatrachlordibenzo-p-dioxin (Vajda & Norris, 2005), as well as methoprene acid, a metabolite of the insecticide methoprene, and the closely related developmental regulator, retinoic acid (Lopez, 2003), at environmentally relevant concentrations.

The synthesis of the yolk precursor protein vitellogenin (Vtg) by the amphibian liver is under the control of E_2, as it is in fishes, reptiles, and birds (see Volume 1, Chapter 4; Volume 3, Chapter 4; and Volume 4, Chapter 3). In maturing females, Vtg is released into the blood for transport to the ovaries, where GTHs stimulate its incorporation into growing oocytes. Normally, E_2 levels are only high enough in females to significantly stimulate Vtg synthesis. Treatment of males with E_2 or exposure to estrogenic EDCs will result in enhanced production of Vtg and its appearance in the circulation, where it can readily be measured. Production of Vtg mRNA by liver cells harvested from *X. laevis* and grown in culture has been developed as a sensitive bioassay for exposure to estrogenic chemicals (Kloas, Lutz, & Einspanier, 1999). Secretion of Vtg by cultured liver cells from *X. laevis* can also be used to detect estrogenic chemicals (Mitsui, Tooi, & Kawahara, 2007). Since only estrogenic hormones and their chemical mimics are known to stimulate Vtg synthesis, elevation of this protein in the circulation of a male amphibian can be considered evidence of exposure to estrogenic chemicals in excess of normal endogenous levels, as also established for fishes (Sherry et al., 1999).

Bioassays using genetically engineered animals are being developed and soon may become part of the arsenal for detecting environmentally relevant levels of pollutants. One such bioassay employs transgenic tadpoles of *X. laevis* with a reporter gene linked to enhanced green fluorescent protein (eGFP) coupled with fluorescence cytometry (Fini et al., 2009), which has promise for detecting a wide variety of pollutants including estrogenic ones.

6. ENDOCRINE-DISRUPTING CHEMICAL (EDC) EFFECTS IN AMPHIBIANS

As a consequence of spending part or all of their lives in water, many amphibians are exposed to the same EDCs as

fishes and have been shown to be sensitive to the same pesticides, for example methoxychlor (Pickford & Morris, 1999; 2003; Fort et al., 2004), DDT (Clark, Norris, & Jones, 1998; Noriega & Hayes, 2000), and carbaryl (Relyea, 2003; Vonesh & Buck, 2007); pharmaceuticals, such as EE_2 (Park & Kidd, 2005; Urbatzka, Bottero, Mandich, Lutz, & Kloas, 2007; Cevasco et al., 2008; Hogan, Duarte, Wade, Lean, & Trudeau, 2008), and diethylstilbestrol (Adams, 1946); plastic components, such as BPA (Levy, Lutz, Krüger, & Kloas, 2004); and polycyclic aromatic hydrocarbons, such as dioxins (Vajda & Norris, 2005a) and PCBs (Qin et al., 2007). Additionally, adult amphibians, especially frogs and toads, often are subjected to terrestrial pollutants including pesticides, commercial fertilizers, and biosolids applied as fertilizer. As a consequence, proximity to agricultural operations often is correlated with reproductive disruption in frogs (McDaniel et al., 2008) and toads (McCoy et al., 2008).

Atrazine is the second most commonly used pesticide in North America and is applied primarily to crops of corn, sorghum, and sugar cane at the rate of 70−80 million pounds per year. It is relatively resistant to degradation with a halflife of up to 244 days in soil, 742 days in surface water, and 330 days in stream and lake sediments (Soloman et al. 2006). Atrazine leaches out from agricultural sites in normal runoff at levels of up to 6.7 ppb (Hayes et al., 2003) and as much as 480 ppb following storms (Huber, 1993). It has been found in surface waters (Kolpin et al., 2002) and has been reported as a common contaminant in drinking water (Benotti et al., 2009). Atrazine also has been reported in rainfall at levels of 40 ppb (Nations & Hallberg, 1992). This herbicide usually is applied in the spring prior to planting, at the same time as when amphibians are breeding and young tadpoles are undergoing early development. Atrazine exposure of *X. laevis* tadpoles in the laboratory to environmentally relevant doses alters later sexual development (Hayes et al., 2002; Carr et al., 2003; Hecker et al., 2005; Hayes et al., 2006; Orton, Carr, & Handy, 2006). These observations include the occasional presence of intersex gonads containing both sperm and eggs. Similar effects have been reported for amphibian tissues *in vitro* (Tavera-Mendoza et al., 2002a; 2002b). Additionally, even males without evidence of intersex exhibit a marked reduction in sperm production (Hayes et al., 2002). Hayes et al. (2003) presented observations of intersex in wild-caught *Rana pipiens* in areas of high atrazine use but not in areas where atrazine was not being used. Although Reeder et al. (1998) reported testicular oocytes in treefrogs inhabiting agricultural areas in Minnesota, examination of museum specimens from that region dating back to the 19th century showed a similar incidence of testicular oocytes (Reeder et al., 2005). Other laboratory (Hecker et al., 2005; Oka et al., 2008), microcosm (Jooste et al., 2005), and field studies (Murphy et al., 2006a; 2006b) did not find reproductive effects of atrazine in *X. laevis* or in native frogs at low doses, however. It is difficult to directly compare these diverse studies as there are numerous differences in experimental designs, sources of animals, background atrazine levels, methods of animal husbandry, mortality levels, etc. (e.g., see Hayes, 2004). It is of interest to note that some studies have found in some species of anuran (*Rana sylvatica*, *Bufo americanus*) and urodele (*Ambystoma barbouri*) that groups exposed to the lowest doses of atrazine (3−4 ppb) exhibit the highest mortality levels (Storrs & Kiesecker, 2004; Rohr, Sager, Sesterhenn, & Palmer, 2006).

Hayes et al. (2006) proposed that the effects of atrazine observed by their laboratory were a consequence of increases in aromatization and the resultant increase in estrogen levels in males. Although atrazine has been shown to affect aromatase ($P450_{aro}$) in mammals (Sanderson, Seinen, Giesy, & van den Berg, 2000; Sanderson, Letcher, Heneweer, Giesy, & van den Berg, 2001; Fan et al., 2007a; 2007b), several field and laboratory studies on a variety of species found no correlation between atrazine levels and activity of $P450_{aro}$, the enzyme responsible for converting androgens into estrogens (Coady et al., 2005; Hecker et al., 2005; Murphy et al., 2006b; Oka et al., 2008; Kloas et al., 2009). Further, evidence shows that atrazine's mechanism of action is not manifest via ERs (Roberge, Hakk, & Larsen, 2004). However, $P450_{aro}$ can be activated either by estrogens working through ERs or via the orphan nuclear receptor steroidogenic factor 1 (SF-1) in mammals (Fan et al., 2007a; 2007b; Suzawa & Ingraham, 2008) and fishes (Suzawa & Ingraham, 2008), and SF-1 stimulation of $P450_{aro}$ is enhanced by atrazine in both fishes and mammals. In addition to effects on timing of metamorphosis and on body size at metamorphosis (Larson, McDonald, Fivizzani, Newton, & Hamilton, 1998; Diana, Resetarits, Schaeffer, Beckmen, & Beasley, 2000; Sullivan & Spence, 2003; Forson & Storfer, 2006; no effects seen by Carr et al., 2003; Oka et al., 2008), which could contribute to declines in population size, atrazine impairs the immune system and increases the sensitivity of amphibians to infections (Christin et al., 2004; Brodkin, Madhoun, Rameswaran, & Vatnick, 2007; Koprivnikar, Forbes, & Baker, 2007; Rohr et al., 2008).

It is important not to lose sight of the obvious fact that atrazine is a potentially dangerous EDC that can produce lethal as well as developmental and reproductive effects of ecological significance in amphibians and other animals following exposures to environmentally relevant concentrations. The differences in scientific opinion due to methodologies and arguments over effects related to methodologies and responses to dosage differences that all fall within an environmentally relevant range should not be construed as a failure to show the potential harm of this herbicide to biological systems.

7. FUTURE STUDIES

Amphibians worldwide exist in a precarious balance, as evidenced by documented declines in the past few decades. Many anthropogenic factors, ranging from physical to chemical in nature, are involved in amphibian declines, but it is clear that EDCs are an important parameter that must be factored into the explanations. Continued additions of EDCs may prove to be the final push that sends many populations into a downward spiral and extinction. Laboratory studies already have demonstrated considerable sensitivity of amphibians to EDCs, which is supported by at least some of the field studies.

The practice of applying biosolids (sludge) obtained from wastewater treatment plants to agricultural fields as fertilizer has become a fairly common practice in recent years. These biosolids contain many of the pollutants that enter the wastewater plant via domestic and/or industrial sewers and concentrate in the biosolids. Although some research is underway to determine the fate of various compounds present in these biosolids, there are no published reports of their possible impacts on the amphibians that are associated with these agricultural ecosystems. This is an area that needs careful investigation.

The majority of published studies on amphibians have examined only one potential EDC at a series of environmentally relevant dosages. Although investigations of exposures to a single chemical are necessary to determine the range of potential effects of a suspected EDC, single chemical studies are far from the reality of the complex chemical mixtures to which organisms are exposed in nature today. Consequently, careful studies are needed that look at complex mixtures where the doses of the component chemicals are varied independently.

Not only is it important to examine effects of dose, but investigators must take into account the timing of exposures with respect to developmental age as well as the duration of the exposure. Careful laboratory studies in mammals have shown that fetal exposures can lead to manifestations much later in life and even result in transgenerational effects due to epigenetic changes (see Anway, Cupp, Uzumcu, & Skinner, 2005; Diamanti-Kandarakis et al., 2009). Few amphibian studies have been designed in this way; see Rohr and Palmer (2005) for an example of larval exposure to atrazine with effects manifest in a physiological process later in life.

Studies of wild species should be paralleled with studies in a universal test species such as *X. laevis* under uniform conditions (e.g., the FETAX bioassay) to facilitate comparisons from laboratory to laboratory and field site to field site. Further, when conducting future field studies, investigators must recognize that there are no wild 'control' populations in the 21st century and that all environments are contaminated by a mixture of chemicals that may influence our observations. Thus, we should take care to better characterize the chemical environments of 'reference' populations to ensure they are free of other disrupting agents in addition to the one or ones we think we are studying.

Finally, it is important to be mindful of the obvious decline of amphibian populations and entire species and the need for conservation. Understanding the reproductive processes of amphibians and their disruption by EDCs is important to knowledgeable management of wild populations. Consequently, it is imperative that our investigations and surveys are designed and conducted so as not to contribute further to the decline.

ABBREVIATIONS

11-KT	11-Ketotestosterone
ACTH	Corticotropin
BPA	Bisphenol A
CORT	Corticosterone
CRF	Corticotropin-releasing factor
DDT	Dichlorodiphenyltrichloroethane
DEHP	Diethyl phthalate
DHT	5α-Dihydrotestosterone
E_1	Estrone
E_2	17β-estradiol
EAC	Endocrine-active chemical
EC	Emerging contaminant
EDC	Endocrine-disrupting chemical
EDSP	Endocrine Disruptor Screening Program
EDSTAC	Endocrine Disruptor Screening Testing and Advisory Committee
EE_2	Ethinylestradiol
eGFP	Enhanced green fluorescent protein
ER	Estrogen receptor
FETAX	Frog embryo teratogenesis assay—*Xenopus*
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-releasing hormone
GTH	Gonadotropin
HPA	Hypothalamus−pituitary−adrenal
HPG	Hypothalmaus−pituitary−gonad
HPT	Hypothalamus−pituitary−thyroid
LH	Luteinizing hormone
NP	Nonylphenol
P_4	Progesterone
$P450_{aro}$	Aromatase
PBDE	Polybrominated biphenyl ether
PCB	Polychlorinated biphenyl
ppb	Parts per billion
ppt	Parts per trillion ppt
PRL	Prolactin
SF-1	Steroidogenic factor 1
T	Testosterone
T_3	Triiodothyronine
T_4	Thyroxine

TSH Thyroid-stimulating hormone
USEPA United States Environmental Protection Agency
Vtg Vitellogenin
WWTP Wastewater treatment plant

REFERENCES

Adams, A. E. (1946). Sexual conditions in *Triturus viridescens*. IV. The effect of administration of diethylstilbestrol on adult normal and castrated males. *J. Exp. Zool, 101*, 1−39.

Anway, M. D., Cupp, A. S., Uzumcu, M., & Skinner, M. K. (2005). Epigenetic transgenerational actions of endocrine disruptors and male fertility. *Science, 308*, 1466−1469.

Balls, M., & Bownes, M. (1985). *Metamorphosis*. New York: Oxford University Press. 439.

Benotti, M. J., Trenholm, R. A., Vanderford, B. J., Holady, J. C., Stanford, B. D., & Snyder, S. A. (2009). Pharmaceuticals and endocrine disrupting compounds in U.S. drinking water. *Environ. Sci. Technol, 43*, 597−603.

Brodkin, M. A., Madhoun, H., Rameswaran, M., & Vatnick, I. (2007). Atrazine is an immune disruptor in adult northern leopard frogs (*Rana pipiens*). *Environ. Toxicol. Chem., 26*, 80−84.

Carr, J. A., & Norris, D. O. (2006a). The thyroid gland. In D. O. Norris, & J. A. Carr (Eds.), *"Endocrine Disruption: Biological Bases for Health Effects in Wildlife and Humans"* (pp. 87−110). New York: Oxford University Press.

Carr, J. A., & Norris, D. O. (2006b). The adrenal gland. In D. O. Norris, & J. A. Carr (Eds.), *"Endocrine Disruption: Biological Bases for Health Effects in Wildlife and Humans"* (pp. 111−134). New York: Oxford University Press.

Carr, J. A., Gentles, A., Smith, E. E., Goleman, W. L., Urquidi, L. J., et al. (2003). Response of larval *Xenopus laevis* to atrazine: Assessment of growth, metamorphosis, and gonadal and laryngeal morphology. *Environ. Toxicol. Chem., 22*, 396−405.

Carson, R. (1962). *Silent Spring*. New York: Houghton Mifflin.

Cevasco, A., Urbatzka, R., Bottero, S., Massari, A., Pedemonte, F., Kloas, W., & Mandich, A. (2008). Endocrine disrupting chemicals (EDC) with (anti)estrogenic and (anti)androgenic modes of action affecting reproductive biology of *Xenopus laevis*: II. Effects on gonad histomorphology. *Comp. Biochem. Physiol., C147*, 241−251.

Christin, M. S., Menard, L., Gendron, A. D., Ruby, S., Cyr, D., Marcogliese, D. J., Rollins-Smith, L., & Fournier, M. (2003). Effects of agricultural pesticides on the immune system of *Xenopus laevis* and. *Rana pipiens. Aquat. Toxicol., 67*, 33−43.

Clark, E. J., Norris, D. O., & Jones, R. E. (1998). Interactions of gonadal steroids and pesticides (DDT, DDE) on gonaduct growth of larval tiger salamanders. *Ambystoma tigrinum. Gen. Comp. Endocrinol., 109*, 94−105.

Coady, K. K., Murphy, M. B., Villeneuve, D. L., Hecker, M., Jones, P. D., Carr, J. A., et al. (2005). Effects of atrazine on metamorphosis, growth, laryngeal and gonadal development, aromatase activity, and sex steroid concentrations in *Xenopus laevis*. *Ecotoxicol. Environ. Saft., 62*, 160−173.

Denver, R. J. (1997). Experimental stress as a developmental cue: corticotropin-releasing hormone as a proximate mediator of adaptive plasticity in amphibian metamorphosis. *Horm. Behav., 31*, 169−179.

Denver, R. J. (2008). Stress hormones mediate environment−genotype interactions during amphibian development. *Gen. Comp. Endocrinol, 164*, 20−31.

Denver, R. J., Hopkins, P. M., McCormick, S. D., Propper, C. R., Riddiford, L., Sower, S. A., & Wingfield, J. C. (2010). Comparative Endocrinology in the 21st centrury. *Integr. Comp. Biol.,* In press.

Diamanti-Kandarakis, E., Bourguignon, J.-P., Giudice, L. C., Hauser, R., Prins, G. S., Soto, A. M., Zoeller, R. T., & Gore, A. C. (2009). Endocrine-disrupting chemicals: An Endocrine Society scientific statement. *Endocr. Revs., 30*, 293−342.

Diana, S. G., Resetarits, W. J., Schaeffer, D. J., Beckmen, K. B., & Beasley, V. R. (2000). Effects of atrazine on amphibian growth and survival in artificial aquatic communities. *Environ. Toxicol. Chem., 19*, 2961−2967.

Fan, W., Yanase, T., Moringa, H., Gondo, S., Okabe, T., Nomura, M., et al. (2007a). Herbicide atrazine activates SF-1 by direct affinity and concomitant co-activators recruitments to induce aromatase expression via promotor II. *Biochem. Biophys. Res. Comm., 355*, 1012−1018.

Fan, W., Yanase, T., Moringa, H., Gondo, S., Okabe, T., Nomura, M., et al. (2007b). Atrazine-induced aromatase expression is SF-1 dependent: Implications for endocrine disruption in wildlife and reproductive cancers in humans. *Environ. Health Persp., 115*, 720−727.

Fini, J.-B., Paullud-Mothre, S., Le Mevel, S., Palmier, K., Havens, C. M., Le Brun, M., et al. (2009). An innovative continuous flow system for monitoring heavy metal pollution in water using transgenic *Xenopus laevis* tadpoles. *Environ. Sci. Technol., 43*, 8895−8900.

Forson, D. D., & Storfer, A. (2006). Effects of atrazine and iridovirus infection on survival and life history traits of the long-toed salamander (*Ambystoma macrodactylum*). *Environ. Toxicol. Chem., 25*, 168−173.

Fort, D. J., Thomas, J. H., Rogers, R. L., Noll, A., Spaulding, C. D., Guiney, P. D., & Weeks, J. A. (2004). Evaluation of the development and reproductive toxicity of methoxychlor using an anuran (*Xenopus tropicalis*) chronic exposure model. *Toxicol. Sci., 81*, 443−453.

Gilbert, L. I., & Frieden, E. (1981). *Metamorphosis: A Problem in Developmental Biology.* New York: Plenum Press.

Gore, A. (2007). *Endocrine-Disrupting Chemicals: From Basic Research To Clinical Practice*. Totowa NJ: Humana Press.

Guillette, L., Jr., & Crain, D. A. (2000). *Environmental Endocrine Disrupters*. New York: Taylor & Francis.

Guillette, L. J., Jr., Crain, D. A., Gross, T. S., Masson, G. R., Matter, J. M., Percival, H. F., & Woodward, A. R. (1994). Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. *Environ. Health Perspect., 102*, 680−688.

Guillette, L. J., Jr., Gross, T. S., Pickford, D. B., Crain, D. A., Rooney, A. A., & Percival, H. F. (1996). Reduction in penis size and plasma testosterone concentrations in juvenile alligators living in a contaminated environment. *Gen. Comp. Endocrinol., 101*, 32−42.

Hayes, T. B. (2004). There is no denying this: defusing the confusion about atrazine. *Bioscience, 54*, 1138−1149.

Hayes, T. B., Collins, A., Lee, M., Mendoza, M., Noriega, N., Stuart, A. A., & Vonk, A. (2002). Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. *Proc. Natl. Acad. Sci. U.S., 99*, 5476−5480.

Hayes, T. B., Haston, K., Tsui, M., Hoang, A., Haeffele, C., & Vonk, A. (2003). Atrazine-induced hermaphroditism at 0.1 ppb in American leopard frogs (*Rana pipiens*): Laboratory and field evidence. *Environ. Health Perspect., 111*, 568−575.

Hayes, T. B., Stuart, A. A., Mendoza, M., Collins, A., Noriega, N., Vonk, A., Johnston, G., Liu, R., & Kpodzo, D. (2006). Characterization of atrazine-indiuced gonadal malformations in African clawed frogs (*Xenopus laevis*) and comparison with effects of an androgen antagonist (cyproterone acetate) and exogenous estrogen (17β-estradiol): Suppport for the demasculinization/feminization hypothesis. *Environ. Health Persp., 114*, 134−141.

Hecker, M., Park, J.-W., Murphy, M. B., Jones, P. D., Solomon, K. R., van der Kraak, G., et al. (2005). Effects of atrazine on *CYP19* gene expression and aromatase activity in testes and on plasma sex steroid concentrations of male African clawed frogs (*Xenopus laevis*). *Toxicol. Sci., 86*, 273−280.

Hogan, N. S., Duarte, P., Wade, M. G., Lean, D. R. S., & Trudeau, V. L. (2008). Estrogenic exposure affects metamorphosis and alters sex ratios in the northern leopard frog (*Rana pipiens*): Identifying critically vulnerable periods of development. *Gen. Comp. Endocrinol., 156*, 515−523.

Hoke, R. A., & Ankley, G. T. (2005). Application of frog embryo teratogenesis assay—*Xenopus* to ecological risk assessment. *Environ. Toxicol. Chem., 24*, 2677−2690.

Huber, W. (1993). Ecotoxicological relevance of atrazine in aquatic systems. *Environ. Toxicol. Chem., 12*, 1865−1881.

Jooste, A. M., Du Preez, L. H., Carr, J. A., Giesy, J. P., Gross, T. S., Kendall, R. J., Smith, E. E., van der Kraak, G. L., & Solomon, K. R. (2005). Gonadal development of larval *Xenopus laevis* exposed to atrazine in outdoor microcosms. *Environ. Sci. Technol, 39*, 5255−5261.

Kidd, K. A., Blachfield, P. J., Mills, K. H., Palace, V. P., Evans, R. E., Lazorchak, J. M., & Flick, R. W (2007). Collapse of a fish population after exposure to a synthetic estrogen. *Proc. Natl. Acad. Sci. U.S., 104*, 8897−8901.

Kloas, W., Lutz, I., & Einspanier, R. (1999). Amphibians as a model to study endocrine disruptors: II. Estrogenic activity of environmental chemicals *in vitro* and *in vivo*. *Sci. Total Environ., 225*, 59−68.

Kloas, W., Lutz, I., Urbatzka, R., Springer, T., Krueger, H., Wolf, J., Holden, L., & Hosmer, A. (2009). Does atrazine affect larval development and sexual differentiation of South African clawed frogs? *Ann. N.Y. Acad. Sci., 1163*, 437−440.

Kolpin, D. W., Furlong, E. T., Meyer, M. T., Thurman, E. M., Zaugg, S. D., Barber, L. B., & Buxton, H. T. (2002). Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999−2000: A national reconnaissance. *Environ. Sci. Technol., 36*, 1202−1211.

Koprivnikar, J., Forbes, M. R., & Baker, R. L. (2007). Contaminant effects on host−parasite interactions: Atrazine, frogs, and trematodes. *Environ. Toxicol. Chem., 26*, 2166−2170.

Larson, D. L., McDonald, S., Fivizzani, A. J., Newton, W. E., & Hamilton, S. J. (1998). Effects of the herbicide atrazine on *Ambystoma tigrinum* metamorphosis: Duration, larval growth, and hormonal response. *Physiol. Zool., 71*, 671−679.

Levy, G., Lutz, I., Krüger, A., & Kloas, W. (2004). Bisphenol A induces feminization in *Xenopus laevis* tadpoles. *Environ. Res., 94*, 102−111.

Linder, G., Krest, S. K., & Sparling, D. W. (2003). *Amphibian Decline: An Integrated Analysis of Multiple Stressor Effects*. Pensacola FL: SETAC Press.

Lopez, E. M. (2003). "The actions of methoprene acid on a developing urodele, *Ambystoma mexicanum". MA. Thesis*. University of Colorado at Boulder.

Lutz, I., & Kloas, W. (1999). Amphibians as a model to study endocrine disruptors: I. Environmental pollution and estrogen receptor binding. *Sci. Total Environ, 225*, 49−57.

Mattiessen, P. (1998). Effects on fish of estrogenic substances in English rivers. In R. Kendall, R. Dickerson, J. Geisy, & W. Suk (Eds.), *"Principles and Processes for Evaluating Endocrine Disruption in Wildlife."* (pp. 239−247). Pensacola FL: SETAC Press.

McCoy, K. A., Bortnick, L. J., Campbell, C. M., Hamlin, H. J., Guillette, L. J., Jr., & St. Mary, C. M. (2008). Agriculture alters gonadal form and function in the toad *Bufo marinus*. *Environ. Health Perspect., 116*, 1526−1532.

McDaniel, T. V., Martin, P. A., Struger, J., Sherry, J., Marvin, C. H., McMaster, M. E., Clarence, S., & Tetreault, G. (2008). Potential endocrine disruption of sexual development in free ranging male northern leopard frogs (*Rana pipiens*) and green frogs (*Rana clamitans*) from areas of intensive row crop agriculture. *Aquat. Toxicol, 88*. 230-.

McLachlan, J. A., Guillette, L. J., Iguchi, T., & Toscano, W. A., Jr. (2001). Environmental hormones: The scientific basis of endocrine disruption. *Ann. NY Acad. Sci., 948*, 1−143.

Mitsui, N., Tooi, O., & Kawahara, A. (2007). Vitellogenin-inducing activities of natural, synthetic, and environmental estrogens in primary cultured *Xenopus laevis* hepatocytes. *Comp. Biochem. Physiol. C. Toxicol Pharm., 146*, 581−587.

Murphy, M. B., Hecker, M., Coady, K. K., Tompsett, A. R., Jones, P. D., du Preez, L. H., et al. (2006a). Atrazine concentrations, gonadal gross morphology and histology in ranid frogs collected in Michigan agricultural areas. *Aquat. Toxicol., 76*, 230−245.

Murphy, M. B., Hecker, M., Coady, K. K., Tompsett, A. R., Higley, E. B., Jones, P. D., et al. (2006b). Plasma steroid concentration, aromatase activities and GSI in ranid frogs collected from agricultural and non-agricultural sites in Michigan (USA). *Aquat. Toxicol., 77*, 153−166.

Nations, B. K., & Hallberg, G. R. (1992). Pesticides in Iowa precipitation. *J. Environ. Qual., 21*, 486−492.

Noriega, N. C., & Hayes, T. B. (2000). DDT congener effects on secondary sex coloradtion in the reed frog *Hyperolius argus*: a partial evaluation of the *Hyperolius argus* endocrine screen. *Comp. Biochem. Physiol., B126*, 231−237.

Norris, D. O. (2007). *Vertebrate Endocrinology"*. San Diego: Academic Press.

Norris, D. O., & Carr, J. A. (2006). *Endocrine Disruption: Biological Bases for Health Effects in Wildlife and Humans*. New York: Oxford University Press.

Norris, D. O., Carr., J. A., Summers, C. H., & Featherston, R. (1997). Interactions of androgens and estradiol on sex accessory ducts of larval tiger salamanders, *Ambystoma tigrinum*. *Gen. Comp. Endocrinol., 106*, 348−355.

Oka, T., Tooi, O., Mitsui, N., Miyahara, M., Ohnishi, Y., Takase, M., et al. (2008). Effect of atrazine on metamorphosis and sexual differentiation in *Xenopus laevis*. *Aquat. Toxicol., 87*, 215−226.

Orchinik, M., & Propper, C. (2006). Hormone action on receptors. In D. O. Norris & J. A. Carr (Eds.), *"Endocrine Disruption: Biological Bases for Health Effects in Wildlife and Humans"* (pp. 28−57). New York: Oxford University Press.

Orton, F., Carr, J. A., & Handy, R. D. (2006). Effects of nitrate and atrazine on larval development and sexual differentiation in the northern leopard frog *Rana pipiens*. *Environ. Toxicol. Chem., 25*, 65−71.

Park, B. J., & Kidd, K. (2005). Effects of the synthetic estrogen ethinylestradiol on early life stages of mink frogs and green frogs in the wild and *in situ*. *Environ. Toxicol. Chem., 24*, 2027−2036.

Pickford, D. B., & Morris, I. D. (1999). Effects of endocrine-disrupting contaminants on amphbian oogenesis: Methoxychlor inhibits progesterone-induced maturation of *Xenopus laevis* oocytes *in vitro*. *Environ. Health Perspect., 107*, 285−292.

Pickford, D. B., & Morris, I. D. (2003). Inhibition of gonadotropin-induced oviposition and ovarian steroidogenesis in the African clawed frog (*Xenopus laevis*) by the pesticide methoxychlor. *Aquat. Toxicol., 62*, 179−194.

Qin, Z.-F., Qin, X.-F., Yang, L., Han-Ting, L., Zhao, X.-R., Xu, X.-B. (2007). Feminizing/demasculinizing effects of polychlorinated biphenyls on the secondary development of *Xenopus laevis*. *Aquat. Toxicol*., 84, 321−327.

Reeder, A. L., Foley, G. L., Nicholas, D. K., Hansen, L. G., Wikoff, B., Faeh, S., et al. (1998). Forms and prevalence of intersexuality and effects of environmental contaminants on sexuality in cricket frogs (*Acris crepitans*). *Environ. Health. Perspect., 106*, 261−266.

Reeder, A. L., Ruiz, M. O., Pessier, A., Brown, L. E., Levengood, J. M., Phillips, C. A., et al. (2005). Intersexuality and the cricket frog decline: Historic and geographic trends. *Environ. Health Perspect., 113*, 261−265.

Reylea, R. A. (2003). Predator cues and pesticides: a double dose of danger for amphibians. *Ecol. Appl., 13*, 1515−1521.

Roberge, M., Hakk, H., & Larsen, G. (2004). Atrazine is a competitive inhibitor of phosphodiesterase but does not affect the estrogen receptor. *Toxicol. Lett., 154*, 61−68.

Rohr, J. R., & Palmer, B. D. (2005). Aquatic herbicide exposure increases salamander desiccation risk eight months later in a terrestrial environment. *Environ. Toxicol. Chem., 24*, 1253−1258.

Rohr, J. R., Sager, T., Sesterhenn, T. M., & Palmer, B. D. (2006). Exposure, postexposure, and density-mediated effects of atrazine on amphibians: Breaking down net effects into their parts. *Environ. Health Persp., 114*, 46−50.

Rohr, J. R., Schotthoefer, A. M., Raffel, T. R., Carrick, H. J., Halstead, N., Hoverman, J. T., et al. (2008). Agrochemicals increase trematode infections in a declining amphibian species. *Nature, 455*, 1235−1239.

Sanderson, J. T., Letcher, R. J., Heneweer, M., Giesy, J. P., & van den Berg, M. (2001). Effects of chloro-s-triazine herbicides and metabolites on aromatase activity in various human cell cell lines and on vitellogenin production in male carp hepatocytes. *Environ. Health Persp, 109*, 1027−1031.

Sanderson, J. T., Seinen, W., Giesy, J. P., & van den Berg, M. (2000). 2-Chloro-s-triazine herbicides induce aromatase (CYP19) activity in H295R human adrenocortical carcinoma cells: a novel mechanism of estrogenicity? *Toxicol. Sci., 54*, 121−127.

Schapiro, M. (2007). *Exposed: The Toxic Chemistry of Everyday Products and What's at Stake for American Power*. White River Junction, VT: Chelsea Green Publishing.

Sherry, J., Gamble, A., Fielden, M., Hodson, P., Burnison, B., & Solomon, K. R. (1999). An ELISA for brown trout (*Salmo trutta*) vitellogenin and its use in bioassays for environmental estrogens. *Sci. Total Environ., 225*, 13−31.

Shi, Y.-B. (2000). *Amphibian Metamorphosis: From Morphology to Molecular Biology*. New York: Wiley-Liss.

Sparling, D. W., Linder, G., & Bishop, C. A. (2000). *"Ecotoxicology of Amphibians and Reptiles."*. Pensacola FL: SETAC Press.

Storrs, S. I., & Kisecker, J. M. (2004). Survivorship patterns of larval amphibians exposed to low concentrations of atrazine. *Environ. Health Perspect., 112*, 1054−12057.

Sullivan, K. B., & Spence, K. M. (2003). Effects of sublethal concentrations of atrazine and nitrate on metamorphosis of the African clawed frog. *Environ. Toxicol. Chem., 22*, 627−635.

Suzawa, M. and Ingraham, H.A. (2008). The herbicide atrazine activates endocrine gene networks via non-steroidal NR5A nuclear receptors in fish and mammalian cells. PLoS ONE 3, e2117. doi:10. 1371/journal. pone0002117.

Swan, S. H. (2008). Environmental phthalate exposure in relation to reproductive outcomes and other health endpoints in humans. *Environ. Res., 108*, 177−184.

Swan, S. H., Main, K. M., Liu, F., Stewart, S. L., Kruse, R. L., Calafat, A. M., et al., the Study for Future Families Research Team. (2005). Decreased anogenital distance among male infants with prenatal phthalate exposure. *Environ. Health Perspect., 113*, 1056−1061.

Tata, J. R. (2005). Hormonal control of metamorphosis. In H. Heatwole (Ed.), *"Amphibian Biology", Vol 6* (pp. 2208−2227). Chipping Norton NSW, Australia: Surrey Beatty & Sons.

Tavera-Mendoza, L., Ruby, S., Brousseau, P., Fournier, M., Cyr, D., & Marcogliese, D. (2002a). Response of the amphibian tadpole (*Xenopus laevis*) to atrazine during sexual differentiation of the testis. *Environ. Toxicol. Chem.*, 527−531.

Tavera-Mendoza, L., Ruby, S., Brousseau, P., Fournier, M., Cyr, D., & Marcogliese, D. (2002b). Response of the amphibian tadpole (*Xenopus laevis*) to atrazine during sexual differentiation of the ovary. *Environ. Toxicol. Chem.*, 1264−1267.

Urbatzka, R., Bottero, S., Mandich, A., Lutz, I., & Kloas, W. (2007). Endocrine disrupting chemicals (EDC) with (anti)estrogenic and (anti)androgenic modes of action affecting reproductive biology of *Xenopus laevis*: I. Effects on sex steroid levels and biomarker expression. *Comp. Biochem. Physiol., C144*, 310−318.

Vajda, A. M., & Norris, D. O. (2005). The effects of steroids and dioxin (2,3,7,8-TCDD) on the developing wolffian ducts of the tiger salamander (*Ambystoma tigrinum*). *Gen. Comp. Endocrinol., 141*, 1−11.

Vajda, A. M., & Norris, D. O. (2006). Endocrine-active phytochemicals: Environmental signaling context and mechanisms. In D. O. Norris, & J. A. Carr (Eds.), *"Endocrine Disruption: Biological Bases for Health Effects in Wildlife and Humans"* (pp. 390−423). New York: Oxford University Press.

Vandenberg, L. N., Maffini, M. V., Sonnenschein, C., Rubin, B. S., & Sotop, A. M. (2009). Bisphenol-A and the great divide: A review of controversies in the field of endocrine disruption. *Endocr. Revs., 30*, 75−95.

Vonesh, J. R., & Buck, J. C. (2007). Pesticide alters oviposition site selection in gray treefrogs. *Oecologia, 154*, 219−226.

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Species Index

Subject Index

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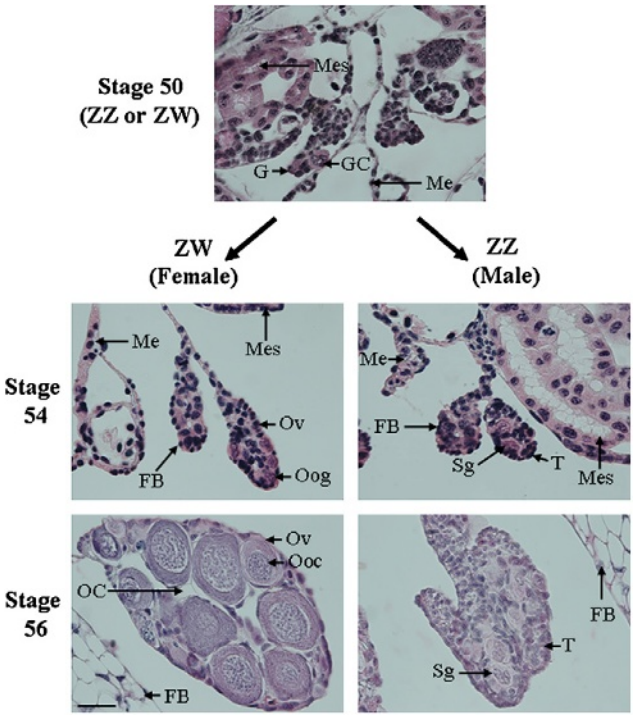

FIGURE 1.2 Development of the gonads during larval life in *Pleurodeles waltl*. At stage 50, the gonad (G) is undifferentiated and has the same aspect in both sexes: it is a small longitudinal organ on each side of the gut mesentary (Me) near the mesonephros (Mes) and it contains a few cortical germ cells (GCs). At stage 54, the ovary (Ov) is characterized by oogonia (Oog) located in the cortex of the gonad and a nascent ovarian cavity (OC), whereas the testis contains spermatogonia (Sg) in its medulla. In both cases, the differentiation of the fat body (FB) has begun. At stage 56 (end of metamorphosis), the ovary contains numerous oocytes (Ooc) that have entered prophase I of meiosis and has begun to accumulate yolk; the ovarian cavity is well developed. In contrast, the testis still contains spermatogonia (Sg) (not yet entered meiosis); in the medulla, spermatogonia are found in lobules and will constitute cysts. The fat body is well differentiated in both ZZ and ZW larvae. Bar = 50 μm; all pictures were obtained at the same magnification.

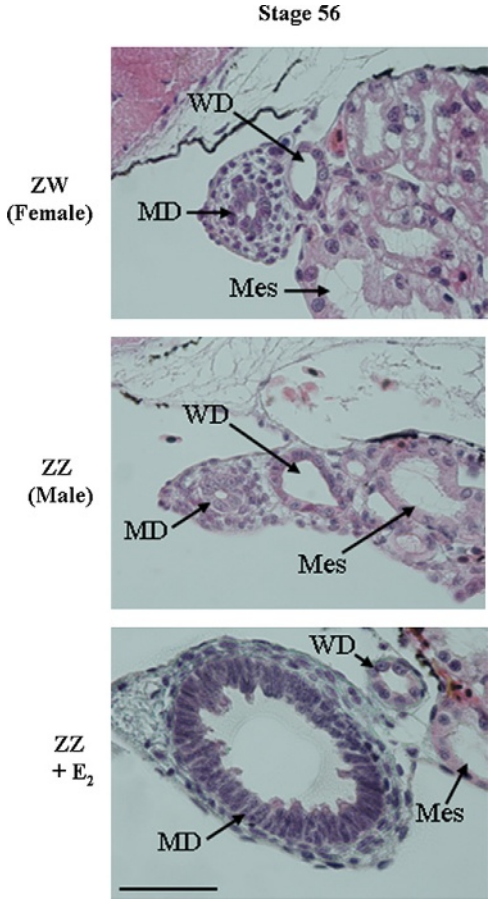

FIGURE 1.3 Urogenital ducts of *Pleurodeles waltl*. At stage 56, male (ZZ) and female (ZW) possess both Wolffian and Müllerian ducts located in the vicinity of the mesonephros (Mes). The Wolffian duct (WD) is used for urine elimination in both sexes, and in males it is also used for sperm transport. The Müllerian duct (MD) will differentiate into the oviduct in females, whereas in males it will be maintained without differentiation but has the capacity to differentiate into oviduct upon estradiol stimulation. Bar = 100 µm; all pictures were obtained at the same magnification.

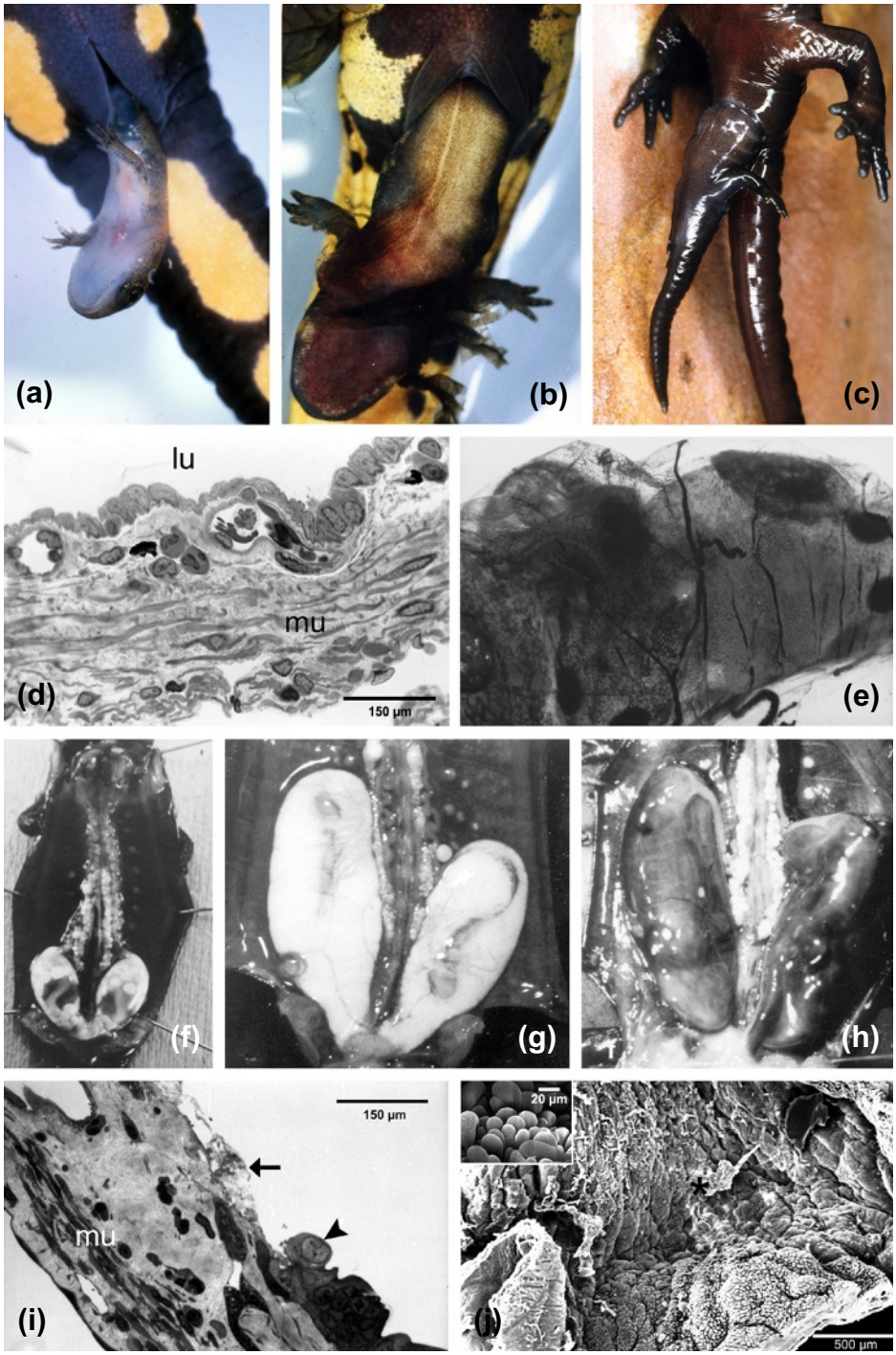

FIGURE 7.1 Oviductal incubation and various 'parities' in *Salamandra* spp. (a) Larviparity in *Salamandra salamandra*. (b) Birth of an advanced larva in *Salamandra salamandra fastuosa*. (c) Pueriparity in *Salamandra atra*. *Copyright of (a–c) retained by W. Sauer, Marburg, Germany.* (d) Uterine epithelium of *S. salamandra* (semithin epoxy section stained with toluidine blue borax). (e) Vascularization of the uterine wall in *S. salamandra* (ink-injected and cleared preparation). (f) Embryos of *S. atra* shortly before hatching, stage 1. (g) Larvae at early stage 2 within the embryotrophic egg mass. (h) Transition from stage 2 to stage 3. Arrowheads indicate the location of the *zona trophica*. *(f–h) from Guex and Greven (1994)*. (i) *Zona trophica* with large cells (arrowhead) and areas devoid of epithelial cells (arrow) in *S. atra* (semithin section stained with toluidine blue borax). (j) *Zona trophica*; note the grazed areas (asterisk). Inset: Cells bulging in the uterine lumen (SEM). l, lumen of the uterus; mu, muscle cells.

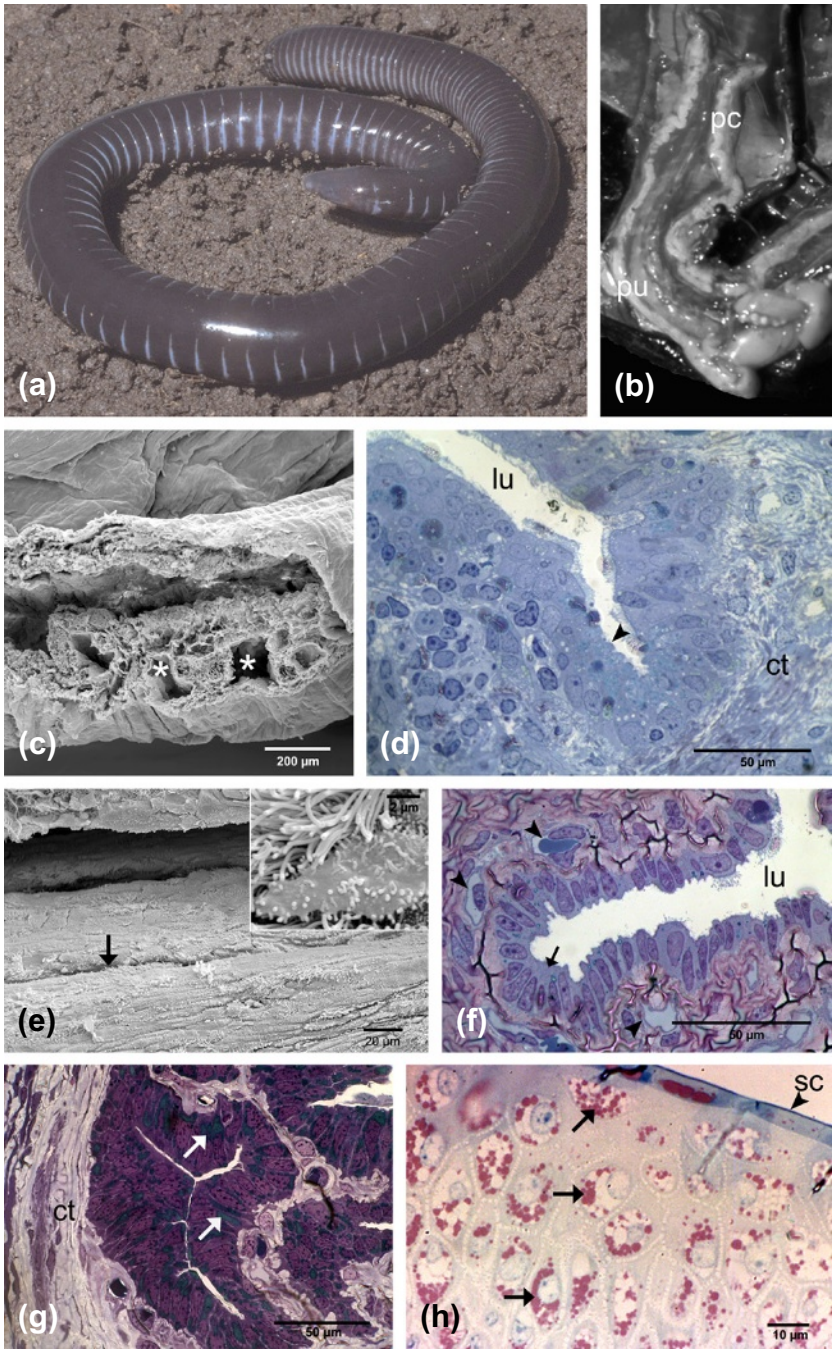

FIGURE 7.2 Oviductal incubation in Gymnophiona. (a) Female of the viviparous *Geotrypetes seraphini*. *Copyright retained by A. Kupfer, Jena, Germany.* (b–f) Oviduct of a non-reproductive female of *Typhlonectes natans*. (b) Pars convoluta (pc) and relatively straight pars uterine (PU). (c) Pars convoluta with large blood vessels (asterisks) (SEM). (d) Pars convoluta epithelium with ciliated cells and non-ciliated moderately secretory cells (arrowhead) on the bottom of a fold. (e) Uterus with flattened folds (arrow) covered with ciliated cells and cells with short microvilli (inset) (SEM). (f) Fold of the uterine epithelium fold; note capillaries (arrowheads) and very few lipid droplets (arrow) (semithin epoxy section, toluidine borax). (g) Uterine fold of a pregnant female of *Chtonerpeton indistinctum* with prismatic cells and accumulations of lipids (arrows) (semithin epoxy section, toluidine borax). (h) Lipid- (arrows) rich epidermis cells of the skin-feeding *Boulengerula taitanus* (semithin section, Nile blue). ct, connective tissue; lu, lumen of the oviduct; sc, stratum corneum.

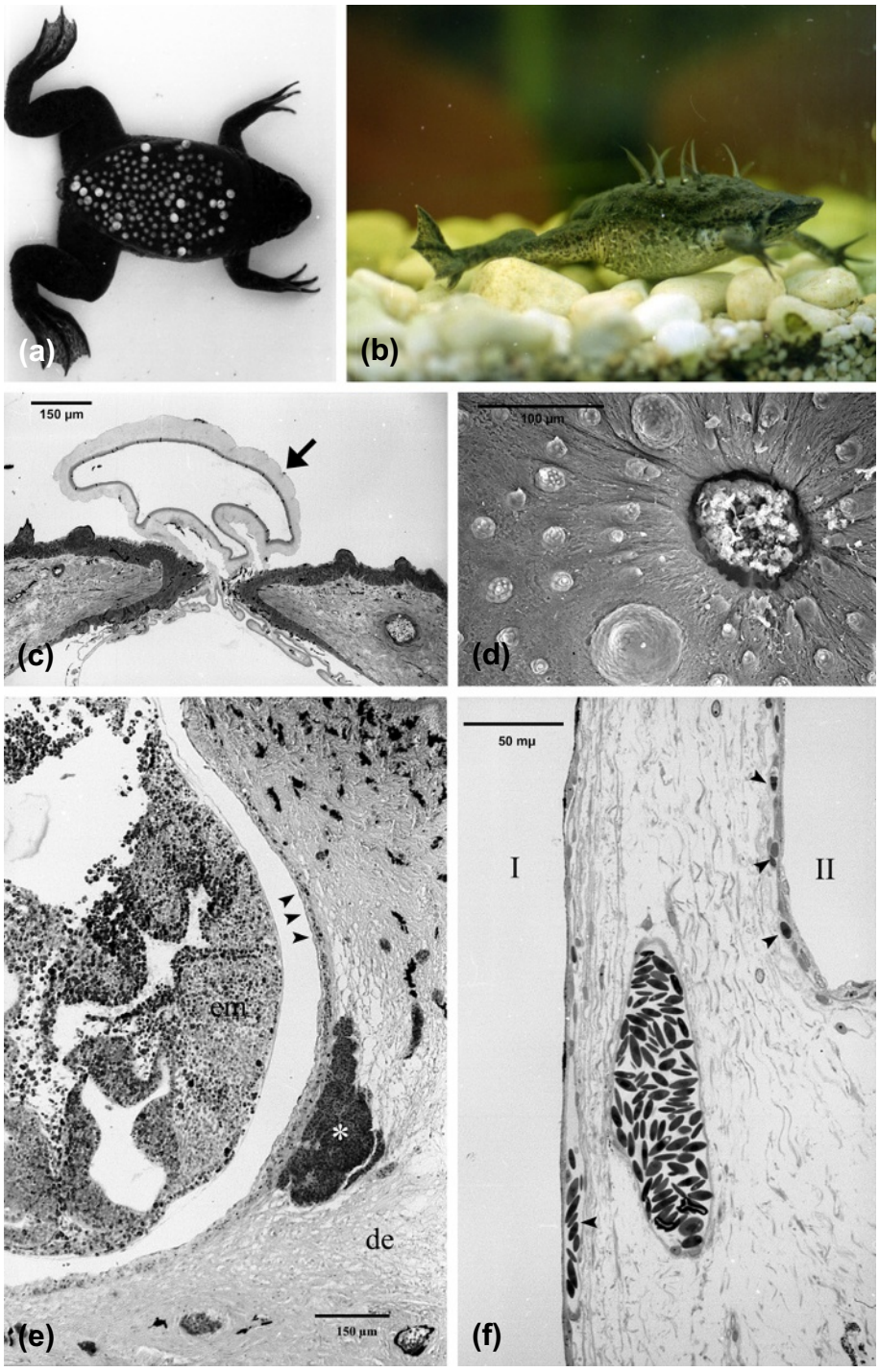

FIGURE 7.3 Skin incubation in *Pipa carvalhoi*. (a) Female a short time after mating with eggs on her back. (b) Birth of tadpoles tail ahead. (c) Pore of a brood chamber with a cup of egg jelly (arrow) (semithin epoxy section, toluidine blue borax). (d) Pore of the brood chamber closed by debris (SEM). (e) Brood chamber with embryo (em) and large dermal (de) blood vessel (asterisk); note thinness of the wall (arrowheads) (paraffin section, trichrome-Goldner). (f) Vascularized connective tissue between two chambers (I, II); note capillaries immediately beneath the very thin brood chamber epithelium (arrowheads).

FIGURE 7.4 Skin incubation in Hemiphractinae. (a) Egg brooding female of *Stefania evansi*. (b) Pregnant female of *Gastrotheca riobambae*. (c) Bell-gills in *Gastrotheca griswaldi*. (d) Birth of a froglet in *G. griswaldi* without the aid of the female. *Copyright of (a–c) retained by Prof. Dr. U. Sinsch, Koblenz, Germany.*

FIGURE 10.4 Morphological forms of *Ambystoma tigrinum*. From left to right, typical paedomorphic larva, cannibal paedomorphic larva, metamorphosed cannibal morph, metamorphosed non-cannibal morph. *Photograph courtesy of Hobart M. Smith.*

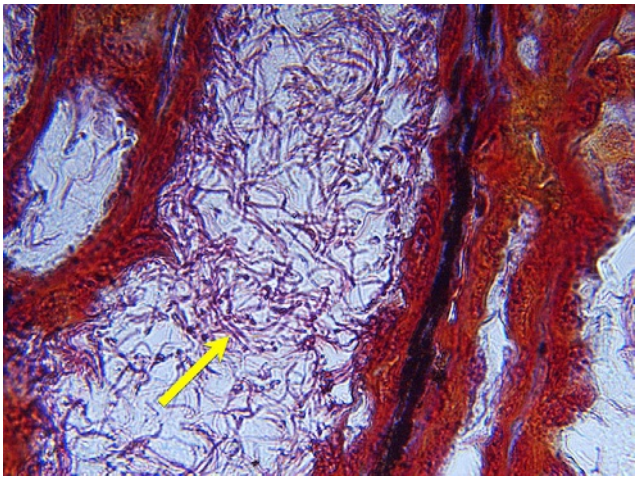

FIGURE 10.5 Presence of sperm in spermatheca of an adult female paedomorphic tiger salamander, *Ambystoma tigrinum*. Sperm (arrow) were observed in the lumina of spermathecal tubules only during the breeding season (late March to early May) and at no other time of year.

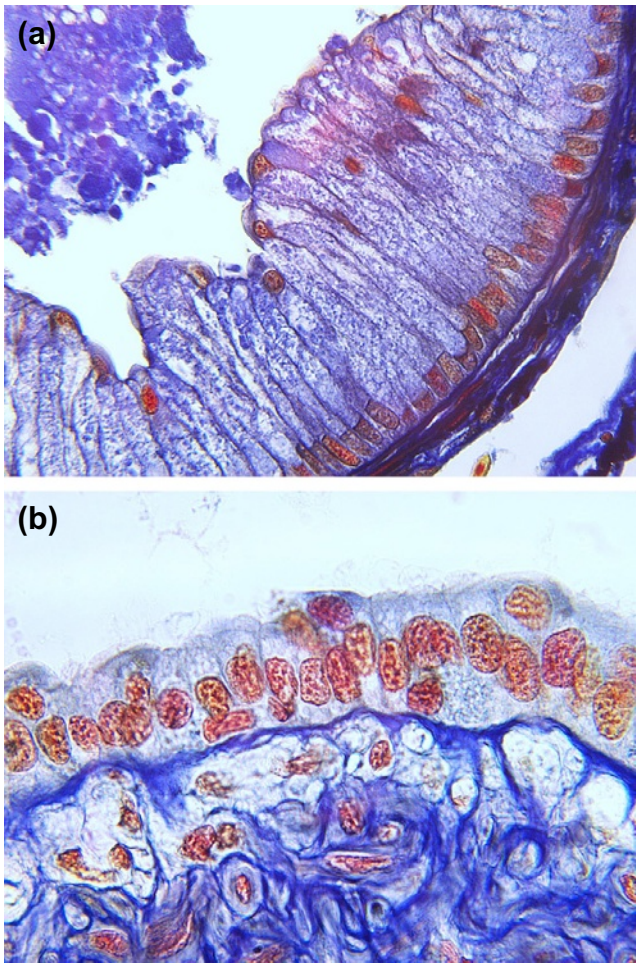

FIGURE 10.7 Vas deferens of paedomorphic tiger salamanders, *Ambystoma tigrinum*. (a) Vas deferens epithelium from a male captured in early November (40X). Secretion is visible in the upper left and at the luminal surface of the columnar epithelium. (b) Vas deferens from a male captured in early June. Note the minimal epithelial thickness and the thick connective tissue layer due to collapse of the vas deferens after evacuation of sperm.