FISH PHYSIOLOGY

Edited by W. S. HOAR, D. J. RANDALL, & E. M. DONALDSON

VOLUME IX

REPRODUCTION

Part A

Endocrine Tissues and Hormones

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PREFACE

The preface to Volume I of "Fish Physiology" noted that a six-volume treatise would attempt to review recent advances in selected areas of fish physiology, to relate these advances to the existing body of literature, and to delineate useful areas for future study. The hope expressed at that time was that the series would serve the biologists of the 1970s as its predecessor "The Physiology of Fishes" (M. E. Brown, editor) has served its readers throughout the 1960s. Our general objectives remain, but with Volumes VII (Locomotion) and VIII (Bioenergetics and Growth) the emphasis has been somewhat altered; these later volumes presented in-depth reviews and assessments of current research in selected areas of fish physiology—especially areas where advances have been particularly rapid during the past decade. In keeping with this concept, we are pleased to add to the series Volumes IXA and IXB on fish reproduction.

When Volume III was published in 1969, the physiology of fish reproduction was reviewed in three chapters. The present treatment in two parts (A and B) attests to the rapid developments in this field. Moreover, Volume IX deals only with selected topics on reproductive physiology, especially the endocrinology, behavior, environmental interactions, and fertility-related topics. Several subjects included in Volume III are not reviewed in these volumes (viviparity, for example), whereas others that now merit consideration in separate chapters were not sufficiently developed to require any comment in Volume III (the hypothalamic hormones and hormone receptors, for example). With the exception of Part A, Chapter 1, which is devoted to the Cyclostomes and Part A, Chapter 2, which is devoted to the Chondrichthyes, the books deal with the much more thoroughly studied teleost fishes.

Volume IX reflects the practical importance of studies in fish reproductive physiology. The control of fertility is now a subject of great economic importance in the manipulation of valuable fisheries resources. Many significant advances and future trends in the research on fertility of teleost fishes are evaluated in several chapters of Part B.

Finally, the editors are happy to express their appreciation to all those who devoted their time to this project; the authors are all active research scientists, and in most cases they had to find the many hours required for writing in an already full program. We are fortunate to have had the pleasant cooperation of the leaders in this rapidly changing area of fish physiology.

W. S. HOAR
D. J. RANDALL
E. M. DONALDSON

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1

REPRODUCTION IN CYCLOSTOME FISHES AND ITS REGULATION

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I. INTRODUCTION

Sexual reproduction is at once the most conservative and yet the most adaptive of functions in the propagation and evolution of species. The cyclostomes illustrate this principle very well. Furthermore, knowledge of how cyclostomes regulate their reproduction offers a basis for understanding the evolution of vertebrate reproductive control mechanisms in general. However, it should be emphasized at the outset that between the two major cyclostome groups the differences in mechanisms regulating reproduction

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appear to be as great or greater than they are among the rest of the vertebrates as a whole. How much of this difference is primitive, and how much is specialized and adaptive remains to be clarified by further study.

The basic differences in reproductive patterns between the two major agnathan groups, together with anatomical and paleontological evidence, indicate that the separation of the petromyzonids, or lampreys, and the myxinoids, or hagfishes, is a very ancient one. Although modern lampreys and hagfish are undoubtedly related groups, the fossil agnathan cephalaspid and heterostracan groups, their probable respective ancient ancestors, diverged during the Pre-Cambrian period more than 600 million years ago (Jarvik, 1968). Hence, there has been as much time available for myxinoids and petromyzonids to evolve adaptive differences from each other as there has been for all of the other vertebrates to evolve into the myriads of modern species.

II. REPRODUCTIVE PATTERNS IN LAMPREYS

All lampreys inhabit fresh water for at least part of their life cycles. In contrast to the direct development of hagfish, a lamprey develops into a blind microphagous larva (the ammocete), which spends a relatively protected burrowing period of several years of slow growth, usually in small streams. At the end of the ammocete phase there is a metamorphosis which involves rapid, important, anatomic changes, especially in the head, that include development of eyes and mouth structures. At the time of metamorphosis the two general types of lampreys become differentiated. In one type (the "nonparasitic" species), gonadal differentiation and metamorphosis occur together. Shortly after the end of the metamorphic period, spawning occurs and the lampreys die. In the "parasitic" lamprey species, metamorphosis yields a sexually immature, eyed form, which migrates downstream into larger bodies of water, generally marine. There its newly developed oral sucker and oral disc of horny teeth enable it to attack large fish, adhering to them while it consumes blood and tissue from the victim. In this "macrophagous" period the parasitic hampreys grow rapidly, and, after a period of 1–2 years, they return to the freshwater streams into which they migrate upstream. Here they stop feeding and complete gonadal maturation. After a nonfeeding interval of several months these animals spawn, usually during the early spring, and die (Larsen, 1980).

It is clear from this summary of the life cycles of parasitic and nonparasitic lamprey species that the fundamental difference between these two groups is the time of maturation of reproductive tissues relative to maturation of somatic tissues. This phenomenon of temporal dissociation or

asynchronous development of the two types of tissue in different lamprey species is heterochrony.

Heterochrony during lamprey development has been discussed eloquently by Hardisty (1979). It is illuminating to consider heterochrony in the context of another feature characteristic of lampreys, the occurrence of paired species. It has been observed by Hubbs and Trautman (1937) and amplified by later researchers (Zanandrea, 1954; Hardisty and Potter, 1971; Potter, 1980) that for a number of individual parasitic species there may be one, or even several, nonparasitic isolated stream species which are closely allied by morphology and by general (nearby) geographic distribution. This, in turn, indicates that the nonparasitic stream lampreys have evolved in relatively restricted freshwater locales from the more freely ranging respective parasitic ancestral species.

Hardisty (1979), in comparing and contrasting paired lamprey species, emphasizes that the total life-span of the two is usually similar. If the nonparasitic species is derived from the parasitic, then they differ mostly in omission of the parasitic phase by stream lampreys. Therefore, Hardisty considers the gonadal maturation phenomenon as occurring "on schedule," but larval life is prolonged in the nonparasitic species and substituted for the parasitic period (Fig. 1). The earlier gonadal maturation relative to the time

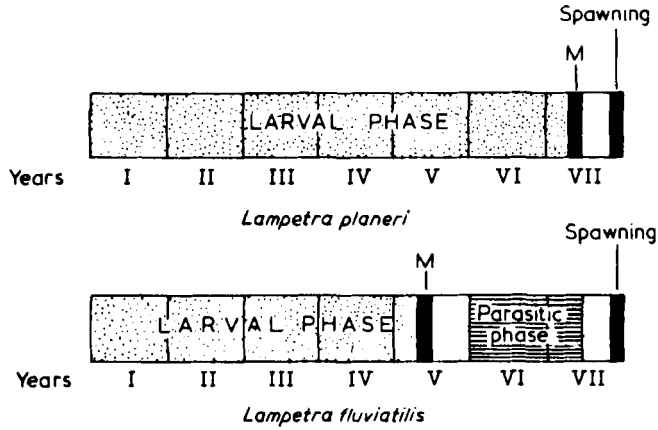


Fig. 1. Diagrammatic summary, year by year, of the time spent as larval (ammocoetes) and metamorphosed adults and sexually mature adults during the life cycles of the paired species of lampreys *Lampetra planeri* (nonparasitic) and *Lampetra fluviatilis* (parasitic). *M* indicates the time of somatic metamorphosis. The period labeled "spawning" is the time of completion of sexual maturation and gametogenesis. The unshaded areas are periods when the animals are not feeding. As Hardisty has emphasized, the total life-spans of the paired species are similar. In the freshwater (nonparasitic) species, *L. planeri*, the parasitic phase has been eliminated and the animal spends the equivalent time period as a larva by delaying metamorphosis until just before spawning. (From Hardisty, 1979.)

of metamorphosis in nonparasitic species may be regarded as a type of neoteny. The adaptive value of the prolongation of larval life by stream lampreys is a more certain survival in a more protected life with a dependable, although limited, microphagous food supply. The penalty of this life is slower growth, metamorphosis at a much smaller body size and, therefore, with smaller and fewer eggs.

The frequency of the paired species phenomen indicates a remarkable adaptive plasticity of lamprey species in general. Because in each instance in which a nonparasitic stream lamprey species has evolved, heterochronic dissociation of gonadal from somatic maturation has occurred, evolution of this feature appears to be a readily achieved phenomenon. Genetically, the paired parasitic and nonparasitic lamprey species are sufficiently similar so that viable hybrids can be produced experimentally between them (Hardisty, 1979).

III. REPRODUCTIVE PATTERNS OF THE MYXINOIDS

The hagfishes are marine organisms, most of which inhabit deep water (50–800 or more m), where one may assume that annual cycles of temperature do not occur. For such species from which appropriate collections have been made, there appears to be no seasonally defined reproductive cycle. That is, at any time of year both males and females in various stages of gametogenesis can be found. Exceptions to this generalization are two species of *Eptatretus, E. burgeri* from Japanese waters and *E. cirrhatus* from New Zealand, which, at least at certain times of the year, migrate into seawater 4–10 m deep. *Eptatretus burgeri*, in fact, regularly migrates into shallow water (about 10 m deep) in the colder months, and into colder water of 50 m depth, or more, during the warmer summer months (July–October). Accordingly, in *E. burgeri*, the breeding cycle appears to be annual and seasonally synchronized, all animals trapped at one time being in the same stage of the reproductive cycle (Kobayashi *et al.*, 1972). Therefore, we must recognize that among the hagfishes although the reproductive cycle may be divorced from seasonal environmental phenomena (light, temperature) in most species, in at least several species seasonally synchronized reproductive cycles can occur.

Although there is little information available concerning development in hagfishes, it appears clear that development is direct, i.e., not involving a larval form as in the lampreys (Price, 1896).

Because of their inaccessability to observation at depths greater than 100 m, reproductive patterns of myxinoids are practically unknown, and only inferred. Even in the relatively shallow species *E. burgeri*, which can be

observed by divers at 10 m, breeding occurs during the period June to October while the animals migrate into deeper waters of more than 50 m. Although occasional photography is possible at these depths, extended study to reveal the precise time of spawning, and the deposition of eggs and their fertilization has not been practical. This is in spite of a long standing interest in these questions. An example of this interest is the prize, a gold medal, offered from 1864 until 1865 by the Danish Academy of Sciences for resolution of several specific questions, posed at that time, concerning the life cycle, reproduction, and development of *Myxine glutinosa* (Anonymous, 1862, 1863). Only one question, regarding whether there is a male in this species, has been resolved, but too late to claim the prize. Other questions remain. There are no external genitalia in myxinoids for transfer of sperm to the female. But it is clear that eggs must encounter a high concentration of sperm for successful fertilization to occur through a single very narrow canal, the micropyle, which is at one point only twice the diameter of a spermatozoan, penetrating the shell at the animal pole end of the large yolky egg. How the hagfish can create such a high concentration of sperm in the proximity of the micropyle is unknown and remains a subject of speculation.

Fertile eggs containing developing embryos have been only rarely encountered for any species of hagfish. Three developing embryos of *M. glutinosa* have been described (Fernholm, 1969); all were advanced embryos. These were recovered from trawls by fishermen. Descriptions of early development of the hagfish are all based on the approximately 150 *Eptatretus stouti* embryos collected in 1896 by Professor Bashford Dean at Monterey, California (Dean *et al.*, 1897). These were used in early research by Dean himself (1899), as well as by von Kupffer (1900) and later by Conel (1931) who studied hagfish brain development. Earlier, G. C. Price (1896) obtained three advanced *E. stouti* embryos in the same area for some cursory studies, which were only briefly described. Despite continuing interest in hagfish development, efforts to obtain additional embryos have yielded nothing, and Dean's success in obtaining such a large a number of embryos has never been repeated.

Another puzzling question about hagfish reproduction derives from the apparently limited supplies of spermatozoa available. The male gonad is a small structure in the posterior extremity of the body cavity. It is composed of follicles that are filled with cells in various stages of spermatogenesis, and few follicles contain ripe spermatozoa (Jespersen, 1975; Hardisty, 1979). Furthermore, because there are no ducts in which spermatozoa can be stored and led to the cloacal aperture, we may presume that as spermatozoal follicles ripen they break open and release spermatozoa into the coelomic space. From here they must be drained toward the exterior through an opening in the cloacal wall. Therefore, it appears that there is no anatomical

basis for sudden release of large amounts of spermatozoa at a given time, e.g., the moment of spawning. Fertilization through a small micropyle in a shelled egg requires a high concentration of sperm, and it is unclear how this can be achieved by hagfish. Jespersen (1975) comments further: "The egg number is very small and the production of sperm is apparently moderate, considering in addition the morphology of the spermatozoa, these facts may indicate some specializations in spawning behaviour." Because hagfish have no copulatory structures, and fertilization must be external, it is difficult to imagine how behavioral specialization can overcome this seemingly difficult problem. In keeping with Jespersen's suggestion, that the answer to these questions is in behavioral modification, we should be aware that the only available methods for capture of hagfish depend on their response to feeding baits. If fully breeding male hagfish are not susceptible to capture because they are nonfeeders, then some of the puzzles concerning hagfish reproduction are explicable, including the frequently high female-to-male sex ratios that have been reported (Walvig, 1963). Reduced or arrested feeding by breeding males would protect the newly laid eggs from cannibalism. It is worth noting that in spawning lampreys (see Fig. 1), neither males or females are feeding. Jespersen (1975) reports that of 1000 specimens of *Myxine glutinosa* collected at several different times of the year in the Oslofjord in Norway in 1971, only 200 were adult males. Of these 200 males only one contained motile sperm. It is clear that the hagfish has solved these problems somehow, even if the reproductive biologist remains mystified.

IV. SEX DIFFERENTIATION

The model embryonic vertebrate gonad is bilateral and bipotential. Primitive sex cells migrate into it, generally from an endodermal or yolk sac source. If they remain lodged in the cortex of the gonad, female differentiation follows; if they penetrate the medulla of the gonad, carried there by the sex cords, male differentiation results. In sex reversal of the gonad, following experimental manipulation (e.g., after sex steroid hormone treatment of teleost embryos), primitive sex cells are caused to associate with the gonadal cortex or medulla regardless of their sex genotype. Genetically male primitive sex cells, if they remain in the cortex, become oocytes. If genetically female sex cells are carried into the medulla by the primary sex cords, they differentiate as spermatozoa.

Neither lampreys nor hagfish conform with this model, and furthermore, each has its own distinct pattern of sex differentiation. In both hagfishes and lampreys the gonads are single (on one side of the dorsal mensentery) and

elongated structures. In neither group is corticomedullary regionalization of male and female potentialities seen.

A. Sex Differentiation in Lampreys

Lampreys have received more study in this respect than hagfish and display what may be called a progynous pattern of sex differentiation. According to Hardisty (1979), in younger ammocoetes of *Lampetra planeri*, there is a proliferation of germ cells followed by growth to primary oocyte status in all animals. They are recognized as oocytes by Hardisty because of their form and nuclear meiotoic changes. At this stage, it is difficult or impossible to recognize any differentiation of sex. In midammocoete stages, the numbers of oocytes are reduced by atresia, but this atresia is almost complete in presumptive testes. In the oldest, premetamorphic *L. planeri* ammocoetes, gonads are completely differentiated. Immature ovaries contain only oocytes, and in testes all oocytes have disappeared and only a few nests of presumptive spermatogenic cells remain. The spermatogenic elements in the premetamorphic testis are believed to have arisen from a few persisting stem cells, not by conversion of the oocytes (Hardisty, 1979).

This interpretation of observable events in sex differentiation in *L. planeri* is not universally accepted. Busson-Mabillot (1965, 1967a,b) has studied the same phenomenon in *L. planeri*, as well as in *Petromyzon marinus*, and has considered the larval oocytes of Hardisty to be "oviform" cells. In the absence of further information, this difference of opinion as to whether both male and female gonads in ammocoetes pass through a transitory female state is largely interpretive. In an attempt to experimentally influence sex differentiation in lampreys, Hardisty and Taylor (1965) exposed young ammocoetes to sex steroids prior to gonad differentiation. No influence was found. Although this experiment suggests that sex steroids may not have a role in gonadal differentiation in lampreys, it leaves completely open the question of what does regulate this phenomenon. It should be noted that although sex steroids will reverse sex in a number of teleostean species, they have little or no influence on the differentiating gonad in many higher vertebrate embryos.

B. Sex Differentiation in Hagfish

With respect to the general model of vertebrate gonadal organization and differentiation, the hagfishes present an immediate important difference. Instead of a corticomedullary localization of potentially male and female structures there is an anteroposterior axis. Ovarian structure is found in the

length of the gonad, from the heart region to a level approximately 15 somites anterior to the cloaca, at the posterior end of the coelomic cavity. The relatively short posterior section of the gonad is always testicular. There is no report in the literature of mixture of male and female germinal structures at a single level. When hermaphroditic gonads are found, functional male and female elements coexist, in series, in the same gonad with a reasonably sharp transition from ovary to testis in *Eptatretus stouti*, at the normal anteroposterior level where testis should normally begin (Fig. 2A, B).

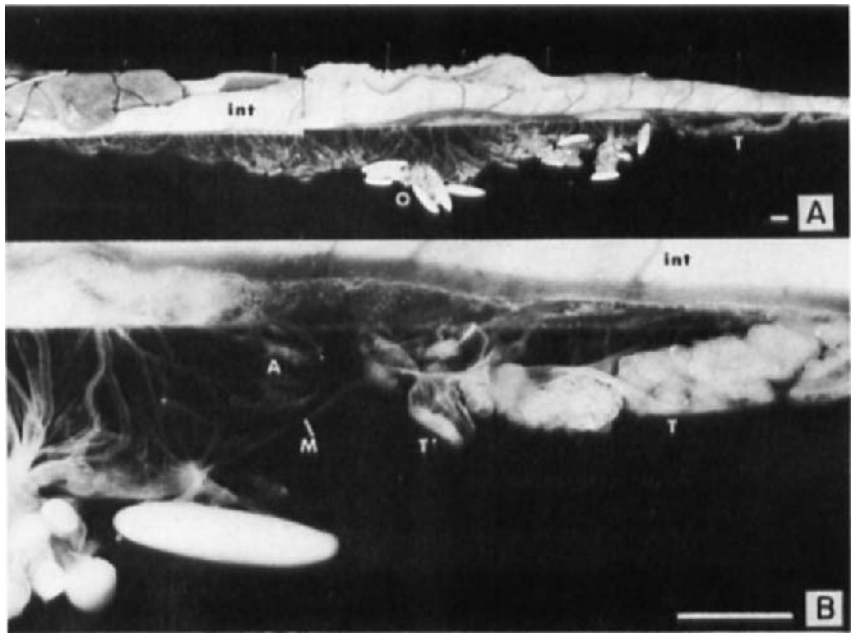
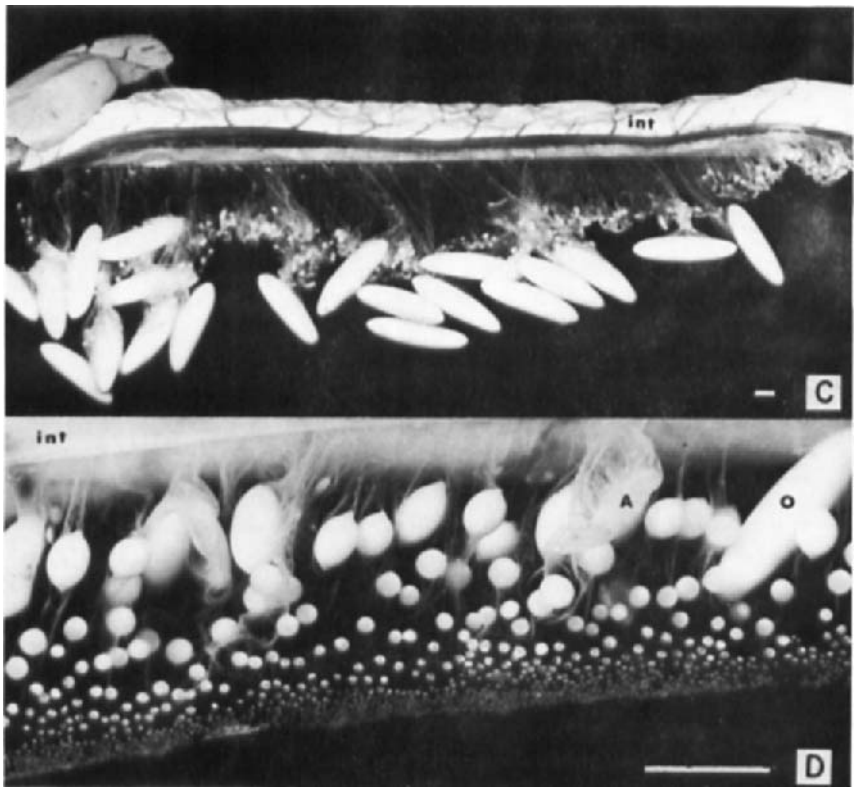


Fig. 2. Gonads of *Eptatretus stouti*, showing the intestine above in each case, with the mesentery of the gonad joined to the dorsal mesentery of the intestine. (A and B) A hermaphrodite, 54 cm long, two magnifications. In each figure the white scale bar at the lower right is equivalent to 5 mm actual size. (C) Adult female (58 cm long) with almost mature eggs, 22–24 mm in length. (D) Small female (43 cm long) apparently forming a first clutch of eggs, the largest egg 9 mm in length. A, atretic follicle; int, intestine; M, mesovarium; O, vitellogenic egg; T, testis. (A, B) In this hermaphrodite the testis, T, occupies a normal posterior position and ovary forms the remainder of the gonad. At higher magnification the sperm follicles are visible in the testis. Between testis and ovary there is a clear interruption of germinal elements although the gonadal mesentery, M, connects the two. The number of large maturing eggs (14–18 mm in length) is unusually small, only nine, divided into two groups. The number of atretic egg follicles is unusually high. (C) In this mature female there are 20 vitellogenic eggs completing their development. Each large egg hangs by a tubular outpocketing of the mesovarium, attached at the more dorsal point from which the egg first developed. There are many

In *Myxine*, a fairly high incidence of gonadal bisexuality or hermaphroditism or sterility (13% of 4000 specimens) was reported by Schreiner (1955; see also Walvig, 1963). In *Eptatretus* incidence of such abnormalities is certainly below 1% (Walvig, 1963).

The frequent observation of high ratios of female-to-male adult hagfish has prompted various interpretations over the years, but few facts support explanatory speculations. An early interpretation was that hagfish (in particular *Myxine*) are protandrous hermaphrodites (Nansen, 1888). Later reex-



small eggs developing at the free ventral edge of the mesovarium, none more than about 4 mm in length. There are no intermediate-sized eggs between 4 mm and the large maturing eggs. In the mesovarium more dorsal than the immature eggs are shadowy atretic follicular structures. (D) In this younger female the largest eggs are 9 mm long. One is shown at the right, marked O. The smallest oocytes are at the free ventral edge of the membranous ovary and larger ones progress dorsally. A, an atretic egg follicle about 4 mm long already shows the outpocketing of mesovarium, as do also several slightly smaller eggs to the left. Between these and the 9-mm eggs, there are no intermediate-sized eggs.

amination of this problem denies this possibility (Schreiner, 1955; Gorbman and Dickhoff, 1978), because males are generally as large as females, and sometimes almost as numerous. Another more likely explanation is that males, especially when sexually ripe, experience behavioral changes, which include cessation of feeding. As mentioned earlier, capture of hagfish almost always depends on the use of baited hooks or traps. However, it should be noted that Gorbman and Dickhoff (1978) have found a high female-to-male ratio even among sexually immature *Eptatretus stouti* in a particular population in Barkley Sound, Vancouver Island, Canada. If nonfeeding is indeed a characteristic of sexually ripe male hagfish it is of interest to know whether this behavioor is under endocrine, possibly androgenic steroid, control.

V. GAMETOGENESIS

A. Lampreys

As in the Pacific salmonid species, which breed once and die, gametogenesis in lampreys is a single event. This being the case, all sex cells mature simultaneously and synchronously. There is a complete commitment of the entire complement of gametogenic cells, no stem ovogonial or spermatogonial cells being withheld for another cycle. Therefore, the process of gametogenesis follows directly the processes of gonadal differentiation described previously.

In nonparasitic brook lampreys, proliferation of gametogenic cells follows shortly after metamorphosis. During this time, as during metamorphosis, feeding behavior is suppressed. In parasitic species gonads remain infantile during the 1–2 year macrophagous period in the sea. In these, too, a nonfeeding period follows return to fresh water and completion of gonadal growth and gametogenesis (Fig. 1). In both stream lampreys and in the anadromous parasitic species, final gonadal growth is rapid and extensive enough to fill the entire abdominal cavity. The ovary experiences maximal vitellogenesis. In *Petromyzon marinus*, for example, the fully developed ovary comprises as much as 20–25% of the total body weight (York and McMillan, 1980). Spawning involves complex behavioral features preceded by building of "nests," shallow depressions created by active removal of stones and some loose gravel. These processes have been amply summarized by Hardisty (1971; see also Lanzing, 1959).

Fecundity, expressed in terms of total number of eggs produced per female lamprey tends to vary with body size (Hardisty, 1971). The largest anadromous species release about 170,000 eggs (*Petromyzon marinus*);

smaller anadromous freshwater species release from 15,000 to 100,000. The landlocked species of *P. marinus*, which matures at a smaller body mass than the anadromous form yields about 60,000 eggs per female. Nonparasitic brook lampreys, which have the smallest relative body mass, produce on the average only from 500 to 2500 eggs. Hardisty (1979) equates the adaptive advantage of the macrophagous parasitic life style with rapid growth, greater body mass, and greater fecundity. The adaptive advantage of permanent stream residence and a protected life in a mud burrow is the elimination of the hazards of catadromous and anadromous migrations and exposure to additional predators.

B. Hagfishes

Although considerable information now has been gathered concerning gametogenesis in the hagfishes, some important questions remain. For example, it is commonly assumed that hagfishes are repetitively cyclic breeders without seasonal limits to their cycles because their normal deep sea habitat excludes seasonal clues. One species, *Eptatretus burgeri*, has been established definitely as a seasonal breeder because it enters shallow waters (10 m) during the colder months but returns to deep water (50 m), presumably to breed in the summer (Kobayashi *et al.*, 1972; Fernholm, 1974; Patzner, 1974, 1978). However, in no myxinoid species, even in *E. burgeri*, has it been proven unequivocally that there is more than one cycle per lifetime. Probably the best evidence of repetitiveness of gametogenic cycles is that, unlike the lampreys, the hagfishes do not commit all stem cells to gametogenesis at one time. In the ovary (Fig. 2C,D) and testis a variety of stages of gamete formation may be found simultaneously, in both the non-seasonally breeding species and as in *E. burgeri*.

Ovogenesis in adult hagfish has been thoroughly described (Schreiner, 1955; Walvig, 1963; Gorbman and Dickhoff, 1978; Patzner, 1978) and is discussed here only to introduce the later section of this chapter in which regulation of the morphogenic phenomena is discussed. The ovary is basically a thin membranous structure attached for most of its length to one side of the dorsal mensentery of the gut. Its free ventral end contains the germinal area from which the supply of primary oocytes continues to proliferate. As oocytes continue to form they appear to move dorsally (Fig. 2). However, no mechanism by means of which such movement could be realized ever has been proposed, and it is just as rational to believe that the ovarian germinal free edge is moving ventrad by continued growth. There is no question but that the ovary becomes deeper dorsoventrally as female hagfish grow in length, therefore, the latter interpretation has merit.

As oocytes grow to a length of 2–3 mm, they are rounded and surrounded by a thin follicle. At 3 mm they begin to grow ovoid, and by 4–5 mm they have the long oval shape that is more or less characteristic of the fully grown eggs. Because growth within the thin confines of the membranous ovary is not possible, the section of the organ containing the growing follicle begins to form a lateral saclike outpocketing from the membranous ovary (Fig. 2). Eventually, as the follicle grows, this sac deepens and elongates to form a tubular stalk which hangs ventrally from its point of attachment to the ovary. This stalk carries blood vessels to the egg at its ventral extremity.

It is significant that atresia is an important feature among ovarian phenomena in adult hagfish. It is clear even from a superficial study of Fig. 2 that there are fewer and fewer representatives of each progressive stage of ovarian follicular growth. This can be explained by either of two alternatives: (1) atresia occurs at all stages of oocyte growth, or (2) there is "selection" of increasingly fewer follicles for further growth as a cycle continues, the rest remaining static and accumulating. This point has not been settled by any of the studies of myxinoid ovaries so far. On the one hand, careful examination of histological sections has shown that atresia is at least in part responsible for the inverse relationship between small (less than 5 mm) follicular size and number. On the other hand, Patzner (1978) favors the interpretation that *Myxine* oocytes stop growing and accumulate at a size of about 2 mm, and he has named these "ova expectantes." In the study of *E. stouti* ovaries Gorbman and Dickhoff (1978) found no evidence of accumulation of oocytes at the 1–2 mm stage. However, they found evenly graded continuity of developing oocytes up to a length of about 4.5 mm. There were no oocytes between 4.5 mm size and ovulation size. Therefore, it may be concluded that at 4.5 mm a selection of about 20–30 follicles occurs forming a "clutch." The ovarian membrane of animals with the largest eggs contains a large number of yellowish or brownish atretic structures. Therefore, it was concluded by Gorbman and Dickhoff (1978) that during growth of the larger selected follicles, others continue to reach the 4.5 mm threshold length, but these become atretic. It should be noted that the mass of eggs less than 4.5 mm is relatively small. However, beyond this point the metabolic demand for vitellogenesis and for deposition in eggs apparently becomes great enough to require considerable acceleration of the process of vitelloprotein synthesis. This is discussed further in Section VI.

A function performed by the myxinoid ovarian follicle that appears to be unique is secretion of a shell after vitellogenic growth has ceased. The shell is not simply a uniform protective covering over the egg, but must also be molded complexly in a morphologic sense. It must be formed with a pore, the micropyle, for sperm entry, and at either end of the egg the shell bears

an array of hooks. In order to secrete the hooks, complex formations of the follicle wall at the poles of the egg are required, but have not yet been well described (see Lyngnes, 1936).

It should be noted that the small number of large yolky eggs of hagfish represent a completely different reproductive strategy from the comparatively enormous number of small eggs produced by lampreys.

The basic structural organization of the testes of hagfish resembles that of lampreys. That is, it is composed of follicles or cysts of developing spermatozoa. The cytology of hagfish spermatogenesis has been adequately described (Schreiner and Schreiner, 1905; Schreiner, 1955; Walvig, 1963; Jespersen, 1975; Alvestad-Graebner and Adam, 1977). Of significance to this discussion is the fact that at any one time spermatocysts in various stages of spermatogenesis can be contemporary, even in the seasonal species. *E. burgeri* (Patzner, 1974). The rarity of finding sexually ripe male *Myxine* and *Eptatretus* with fully developed spermatozoa in the testes, has been mentioned earlier in this chapter.

VI. ENDOCRINE FUNCTIONS OF THE GONADS

Although biological actions for the sex (gonadal) steroids have been found in both lampreys and hagfish (see further discussion), it is remarkable that the demonstrable levels of these steroids in blood plasma, as well as in gonadal tissues, are very low in cyclostomes compared to other vertebrates. Accordingly, efforts to demonstrate that the gonads are indeed capable of producing sex steroids have been conducted along a variety of lines: macro- and micromorphology, identification of steroidogenic enzymes, biochemical analysis of metabolites of sex hormone precursors, and use of sensitive quantitative techniques such as radioimmunoassay and double-isotope derivative assay (DIDA).

A. Morphology

Macroscopically, maturing lamprey gonads provide the clearest evidence of rapid activity because, in both parasitic and nonparasitic forms, there is an intense growth period with a few weeks just prior to spawning (Lanzing, 1959; Larsen, 1974). At this time there is correlated vitellogenesis and appearance of secondary sexual characters (modified anal fin in the female and urogenital papilla and modification of the dorsal fin in males). Before appearance of these external sex hormone-dependent features it is impossible to visually distinguish the sexes.

In hagfish, among which sex is never distinguishable by means of exter-

nal features, the only grossly useful indicator of sexual state is the outline of large ovarian eggs, which cause the abdominal wall to appear characteristically "lumpy."

Because breeding male hagfish have rarely or never been seen, there is little to say concerning macroscopic changes in the testis associated with sexual activity. In females growth of large yolky eggs provides ample evidence of vitellogenesis, which is estrogen activated.

In a histological study, Larsen (1973) observed the appearance of "Leydig cells" in the testes of *Lampetra fluviatilis* in January and February, shortly before secondary sex structures developed. Busson-Mabillot (1967a,b) has noted by electron microscopy the appearance of cells in ovarian follicles with the organellar structure of steroid hormone secretion: well developed smooth endoplasmic reticulum (ER) and mitochondria with tubular cristae.

The testis of hagfish (*E. stouti*) contains no apparently steroidogenic cells until a length of about 40 cm is attained. At that time cells with the ultrastructural features of Leydig cells (e.g., smooth ER, tubular cristae) appear among the spermatogenic follicles (Tsuneki and Gorbman, 1977a). In a study of adult ovaries of *E. stouti*, Tsuneki and Gorbman (1977b) found no cells with the ultrastructure usually considered characteristic of steroid hormone secretion. They examined eggs of all sizes along with their follicular structures, atretic follicles of different age, newly ovulated follicles, and "interstitial" tissue.

B. Sex Hormone Production

As long ago as 1963, by extraction techniques that cannot be considered as definitive as more current methods, Botticelli *et al.* reported the presence of estradiol and progesterone in *Petromyzon* ovaries. Hardisty (1971) cites Symonds (1968) as having extracted *L. fluviatilis* ovaries at intervals through the reproductive cycle. He found "oestrogen" only in March, shortly before spawning. Since then, Weisbart and Youson (1977) did not find any labeled testosterone in the blood of *Petromyzon* injected with radiolabeled progesterone followed by chromatography and recrystallization to isopolarity. However, in the same species, using DIDA, Weisbart *et al.* (1980) found low concentrations, 0.1–0.4 μg/dl of testosterone in pooled blood. Given the technological difficulties, it is not clear whether these differences in measurement relate to technique, differences in state of maturation or to the use of larger pooled blood samples. It is apparent that a careful study of plasma levels of sex steroids of individual lampreys in different phases of the reproductive cycle is needed. Furthermore, better identification of the tissue source of sex steroids is also required.

Callard *et al.* (1980) in a recent study, incubated *in vitro* homogenates of various tissues (e.g., ovary, testis, kidney, liver, muscle, or brain) of sexually ripe *P. marinus* with radiolabeled [^{3}H]androstenedione. Labeled estrone was found formed by ovary, testis and kidney but not by liver, muscle, or brain. Testis also yielded estradiol. All tissues but muscle of those tested had 5α-reductase activity. Gonadal tissue and kidney, but not brain, had aromatase activity (i.e., produced estrogens).

In hagfish the available information is more voluminous but indicative of sex steroid production levels as low as those in *Petromyzon*. Matty *et al.* (1976), using radioimmunoassay (RIA) of blood sera of a series of individual *E. stouti*, found estradiol, testosterone, and progesterone measurable near the lower limits of sensitivity of the technique, picograms per milliliter. In a considerable part of the population studied, estradiol and testosterone, in both sexes, were not measurable at all and progesterone was the most abundant steroid. An interesting inverse relationship was found by Matty *et al.* between body mass of female hagfish and plasma testosterone concentration. That is, as females matured sexually, they produced progressively less testosterone. In an attempt to determine if the gonad itself is capable of producing measurable quantities of sex steroids, Gorbman and Dickhoff (1978) extracted *E. stouti* ovarian follicles containing eggs of various sizes (previtellogenic, early vitellogenic, almost completely mature) and measured their content of dihydrotestosterone, testosterone, and progesterone (Table I).

In absolute terms these values are very low. Although they show a gradual increase in the total amount of each steroid per egg, the concentrations actually decreased, perhaps because of the fact that most of the mass of the follicle is yolk. Reduction in progesterone and testosterone concentrations could reflect estrogen synthesis from these precursors, but unfortunately, estrogens were not measured in this study.

In another type of study of steroidogenesis and steroid metabolism in

Table I[a]

Testosterone, Dihydrotestosterone, and Progesterone in Eggs of Hagfish

Egg size (longest mm)	Number	Testosterone		Dihydrotestosterone		Progesterone	
		pg/egg	pg/gm	pg/egg	pg/gm	pg/egg	pg/gm
2–3	6	1 ± 4.0	245 ± 50	2 ± 0.3	420 ± 70	9 ± 2	1920 ± 365
9–10	4	5 ± 2.0	130 ± 48	3 ± 2.0	70 ± 60	80 ± 22	2000 ± 550
24–26	4	16 ± 4.0	12 ± 3	14 ± 5.0	10 ± 3	111 ± 25	80 ± 18

[a]From Gorbman and Dickhoff (1978).

hagfish, gonadal or other tissues were incubated *in vitro* with radioactive presursors to determine, from the radioactive products obtained, which enzymes are present. In this way Hirose *et al.* (1975) incubated homogenized mature ovary of *E. burgeri* with [^{14}C]pregnenolone. Among the labeled products were progesterone, 17α-hydroxyprogesterone, androstenedione, and some other 5α-reduced products. Labeled androstenedione was a product of both labeled-progesterone and labeled-testosterone incubation. Therefore, among other enzymes, Hirose *et al.* found evidence of 3β-hydroxysteroid dehydrogenase (3β-HSD), an enzyme that Fernholm (1974) could not demonstrate in *Myxine* ovary by cytochemical methods. In a similar experiment in which precursor [^{3}H]androstenedione was incubated with *Myxine* ovary, Lance and Callard (1978) found labeled estradiol and estrone among the radioactive products, but yields were very low. However, Callard *et al.* (1978), in a single experiment, found ovary incubated with labeled androstenedione incapable of producing detectable labeled estrone or estradiol. Kime and Hews (1980) incubated *Myxine* ovarian tissue with ^{3}H-labeled progesterone or testosterone. The only labeled product from progesterone was 5α-pregnanedione unlike Hirose's results in *E. burgeri*. Testosterone was converted (32%) to dihydrotestosterone and other related products, indicating the presence of 5α-reductase. Incubation of *Myxine* testis with ^{3}H-labeled progesterone yielded a small proportion of labeled testosterone (Kime *et al.*, 1980), and other substances which appear to represent further metabolism of this hormone. These authors suggest that the reason Weisbart and Youson (1977) were unable to find testosterone in the adult lamprey testis is that it may be converted to "unusual hydroxylated derivatives." This suggestion is supported by their discovery of such metabolites in *Myxine* testis preparations after incubation with labeled testosterone. However, among labeled derivatives of testosterone were also androstenedione (23%), 6β-hydroxytestosterone and some 5α-reduced substances.

Liver preparations of hagfish also have been examined for their ability to metabolize steroids *in vitro*. Inano *et al.* (1976) found 5α-reductase activity in *E. burgeri* liver (combined microsome and cytosol fractions), labeled testosterone being converted to dihydrotestosterone, and a variety of other products. Hansson *et al.* (1979) incubated labeled androstenedione with *Myxine* liver microsomes and found labeled testosterone, in addition to other reduced steroids.

In sum, it appears that the gonads of both lampreys and hagfish have 3β-HSD enzymatic activity and that ovaries and testes both may produce surprisingly low concentrations of sex steroids (although there is some doubt whether hagfish ovaries are capable of aromatization and production of estrogen). Plasma concentrations of androgen and estrogen, where demonstrable, are low. In part, some of the inconsistencies in results may be attributa-

ble to the fact that age and sexual condition often were not assessed, although they might have an extremely important bearing on interpretation of measurements. Studies of metabolism of steroids by nongonadal tissues are still too few to permit generalizations. However, it is of interest that hagfish liver has been found in metabolic tracer studies to produce testosterone from androstenedione (Hansson *et al.*, 1979) and to reduce testosterone to dihydrotestosterone (Inano *et al.*, 1976; Callard *et al.*, 1980). At least a partial explanation of the low plasma levels of sex steroid in cyclostomes may be based on the fact that in these animals there is little or no demonstrable sex steroid-binding protein (Wingfield, 1980).

VII. BIOLOGICAL ACTIONS OF SEX HORMONES IN CYCLOSTOMES

Actions of the sex steroids have been studied in cyclostomes principally by use of two classical procedures: gonadectomy and hormone administration. Neither the lampreys nor hagfishes have sex ducts to lead gametes from the gonads to the cloaca, therefore these, and their associated glandular structures are not available to assess sex hormone action. In fact, in hagfishes, aside from the gonads themselves, there appears to be little or no known sexual dimorphism. In lampreys there are several so-called "secondary sexual characters," which are the modified anal fin and cloacal swelling of females, and the urogenital papilla and modified dorsal fin structures of males. These appear only at the time of maximal development of the gonads. In addition, there are several other phenomena, common to both sexes, that are coincident with the prespawning and spawning period, and therefore suspected of being targets of sex hormone action. These latter include atrophy of the gut, a related phenomenon, "green liver," attributable to bile stasis consequent to atrophy of bile ducts and gall bladder, hepatic vitellogenesis and loss of ability for osmoregulation in sea water. Hepatic vitellogenesis has been shown to be a sex steroid (estrogenic) hormone-responsive phenomenon in hagfish of both sexes (Yu *et al.*, 1981).

In earlier experiments, Hardisty and Taylor (1965) found that immersion of sexually undifferentiated ammocoete larvae for 6 months in estradiol or testosterone solutions had minimal action on gonad differentiation. Therefore, sex steroids apparently have no important role in this phenomenon. Evennett and Dodd (1963) established that gonadectomy (or hypophysectomy) of *Lampetra fluviatilis* before secondary sex features appeared prevented their development. Intraperitoneal insertion of a 25-mg pellet of testosterone into hypophysectomized males in October permitted development of secondary sexual characters in the following May. Hypophysec-

tomized females so treated developed male sex secondaries (urogenital papilla). These experiments not only demonstrated the sex steroid dependence of the lamprey secondary sex structures, but demonstrated also that they are sex hormone specific. That is, the hormonal receptors in the respective secondary sex organs probably recognize only the appropriate sex hormone. It is interesting that the female type of sexually differentiated anal fin was found by Larsen (1974) to be responsive to estrogens in both males and females.

Of the nonsexual changes at the time of prespawning sexual ripening, atrophy of the gut has attracted the most investigative attention. The fact that gonadectomy (ovary or testis) prevents the normal involution of the intestine in anadromous prespawning age adult *L. fluviatilis*, or that it may even favor rehypertrophy after partial atrophy, has been confirmed by a number of authors (Larsen, 1969, 1972, 1973, 1974; Dockray and Pickering, 1972; Pickering, 1976a,b). However, there has been some apparent disagreement as to whether implants of 25-mg pellets of sex steroid will themselves induce intestinal atrophy in normal or castrate lampreys. Larsen (1972, 1974), for example, found that testosterone pellets will not prevent the postcastrational hypertrophy of the gut. However, Pickering (1976a,b), found that 25-mg pellets of estradiol or testosterone given early enough (August–October) have such action, but not if they are administered later (January). Pickering concludes that these apparently conflicting results are reconcilable if one postulates a loss of target cell receptors for sex steroids after January, making these cells unresponsive to the steroids after that time.

Larsen (1969) has reported that gonadectomy of returned anadromous *L. fluviatilis* in January increased survival time and either slowed intestinal atrophy or caused hypertrophy. However, it is interesting that "green liver" caused by biliary degeneration proceeded in these experiments as in intact control lampreys. The morphological changes involved in atrophy of bile ducts and gall bladder have been described by Youson and Sidon (1978) in *Petromyzon marinus*.

Less well studied has been the loss of ability to osmoregulate in seawater after *L. fluviatilis* have returned to fresh water. Pickering and Dockray (1972) found that gonadectomy prolonged the ability to osmoregulate in 50% sea water, and the presence of the maturing gonad is in some way influential in these changes in ion regulation. However, the mechanisms involved remain to be further characterized.

As mentioned previously, efforts to describe the biological actions of sex steroids in hagfish have been limited because the only experimentally established action is that of estrogens on hepatic vitellogenesis (Yu *et al.*, 1980, 1981). Pickering (1976a) has shown in the lamprey, *L. fluviatilis*, that estrogens have the same hepatic action. A shared property between myxinoids

and petromyzonids like this appears to show that estrogenic control over vitelloprotein synthesis in the liver is a phylogenetically ancient function in vertebrates, because divergence of the two groups is itself ancient. Turner *et al.* (1982) found a single class of high-affinity nuclear receptor for estrogens in liver cells (*Eptatretus stouti*) taken from adult females with large eggs. Livers from females with small previtellogenic eggs had only about 20% of the specific estrogen binding sites as did vitellogenic females. Sexually immature hagfish livers had no detectable specific estrogen binding ability. Estrone, estriol, and diethyl stilbestrol bound to the receptor as well as estradiol; progesterone and testosterone bound poorly. The affinity of the hepatic receptor for estrone correlates with the finding by Yu *et al.* (1981) that estrone stimulates vitellogenesis in *E. stouti* as well as estradiol. It is worth noting that the high affinity of liver receptors would make it possible for estrogen to exert its effect on vitellogenesis in hagfish despite the extremely low plasma levels of estradiol (Matty *et al.*, 1976).

VIII. PITUITARY GONADOTROPIC ACTIVITY

Experimental efforts to demonstrate that the pituitary gland is involved in reproductive function generally depend on hypophysectomy and/or administration of pituitary hormones. For hagfish the only extensive study of this type is that of Matty *et al.* (1976) who hypophysectomized about 150 adult hagfish. They found no evidence that hypophysectomy discernably affects gonadal function in either male or female *E. stouti*. In fact, after as long 8 months after complete pituitary removal, some females were still laying eggs. Therefore, completion of vitellogenesis, ovulation and laying of apparently normal eggs is not dependent on a hypophysial gonadotropin in these hagfish.

More extensive research along these lines has been done with lampreys, largely by Dodd (1972) and his co-workers (Dodd *et al.*, 1960) and by L. O. Larsen.

A. Hypophysectomy

Evennett and Dodd (1963) hypophysectomized 80 lampreys (*L. fluviatilis*) of both sexes between October (shortly after their re-entry into fresh water and before initiation of gametogenic meiosis) and March (shortly before normal spawning). No hypophysectomized animals developed steroid hormone-dependent secondary sex features, showing that removal of a presumed gonadotropin inhibited sex steroid synthesis. In the morphological sense, Larsen and Rothwell (1972) note that hypophysectomy does not ap-

preciably alter the appearance of testicular interstitial cells, or suppress the histochemical 3β-HSD reaction of these cells.

In the testis of lampreys hypophysectomized in October, spermatogenesis proceeded through spermiogenesis, although at a slightly decelerated pace. However, histological study of spermatozoa "suggested that they were incompletely developed." Larsen (1973) made essentially similar observations of spermatogenesis in hypophysectomized *L. fluviatilis* and stated, in addition, that there was no release of spermatozoa from the testis. Therefore, it would appear that the relationship between spermatogenesis and the pars distalis of the hypophysis of lampreys is different from that of all other vertebrates. In other vertebrates hypophysectomy almost completely arrests spermatogenesis. In Evennett and Dodd's later hypophysectomies (January and February, when testicular germ cells are in later prophase), there was little, if any, effect on spermatogenesis; therefore, these authors concluded "that spermatogenesis and spermiogenesis in lampreys, unlike other vertrebrates, are autonomous processes. . . ."

According to Evennett and Dodd (1963), the ovaries of hypophysectomized *L. fluviatilis* responded in a more complex manner. Oocytes developed in a normal manner until January, but, at the stage in which maximal preovulatory growth should occur, presumably by yolk deposition, there was no further growth in hypophysectomized *L. fluviatilis*. However, although egg growth plateaued, there was no ovarian atresia as occurs in ovaries of other hypophysectomized vertebrates. In a similar experiment Larsen (1973) hypophysectomized 17 female *L. fluviatilis* in November. Some were completely adenohypophysectomized; in some only the rostral pars distalis was removed, while in others only the proximal pars distalis was taken. Interpretation of this experiment is made complex not only by the protocol, but also by the variability of results. In general, removal of the proximal pars distalis had a greater inhibitory effect on ovarian growth than removal of the rostral pars distalis. However, because there were exceptions, it could not be concluded that "gonadotropic activity" is limited to the proximal zone. Undoubtedly, the precision of selectively removing one lobe of the pars distalis alone cannot be absolute. Nevertheless, it was concluded by Larsen (1973) that even in those instances in which there was complete removal of the pars distalis, growth of the ovary continued, although at a slower pace, unlike the results of Evennett and Dodd (1963). Ovulation failed to occur in Larsen's *L. fluviatilis* hypophysectomized in November.

Despite the slight differences between the reported data of Evennett and Dodd (1963) and Larsen (1973), one can conclude that much of the process of ovogenesis, as well as spermatogenesis, is independent of a presumptive pituitary gonadotropin. Sex hormone synthesis, on the other hand, responds much more consistently to hypophysectomy, to judge by the failure of sec-

ondary sex structures to develop. However, Larsen's experiments produced some exceptions in which secondary sex structures differentiated at least partially in hypophysectomized animals. It must be emphasized that no actual measurements of plasma levels of sex steroids have been made in hypophysectomized lampreys. This information is needed for better interpretation of existing data in this field. For example, if it is true that vitellogenesis is arrested or inhibited by hypophysectomy of *L. fluviatilis* females, this could be a consequence of low plasma estrogen values.

B. Administration of Exogenous Pituitary Preparations

In an early experiment involving injection of hypophysial, or other gonadotropic preparations, Evennett and Dodd (1963) gave the following to hypophysectomized or intact *L. fluviatilis*, once every 2 weeks for more than 3 months between December and March: three whole lamprey pituitaries in saline solution, 200 IU of pregnant mare's serum gonadotropin (PMSG), and/or 300 IU of human chorionic gonadotropin (HCG). Apparently, all the preparations had gonadotropic activity; however, lamprey pituitary, in the dose given, was not as active as PMSG or HCG. The hypophysectomized animals developed secondary sex characters, fully differentiated spermatozoa, and the females ovulated (presumably this was determined by stripping or direct inspection after decapitation). Thus, these preparations corrected the several defects in reproductive development caused by hypophysectomy. In the intact lampreys, there was gonadal stimulation, and secondary sexual features developed in February, ahead of the normal schedule. Evennett and Dodd, noting that secondary sex structure appearance (indicative of stimulation of sex steroid secretion) did not occur until February, although gonadotropic hormone had been given since November, commented: "This suggests that the tissues concerned are insensitive to hormonal stimulation until shortly before the time at which the secondary sexual characters normally develop." Sensitivity to hormones, in the current context, is equated with possession of receptors for a given hormone. Therefore, Evennett and Dodd, suggest that receptors develop on some type of normal developmental seasonal schedule and that either gonadotropic receptors or sex steroid receptors appear relatively late in development. It should be noted that for the intestine, an organ that degenerates as development proceeds, Pickering (1976a,b) has speculated that an opposite developmental program exists. That is, sex steroid receptor may be available early, but it decreases or is lost at later times. Here, again, it is clear that measurements of plasma levels of sex steroids correlated (or not) with the various observed phenomena would greatly aid in interpretation of the mechanisms regulating these phenomena.

IX. REGULATION OF REPRODUCTION THROUGH THE BRAIN

In the higher vertebrates the regulation of pituitary gonadotropic function through centers in the hypothalamus is clearly a function with high adaptive value in the temporal as well as evolutionary sense. The hypothalamic centers receive innervation relaying virtually any kind of sensory information to them. By this means, the brain becomes the transducer for relating sensory-detected environmental changes with subsequent endocrine phenomena. Of special interest here is that sensory signals from olfactory, optic, auditory, tactile, taste, temperature, and even other sensory modes can, and do, influence reproduction in many particular species. To what extent is such central nervous system-mediated control exerted on the pituitary and gonads in cyclostomes? Answers to this question can be inferred, at least, from a variety of data: (1) correlations between season or particular environmental changes and reproductive changes; (2) demonstration of anatomical links (nervous, vascular) between brain and pituitary (adenohypophysis) by means of which an influence from the brain can reach the pituitary; (3) demonstration of the presence or absence of gonadotropin-releasing peptides in the brain; (4) demonstration of responsiveness of cyclostome pituitary gonadotropic secretion to gonadotropin-releasing peptides or hormones; (5) a variety of other experimental approaches such as brain lesioning, stereotoxically localized electrical stimulation or recording that have not yet been applied in studies of cyclostomes.

Because no gonadotropic function can be detected in hagfish, as discussed previously, there can hardly be any useful study of its control. Nevertheless, it is clear from histological studies and from studies of the vascular supply of hagfish pituitaries, that no nervous connection or portal blood vessel supply exists in *Eptatretus* or *Petromyzon* whereby control can be exerted by the brain over the adenohypophysis as it is in higher vertebrates (Gorbman, 1965; Crim *et al.*, 1978). Furthermore, Crim *et al.* (1979a) have shown that there is no immunocytochemical evidence for the presence of gonadotropin releasing hormone (LHRH) in the brain of *Eptatretus stouti.* Nozaki and Kobayashi (1979) have shown that there is the same lack of immunoreactive LHRH in the brain in *Eptatretus burgeri.* The case of *E. burgeri* is puzzling because it has been found to be a seasonal breeder with an apparent annual migration between deeper and shallower waters. Unless this migration and annual breeding cycle are direct responses to temperature or photoperiod, without intervention of pituitary gonadotropic function, there is at present no way to explain the breeding cyclicity in *E. burgeri.*

In the lampreys, it is clear that migrations, gonadal maturation, gamete release, and spawning occur in season, at particular phases of a life cycle.

Thus, there is reason to believe that seasonal environmental physical cues may be linked to endocrine-mediated reproductive function by appropriate sensory-evoked pathways in the brain. Furthermore, unlike the hagfishes, some evidence for a gonadotropic function in the lamprey adenohypophysis exists, although its role appears to be a more modest one than in higher vertebrates. Beyond this, Crim *et al.* (1979a,b) has shown in two lamprey species (*L. tridentata, L. richardsoni*) that there is immunoreactive LHRH in the hypothalamus (largely, the preoptic nuclear area), and that there is more of it in sexually mature animals contrasted with immature ones. Therefore, the case may be made that a primitive type of hypothalamic–hypophysial–gonad axis exists in the lampreys. In opposition to this is the fact that neither nervous nor vascular structures can be found connecting the brain to the pars distalis of lampreys (Gorbman, 1965; Tsuneki and Gorbman, 1975a,b). Accordingly, if LHRH moves from the brain to the pars distalis, it would have to do this by diffusion across the thin connective tissue barrier between them.

Larsen (1974) has found that there is no "feedback" between the gonadal hormones and the presumed hypothalamic–hypophysial axis. That is, large doses of estradiol or testosterone do not have an apparent inhibitory action on the pituitary or gonad as they do in higher vertebrates where negative feedback is part of the interlocking mechanism. Tsuneki (1976) found the same to be true in hagfish (*E. burgeri*). This still does not invalidate the hypothesis that there is control of gonadotropin secretion by the brain, but a more serious negative point stems from experiments by Larsen (1973; cited in Larsen, 1969, 1978) in which lamprey (*L. fluviatilis*) recently returned to fresh water, continued normal sexual development although they had been kept for "many months" in darkness at constant temperature. She concluded from this that "external clues, as increase in temperature and increase in day length, seem of little importance." If these two basic and common physical environmental clues can be ignored by the seasonally limited reproductive process of the lamprey, it may be difficult to identify the ones that do tie them to season.

X. GENERALIZATIONS, PROJECTIONS, SPECULATIONS

As summarized here, there are some key differences between reproduction in cyclostomes, and its regulation, from other vertebrate groups. First, as regards gonadal differentiation, neither hagfish nor lampreys follow the general vertebrate pattern of corticomedullary organization and interaction in the primitive developing gonad. Furthermore, the myxinoid and petromyzonid patterns bear little resemblance to each other. In developing

lamprey, if one accepts Hardisty's interpretation of morphological events, both sexes go through a proterogynous phase of ovogonial and oocyte multiplication, followed by partial involution in the female and complete ovocyte suppression in the male. The processes of ovogenesis and spermatogenesis themselves appear to be quite orthodox in both cyclostome groups, with the difference between the two being complete commitment to a single cycle in lampreys in contrast to apparently repeated cycles in hagfishes. The single cycle of lampreys, followed by death, is tied to season, although in most hagfishes it is not.

Knowledge about the endocrine management of the reproductive processes in cyclostomes at present raises as many questions as it has answered. For example, in both groups, plasma levels of sex steroids are remarkably low, differing by two or three orders of magnitude from most other vertebrates. Yet, in both groups, sex hormone-dependent phenomena that are integral in the reproductive process have been identified. The low plasma levels of sex steroids are partially explained by the absence of specific high-affinity sex steroid-binding proteins in blood. In the few measurements that have been made of gonadal sex steroid production, this too seems to be extremely slight or slow. Whether or not there are surges or cycles of sex hormone production in individual cyclostomes that may be correlated with specific phases of reproduction is not known because appropriate measurements have not been taken.

Control over gametogenesis in both lampreys and hagfishes by the pituitary gland (pars distalis) in the light of present information appears limited or doubtful. In any case, it is certainly different from the close involvement of pituitary gonadotropins with gametogenesis in all other vertebrates. Better information exists in lampreys that ties function of pars distalis to sex steroid synthesis, presumably by the gonad. The failures of later stages of gamete formation (i.e., spermiation in males, completion of vitellogenesis and ovulation in females) in hypophysectomized lampreys might be actually caused by a lack of sex steroids or other steroids rather than by absence of a gonadotropin. A relationship of the pars distalis of hagfish to any phase of gonadal activity is not even suggested by any currently available information.

How the lampreys relate their reproductive functions to season remains a mystery. No obvious anatomical means exists for tying afferent sensory information concerning external conditions to endocrine and reproductive events. Furthermore, some experiments by Larsen (1973) indicate that sexual ripening of lampreys can occur at constant low temperature in constant darkness. She also has shown that the pituitary of sexually ripening lampreys functions in the gonadotropic sense even when transplanted to a site (pharyngial muscles) far from the brain and the possible diffusion of factors from

the brain (Larsen, 1969, 1973). Thus, the manner of recognition of season by lampreys is inexplicable at present. Furthermore, if the pars distalis can secrete gonadotropins at an anatomical locus that is completely isolated from central nervous system integrators of sensory information, then how is environmental seasonal change communicated to it, or to the gonad?

One possibility is that gonadotropin-releasing hormone, which is known to occur in the lamprey hypothalamus, reaches the pituitary via the general circulation rather than via diffusion from the neighboring neurohypophysis of lampreys. If this is so, it is pertinent to ask in this representative of the most primitive offshoot line of vertebrates: why has the pars distalis evolved at all in the juxtaneural locus it occupies in lampreys?

Obviously, the unanswered questions are numerous and basic for the understanding of the evolution of the hypothalamic–hypophysial–gonadal axis. More research will supply the means for better limiting or defining some of these fascinating questions. One large uncertainly is in deciding whether the anatomical and functional properties of the hypothalamopituitary system seen in lampreys and hagfish are truly primitive or whether they represent degeneration from a previously more highly evolved state. There is little factual basis for a direct rational decision between these alternatives, but a possibility has been suggested recently (Gorbman, 1980). The cyclostome pituitary gland differs anatomically from that of all other vertebrates in lacking completely the portal blood vessels that carry the hypothalamic releasing hormones from the brain to the pars distalis. There are three groups of fishes, the holocephalians, the elasmobranchs, and the coelacanths, which evolved from the vertebrate line shortly after the divergence of the cyclostomes, that have a pars distalis that is partly supplied with such a portal blood circulation. If this partial portal vascularization is taken as an intermediate phase in evolution of the complete hypophysial portal system, then the cyclostome pattern can be taken as primitive. In this concept, the original evolutionary adaptive value of bringing the epithelial pituitary into contact with the nervous pituitary and brain was to serve the functions of the pars intermedia in skin pigmentary changes in concert with environmental background light changes. In such a scheme, the involvement of the "stalk," which carried in the pars intermedia and which eventually became the source of gonadotropins and other hormones of the pars distalis, was a later development.

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2

REPRODUCTION IN CARTILAGINOUS FISHES (CHONDRICHTHYES)

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I. INTRODUCTION

Contemporary cartilaginous fishes (Chondrichthyes) are assigned to two subclasses of very unequal size: Elasmobranchii, with 128 genera and about 600 species, and Holocephali with 6 genera and about 28 species (Nelson, 1976). The former comprise two orders, Squaliformes (sharks and dogfishes) with 16 families and Raiiformes (skates and rays) with 12 families (Breder and Rosen, 1966). The affinities and phylogeny of the two subclasses are subjects of contention (Dodd and Dodd, 1984), although the view of Zangerl and Case (1973) that they are sister groups sharing a common ancestor is now widely accepted (C. Patterson, personal communication).

The reproductive biology of both groups is of exceptional interest because of their long phylogenetic history (at least 350,000,000 years), their long separation from each other, and the diverse range of reproductive

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specializations that they have adopted. Unfortunately, partly because cartilaginous fishes are of less economic importance than teleosts and partly because they are often difficult to obtain and demanding as experimental animals, rather little is known about them; this is especially true of the Holocephali. Of the 600 available elasmobranch species, virtually nothing is known of two-thirds, and most of the information available on the rest is selective, descriptive, and fragmentary. This applies particularly to physiological aspects of chondrichthyan reproduction, experimental investigation being virtually restricted to a single species, *Scyliorhinus canicula*, the lesser spotted dogfish. The generalizations made in this chapter are vulnerable and must be assessed in the light of this reservation.

Many aspects of reproduction in elasmobranchs, including gonadogenesis, sex determination, origin of the reproductive ducts, and low ovarian fecundity, are more similar to amphibians and amniotes than to the teleosts. Further, their approach to viviparity, in which, unlike the situation in teleosts, gestation takes place in a modified region of the oviduct, rather than in the ovary, is closely related to that of amphibians and amniotes.

It is widely accepted that viviparity has evolved independently many times in vertebrates, and it is the commonest mode of reproduction in elasmobranchs. It may or may not be placental, but it is usually associated with cyclical breeding. In consequence, many of the cartilaginous fishes have sophisticated life cycles and these are often associated with a migration. A good deal is known about cyclicity in littoral species though it is not always realized that these too migrate and populations are never static. This and other factors discussed in following pages have caused some confusion in the delineation of reproductive cycles, especially in species in which the egg-laying period is lengthy.

Extant cartilaginous fishes are often said to be primitive, and some of their morphological characters may well have retained a primitive condition [witness the recent, albeit tentative, identification of two species of *Scyliorhinus*, from the Cretaceous, that lived approximately 100,000,000 years ago (Cappetta, 1980)], but the physiology of contemporary species is often highly sophisticated. Unfortunately, little is known of reproductive physiology in elasmobranchs and nothing in holocephalans. The little that is known concerning pituitary–gonad relationships suggests that any differences between these fish and so-called "higher" vertebrates may be superficial, although there may well be surprises from further research.

Reviews on various aspects of chondrichthyan reproduction include the following: Dean (1906), Ranzi (1932, 1934), Needham (1942), Budker (1958), Amoroso (1960), Chieffi (1967), Hoar (1969), Dodd (1960a, 1972, 1975), Dodd and Sumpter (1982), and the comprehensive recent review of Wourms (1977) on wide ranging aspects of chondrichthyan reproductive biology.

II. STRUCTURES AND FUNCTIONS ASSOCIATED WITH REPRODUCTION

A. Differentiation of the Gonads and Sex Determination

As in amphibia and amniotes, but unlike teleosts, the gonads of elasmobranchs develop by the migration of primordial germ cells from extra-embryonic endoderm into paired genital ridges consisting of two distinct regions, cortex and medulla, which originate from peritoneal epithelium and interrenal anlage, respectively (see Chieffi, 1967, for a review). In genetic males, the primordial gonia come to rest and develop in the medulla, but in females they develop in the cortex (Chieffi, 1949, *Torpedo ocellata;* Chieffi, 1950, *Scyliorhinus canicula;* Picon, 1962, *Leptocharias smithii*). According to Beard (1900, 1902, *Pristiurus* sp., *Raia batis*) and Woods (1902, *Squalus acanthias*) the numbers of primordial germ cells are unusually high; estimates have varied between 127 and 327, whereas in teleosts they appear to vary between 30 and 70 (Hardisty, 1978). The onset of meiosis is believed to be precocious. The entire crop of germ cells enters the first meiotic prophase and the germ cells become primary oocytes before sexual maturation (Franchi *et al.*, 1962).

Sex determination seems to be more stable in elasmobranchs than in many other vertebrates (Dodd, 1960b); nothing is known of it in Holocephali. Experiments by Thiebold (1964), in which developing gonads, prior to sexual differentiation, were transplanted and attached to epithelium of the extra-embryonic coelom, have shown that sex is determined early because the gonads develop according to their genetic sex regardless of the sex of the host. Furthermore, there is no antagonism between a testis and an ovary developing side by side. Chieffi (1967) has summarized his earlier work in which the effects of a range of steroids, injected into the yolk sac of *S. canicula* eggs, well before the stage of sexual differentiation, were investigated. Testosterone propionate, 17β-estradiol, progesterone, and deoxycorticosterone acetate were injected separately and all, at the doses used, had a feminizing influence on sexual differentiation. This is characteristic of species in which the female is the heterogametic sex.

B. The Ovary and Oogenesis

The developing ovary in elasmobranchs is paired and symmetrical, but in the majority of the few genera that have been examined [*Scyliorhinus, Pristiophorus, Carcharhinus, Galeus, Mustelus, Sphyrna* (Daniel, 1928);

Cetorhinus (Matthews, 1950)], only the right ovary becomes functional. In some cases, the left ovary is the only one to develop [*Urolophus, Dasyatis* (Babel, 1967)], and in yet others both ovaries function [oviparous skates (Wourms, 1977), *Squalus acanthias* (Hisaw and Albert, 1947), *S. brevirostris* (Kudo, 1956); *Scoliodon sorrakowah, S. palasorra* (Mahadevan, 1940)]. Whether or not these examples are representative of the extant genera is impossible to say, but it is clear that the left, or the right, or both ovaries may develop and Thiébold (1964) has shown that if the left gonad of female embryos of *S. canicula* is transplanted into the extra-embryonic coelom before sexual differentiation it does not atrophy. There is similar variability in the development of the oviducts although usually both are present and functional.

The morphology of the mature ovary is extremely variable, but the differences are superficial and attributable largely to the numbers and sizes of the yolky eggs it contains. This in turn is largely a function of the stage of the reproductive cycle at which the ovary is examined, although it is also closely correlated with the mode of reproduction of the species (i.e., whether it is oviparous or viviparous) and the degree to which the developing young are dependent on the female for nutrients.

The ovary is suspended from the dorsal body wall by a broad mesovarium which carries blood vessels and nerves. It consists of stroma, previtellogenic oocytes, and a variable number of yolky oocytes of various sizes. Sometimes the latter, even when ready for ovulation, are small (1.0 mm in *Scoliodon sorrakowah;* Ranzi, 1934), but in *Chlamydoselachus* and *Ginglymostoma* they may reach 100 mm or more (Gudger, 1940). At certain times in the reproductive cycle, "corpora lutea" and corpora atretica also are present in the mature ovary. All of these elements are embedded in a lymphomyeloid hemopoetic organ called the epigonal organ (Matthews, 1950; Fänge and Mattisson, 1981). The ovary, which is gymnovarian (Hoar, 1969), is not continuous with the oviducts. The oocytes are ovulated into the peritoneal cavity and carried to the oviducts by cilia. The number of primed previtellogenic oocytes from which eggs will develop varies with the season and with the species. Hisaw and Albert (1947) state that in *S. acanthias* approximately 50 eggs develop and they are white in color, pea-sized, and weigh less than 1 g; at this stage there are no yolky eggs. Several, possibly all 50, follicles begin to undergo vitellogenesis but only two or three in each ovary persist; the rest are completely removed by atresia before the chosen oocytes, which reach a weight of about 50 g, are ovulated. Thus at the time of ovulation, *S. acanthias* ovaries do not contain corpora atretica, an unusual situation. In *Mustelus canis*, 12–24 eggs reach ovulable size (Te Winkel, 1950), but in *Carcharhinus dussumieri* only a few grow, of which only two are ovulated (Teshima and Mizue, 1972).

1. OVARY OF THE DOGFISH, *SCYLIORHINUS CANICULA*

The ovary of *S. canicula* (Fig. 1A–D), which has been described by Metten (1939), Dodd (1977), and Dodd and Sumpter (1982), is probably typical of oviparous and many viviparous species. During the active egg-laying season (winter and spring) it contains a hierarchy of paired yolky follicles varying in weight from about 3.0 g through smaller yolky follicles to small translucent yolk-free oocytes of a few milligrams (Fig. 1B). Corpora atretica (Fig. 1C) are also usually present, especially in late summer; they probably represent yolky oocytes which for some reason have failed to be ovulated. Postovulatory follicles or "corpora lutea" (Fig. 1A) are also found in egg-laying fish. The vitellogenic follicles have outer walls consisting of peritoneal epithelium, underlain by theca externa, theca interna, basement membrane, granulosa, and a zona radiata which abuts on the vitelline membrane of the oocyte and consists of interlocking villi from oocyte and granulosa. The granulosa in large yolky follicles of *S. canicula* is single layered (Fig. 2A), as in most squaliform species (Wallace, 1904; Samuel, 1943; Chieffi and Botte, 1961), whereas in raiiformes it is said to consist of large yolk-secreting cells and smaller columnar cells (Samuel, 1943; Chieffi, 1961; Botte, 1963).

The only ultrastructural investigation of ovarian follicular structure in an elasmobranch is that of Dodd and Dodd (1980) in *S. canicula* (Fig. 2C). In mature and maturing follicles the theca externa consists of a single layer of apparently active cuboidal or flattened cells which have a good deal of smooth endoplasmic reticulum, often arranged in whorls associated with a lipid droplet. Vesicles and many mitochondria with tubular cristae are also present. The theca interna lies immediately below the externa and consists of 4–6 rows of flattened elongated cells, the walls of which are extended outward into processes. The cells are separated from each other by an interlocking meshwork of collagen fibers and collagenocytes which constitute the main supporting structure of large oocytes. Theca interna cells show signs of secretory activity; they contain parallel arrays of rough endoplasmic reticulum, sometimes with distended cisternae and abundant mitochondria. Intracellular microfibrils, which may be contractile and associated with ovulation, are also visible. The theca interna is separated from the granulosa by a thick granular basement membrane. In all vitellogenic follicles there is an extensive system of blood vessels between theca interna and basement membrane; the vessels have fine fenestrations. Striations are sometimes visible in the basement membrane, and it is presumed that they represent channels through which plasma proteins (vitellogenins) pass to become electron-dense accumulations in the intercellular channels between contiguous granulosa cells. These channels form an extensive series of spaces between

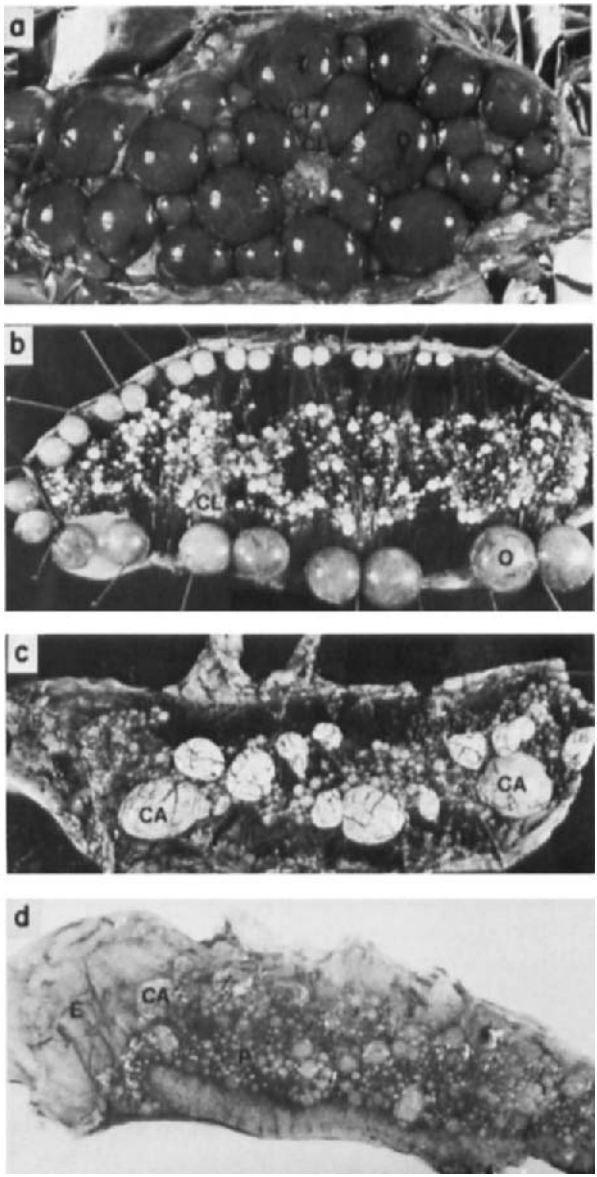


Fig. 1. The ovary of *S. canicula*. (a) Surface view of partly dissected ovary. (b) Vitellogenic oocytes removed and placed peripherally to demonstrate paired size hierarchy. (c) Ovary after thyroidectomy in May. Note that all large follicles are atretic and none of the smaller follicles is undergoing vitellogenesis. (d) Ovary in late atresia, 11 months after removal of VL (CA, corpus atreticum; CL, corpus luteum; E, epigonal organ; O, large vitellogenic oocyte, ovulatable size; P, previtellogenic follicle). (From Dodd, 1972.)

the basement membrane and the zona radiata. The putative vitellogenin lies between the villi of the zona radiata and is incorporated into the oocyte by pinocytosis. The granulosa in immature follicles is multilayered, but in mature and maturing follicles it consists of a single layer of columnar cells which appear very active and have many electron-dense inclusions, especially in their apical regions, and abundant rough endoplasmic reticulum associated with mitochondria. The cells are probably involved in a wide range of secretory activities.

2. Ovary of the Basking Shark, *Cetorhinus maximus*

The ovary of the basking shark has been described in considerable detail by Matthews (1950), and it is a remarkable structure more similar, as Matthews points out, to that of an oviparous teleost then to any known elasmobranch. It is single, developing from the right gonadal primordium and about 50 cm long. A tough fibrous outer coat encloses the sparse and loose stromal tissue in which are embedded several million small oocytes. A large aperture in the fibrous coat leads into a so-called pocket. This in turn opens into a complicated system of branching ciliated tubes which ramify throughout the stroma. The oocytes lie within the walls of these tubes. Matthews has calculated that the ovary contains at least 6 million ova of 0.5–5.0 mm diameter; these already contain yolk. He believes that the largest eggs in the *Cetorhinus* ovary do not attain a diameter greater than 5.0 mm because anything larger would be unable to negotiate the finer branches of the stromal canals into which they pass at ovulation.

In addition to the oocytes there are large numbers of corpora lutea which are lenticular in form, opaque, and lighter colored than the ova. They are of two sizes, the largest being about 4.0 mm in diameter and less numerous than the smaller ones. Matthews (1950) believes that the large bodies are postovulatory corpora lutea; the smaller ones are produced by atresia of follicles of about 1.0 mm (corpora atretica), and Matthews describes their quite different structures.

As in all elasmobranchs examined, although not in holocephalans, the gonad is associated with a haemopoietic epigonal organ, which in *Cetorhinus* is paired, the one on the right side lying posterior and slightly dorsal to the ovary and suspended by a posterior extension of the mesovarium. It measures about 60 cm in length and has a diameter of 20 cm. The anterior region is intimately fused with the posterior one-third of the ovary. The left epigonal organ is similar in size and shape although not associated with a gonad.

Stanley (1963) has described both the developing and the mature ovaries in *H. colliei* and Vu Tan Tue (1972) has discussed the structure of the ovaries

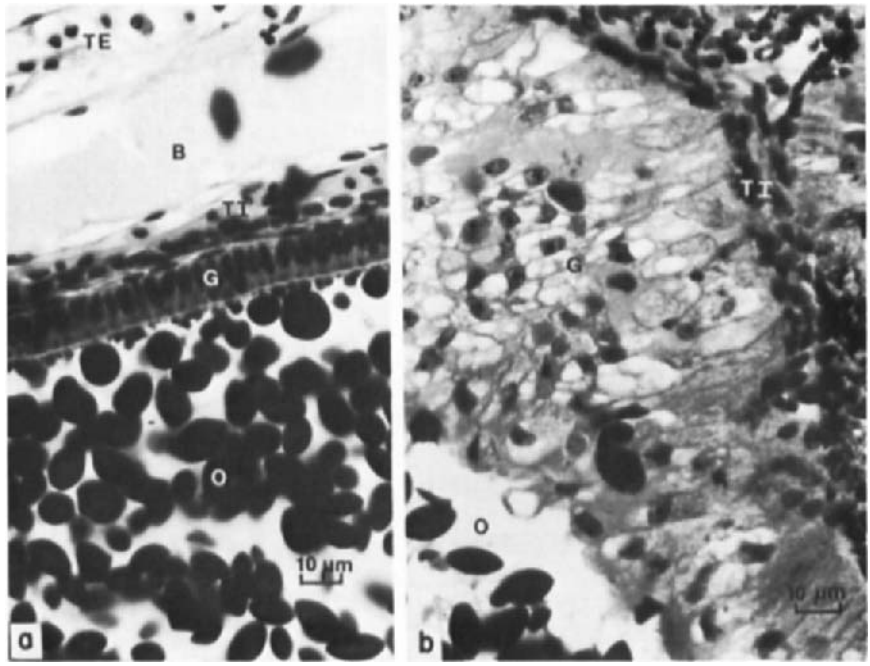
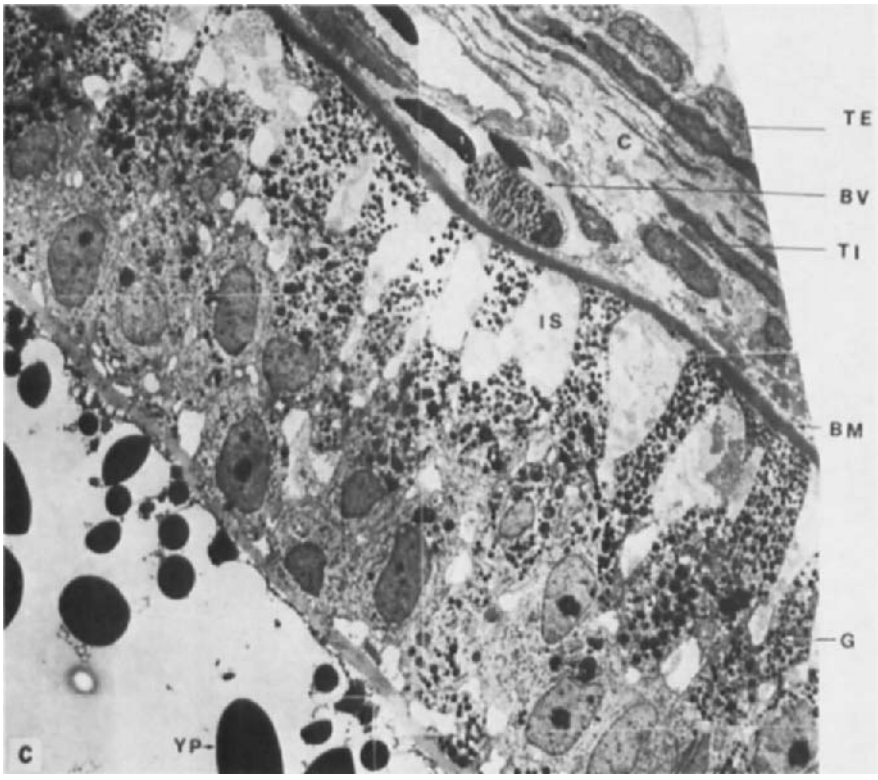


Fig. 2. Follicular structure in *S. canicula*. (a) Transverse section of portion of the wall of a vitellogenic follicle. (b) Transverse section of an early corpus atreticum (B, blood vessel; G, granulosa; O, oocyte with yolk platelets; TE, theca externa; TI, theca interna). (From Dodd, 1977.) (c) Electron micrograph of a transverse section of a portion of the wall of a vitellogenic follicle (BM, basement membrane; BV, blood vessel; C, collagen fibers; G, granulosa; IS,

of *Chimaera monstrosa* and described cyclical changes in them. In *H. colliei* the largest ova are about 20 mm in diameter and there are occasional large corpora atretica (Hisaw and Hisaw, 1959). The follicle wall has not been described in detail and there have been no ultrastructural studies. Both ovaries are functional and they may differ markedly in weight although this does not appear to represent a fundamental asymmetry such as that which is found in many elasmobranchs.

3. Corpora Atretica and Corpora Lutea

In common with all other vertebrates the elasmobranch ovary may contain a number of structures, often bright yellow in color, in various stages of development or degeneration (Saidapur, 1978). The latter are follicles in which the enclosed oocyte has lost its integrity for some reason and is being removed by invading granulosa cells supported by ingrowths from the theca (Figs. 1C and 2B). Such structures, which are sometimes called preovulatory



intercellular space; TE, theca externa; TI, theca interna; YP, yolk platelet (from Dodd and Dodd, 1979).

corpora lutea, are here referred to as corpora atretica. The postovulatory follicles are more variable both in degree of development and in structure. They arise from what remains of the follicle after ovulation and are here called corpora lutea because of their analogy with these structures in mammals. Admittedly, it is difficult to defend the use of this term because nothing is known of their functions, and in some cases (*Squalus acanthias; S. canicula*) the follicular remnants appear to be removed, presumably by phagocytic action, with the minimum of postovulatory development.

Atresia has been described in a number of elasmobranchs, some oviparous and some viviparous, both aplacental and placental: *Cetorhinus maximus* (Matthews, 1950); *Raia binoculata, R. erinacea, Squalus acanthias, S. suckleyi, Mustelus canis* (Hisaw & Hisaw, 1959); *S. canicula, S. stellaris, Torpedo marmorata, T. ocellata* (Chieffi, 1962); *S. acanthias* (Lance & Callard, 1969); *Scoliodon sorrakowah* (Guraya, 1972); *Mustelus canis* (Te Winkel, 1972). Lance and Callard (1969) have described four stages in the

formation of the atretic follicles as follows: (1) In stage 1, the granulosa is highly folded and invaginated, the villuslike processes being supported by a scaffolding of thecal cells; phagocytosis of yolk is under way. (2) In stage 2, yolk phagocytosis is complete; hypertrophied granulosa cells fill the structure. (3) In stage 3, yolk granules are no longer present; the follicle is thin walled and solid, and involution has begun. (4) In stage 4, involution is well advanced; the granulosa has degenerated and is infiltrated by connective tissue. Lance and Callard have also described four stages in the formation of the corpus luteum in *S. acanthias*. Stages 1 and 2 result from the collapse of the follicle wall after ovulation, resulting in an apparent thickening of theca and granulosa, the latter filling the cavity. Stages 3 and 4 are similar to the corresponding stages of atresia. Putative steroidogenesis in both types of structure is discussed further on.

Copora lutea and corpora atretica in the holocephalan ovary have been described by Hisaw and Hisaw (1959). They are similar to those found in elasmobranchs, but their function is unknown.

Therefore, the situation regarding the relative development of corpora lutea and corpora atretica varies with the species and their function remains obscure. Hisaw and Hisaw (1959) have concluded that the elimination of yolk from atretic follicles and of debris from ruptured follicles is a primitive function of the granulosa in both corpora atretica and corpora lutea. Luteinisation of the granulosa by the pituitary and secretion of progesterone are later developments. Certainly, it has never been demonstrated that the formation of corpora lutea in elasmobranchs is in any way stimulated by the pituitary, and, as Dodd (1972) has emphasized, the best way to produce corpora atretica experimentally is to remove the gonadotropic region of the pituitary.

However, Chieffi (1962) believes that "true corpora lutea" (i.e., in the mammalian functional sense) are found in both oviparous and viviparous elasmobranchs. In the former (*S. stellaris*) they are believed to develop from ovulated follicles ("corpora lutea") and in the latter (*T. marmorata*) by follicular atresia (corpora atretica). However, Lance and Callard (1969) in the viviparous aplacental *S. acanthias* identified 3β-hydroxysteroid dehydrogenase (3β-HSD) only in the corpora lutea and this decreased in intensity as gestation progressed. Chieffi (1961) found that corpora atretica increase in number at the start of gestation in *T. marmorata*. This correlates well with the lengthening of the uterine folds as gestation proceeds and from this Chieffi suggests that the atretic follicles in this species are the source of steroids involved in the maintenance of gestation. However, it may be noted that all yolky eggs not ovulated with the ones that led to the pregnancy would be expected as in other vertebrates, to be removed from the ovary by atresia prior to the development of the next clutch. Mellinger (1974), unlike

Chieffi (1961), believes that this is the explanation of choice to account for the appearance of corpora atretica after ovulation in *T. marmorata*.

The problem of assigning a function to corpora atretica and corpora lutea is exacerbated by the fact that all the evidence is indirect and based on either putative correlations between changes in their secretory activity (also measured indirectly) and changes in the reproductive system during gestation, or by the presence in them of 3β-HSD. Lance and Callard (1969) have concluded that the admittedly sparse evidence available does not support the idea that either corpora atretica or corpora lutea play a role in the maintenance of pregnancy in the aplacental *S. acanthias*. There is a need for more research on possible ovarian and pituitary involvement in gestation in placental species.

4. VITELLOGENESIS

In oviparous and ovoviviparous vertebrates it has long been known that the blood plasma of mature females contains a calcium-binding lipophosphoprotein, vitellogenin, which is synthesized in the liver under the stimulation of female sex steroids. It passes from the plasma into the oocytes in which it gives rise to the yolk proteins lipovitellin and phosvitin (Wallace, 1978). Until recently, it was not clear whether the elasmobranchs conformed with this general situation. Urist and Schjeide (1961) were unable to induce vitellogenin synthesis by injection of estrone in two sharks *Triakis semifasciatus* and *Heterodontus francisci*. However, Woodhead (1969) demonstrated that estradiol treatment raises plasma calcium in *S. canicula*, and concluded from this indirect evidence that in this respect, vitellogenesis in the dogfish is similar to that found in other vertebrates. Furthermore, Fujii (1960) obtained a lipovitellinlike protein from the eggs of *S. stellaris*. The situation has been largely resolved by recent comprehensive studies by Craik (1978a-d) on *S. canicula*. Craik demonstrated that in this species at any rate, vitellogenesis does not differ fundamentally from the process in other vertebrates that produce yolky eggs.

Craik (1978a), using a radioimmunoassay based on an antiserum raised in rabbits against a 5% solution of yolk granules, and an isotopic technique for measuring plasma phosphoprotein, has shown that a yolk granulelike phosphoprotein occurs in the plasma of mature female *S. canicula*, although it has not as yet been isolated and characterized. A mean plasma level of 0.4 mg/ml was recorded with little seasonal change other than a brief decline in October. This level is between one and two orders of magnitude lower than that found in most other vertebrates that produce yolky eggs. Further, it may reflect the unusually extended egg-laying period of the dogfish in which two eggs mature and are ovulated at intervals of unknown length (estimated

to be between 10 days and 3 weeks) over a number of months. This may well account for the low levels of vitellogenin encountered in *S. canicula*, although the situation might well be different in a species with a more concentrated pattern of vitellogenesis.

In a study of the kinetics of vitellogenin metabolism, Craik (1978b) measured the rates at which vitellogenin is synthesized and converted into yolk granules. The rate of synthesis varies widely in individual fish, as does the half-life of the protein in the plasma, which ranges between 132 and 303 hr (mean 216 hr) at 7 ± 2°C. Both males and females synthesize phosphoprotein, but the low level in males is believed to represent a zero level of vitellogenin synthesis. A point of considerable interest, in view of the fact that egg laying in dogfish is most active in winter, is the finding that low temperatures, but not winter photoperiods, stimulate vitellogenin synthesis in midsummer. Craik concludes from this that temperature is the proximate factor controlling ovarian activity. Craik (1978c) has also identified an annual cycle of vitellogenin production, but mature females appear to synthesize measurable quantities throughout the year. Comparison of measurements taken between March and August with those taken between September and February shows that the latter are significantly higher. As a result of these investigations, Craik (1978c) summarized the times at which the various factors associated with vitellogenesis start to recover from their minima: plasma estradiol levels, July; hepatosomatic index, July; rate of vitellogenin synthesis, August; gonosomatic index, September; oviposition, October–November.

The stimulatory effect of estrogens on vitellogenesis in vertebrates is well documented for only a few species. The steroids act on the liver to increase its production of vitellogenin. Concomitant rises are observed in phosphoprotein, calcium, total lipid, and total protein (see Craik, 1978d, for a review). Craik (1978d) demonstrated, by treating *S. canicula* with estrogens, that the responses are similar to those observed in other vertebrates. Intramuscular injection of 17β-estradiol (3 mg/kg) increases plasma phosphoprotein levels by 9.5 mg protein phosphorus per 100 ml, after 25 days. This represents a 17-fold increase above that found at the time of capture. Circulating levels of phospholipid, total lipid, calcium and protein also were significantly elevated. Plasma levels of estrogen in the injected fish after 14 days were 103 ± 8 ng/ml, i.e., between two and three times higher than fish in the sea, and this must be taken into account when evaluating the results, but there seems no reason to doubt that the stimulation of vitellogenesis was both significant and physiological. In line with work on other vertebrates, males treated in the same way had measurable vitellogenin in their plasma. The role of the pituitary in vitellogenesis is considered further on.

Although holocephalan eggs are heavily yolked, nothing is known of vitellogenesis in these fish.

5. STEROIDOGENESIS

Information regarding the endocrine functions of the elasmobranch ovary is available from three main sources: first, from the identification of steroids in ovarian extracts; second, from *in vitro* studies on steroidogenesis, and third, from the histochemical demonstration of steroidogenic enzymes in ovarian tissue. Appreciable quantities (120 μg/kg) of 17β-estradiol and traces of progesterone and estrone were identified in the ovaries of *Squalus suckleyi* by Wotiz *et al.* (1958, 1960). Chieffi and Lupo di Prisco (1963) found progesterone, estriol, and 17β-estradiol in *Torpedo marmorata*, and Simpson *et al.* (1963) and Gottfried (1964) identified 17β-estradiol and estrone in *Scyliorhinus canicula* and *Squalus acanthias* respectively. Using an *in vitro* approach Callard and Leathem (1965) demonstrated that ovarian fragments of *Raia erinacea* and *Squalus acanthias* can synthesize progesterone from [^{14}C]pregnenolone and Lupo di Prisco *et al.* (1966) obtained similar results with the ovaries of *Scyliorhinus stellaris* and *Torpedo marmorata*. The detailed studies of Lance and Callard (1969, 1978a) on the occurrence of enzymes implicated in steroidogenesis in the ovary of *S. acanthias* established not only the presence of an extensive range of dehydrogenases including glucose-6-phosphate dehydrogenase (G-6-PDH), 3β-hydroxysteroid dehydrogenase (3β-HSD), 3α-hydroxysteroid dehydrogenase (3α-HSD) 17β-hydroxysteroid dehydrogenase (17β-HSD), and 20β-hydroxysteroid dehydrogenase (20β-HSD), but also their distribution within the ovary. In most of the successful reactions nicotinamide dehydrogenase (NAD) was used as a cofactor. Positive reactions for 3β-HSD were obtained in the granulosa and occasionally in the theca externa of developing follicles of all sizes, although reactions were weak and variable in follicles less than 20 mm in diameter. The enzyme was never found in the theca interna. None of the ovarian tissues tested gave positive reactions for either 17β-HSD or 20β-HSD, but G-6-PDH was universally distributed. Lance and Callard suggested that the intense G-6-PDH activity in the granulosa of developing follicles, together with the presence of lipid and 3β-HSD activity that increases with follicular size, may indicate that this follicular layer is a site of steroid production. Simpson *et al.* (1963) demonstrated that mature follicles of *S. acanthias* on a weight basis contain more 17β-estradiol than immature ones. Lance and Callard (1969) suggest that these data, together with their own, support the view that ovarian steroid production increases prior to ovulation and may prepare the oviducts for their role in gestation.

Further, 3β-HSD was found in corpora lutea but not in corpora atretica, and 3α-HSD was weakly present in the granulosa of late corpora atretica. Chieffi and co-workers have concentrated largely on steroidogenesis in corpora atretica and corpora lutea. In *Torpedo marmorata* and *T. ocellata* (viviparous-aplacental; Chieffi, 1961), the corpora lutea are said to show no signs of secretory activity although the granulosa of the atretic follicles becomes strongly sudanophilic and cholesterol positive. However, the converse is true in *Scyliorhinus stellaris* and several species of *Raia* which are oviparous. Furthermore, histochemical and biochemical studies have shown that the corpora lutea of *S. stellaris*, although not those of *T. marmorata*, are capable of converting pregnenolone and dehydroepiandrosterone into progesterone and androstenedione respectively. Only the atretic follicles in *Torpedo* produce the 3β-HSD necessary for these conversions (Lupo di Prisco *et al.*, 1965). Chieffi (1967), although recognizing that more species must be studied, believes that the functions of the two structures may be related more to the reproductive mode than to the taxonomic affinities and that oviparous species have evolved enzymically active postovulatory follicles but that ovoviviparous species possess corpora atretica that are secretory.

6. Fecundity

Estimates of fecundity are based on the rate of egg production in oviparous species and the number of embryos in the uteri of viviparous species. In the latter, comparisons have sometimes been made between ovarian fecundity and uterine fecundity. Estimates for oviparous species based on data obtained from the occurrence of eggs in dead fish after trawling have recently been disputed by Dodd and Duggan (1982), who have identified what they believe to be induced ovulation, attributable to trawling stress, on the basis of the following results. A total of 472 mature ovulating fish (*S. canicula*) were trawled on four successive trips. On one occasion all the fish were autopsied immediately (group 1); fish from the other three trips were allowed to survive for 20 h before autopsy (groups 2, 3, and 4). Of group 1, 5.5% fish contained fully formed purses and 13.8% had purses in various stages of formation which were believed to be products of trawling stress. In the three groups examined 20 h after trawling the percentages of purses at all stages of formation, including fully formed purses, were 47.3, 33.6, and 38.5, respectively. If it may be assumed that 5.5% of all fish were gravid pretrawl, then 41.8, 28.1 and 33.0% of the 20-h fish were stress ovulated. If all the published records of fecundity in *S. canicula* were obtained from fish examined several hours after trawling, as maybe likely, it is clear that the estimates are too high. Furthermore, our own research has demonstrated that *S. canicula* populations are far from constant in composi-

tion. It may be that the few fish found to have eggs in their oviducts during the summer months have migrated inshore from deeper colder water. Harris (1952), on the basis of a number of unverified assumptions, believed that mature females of *S. canicula* must lay at least 10 eggs per month, and, if as he suggests, the spawning season lasts from November until at least July, this indicates an annual production of 90 eggs, or 120 if it is accepted that eggs are laid throughout the year. However, these estimates must be interpreted in the light of the aforementioned reservations; they are almost certainly too high.

A number of observations have been reported regarding the rate of egg laying in raiiform elasmobranchs kept in captivity, usually for restricted periods, so that total fecundity can only be estimated. Clark (1922) observed that a specimen of *Raia brachyura* kept in an aquarium for 41 days laid an average of 0.61 eggs/day. Libby (1959) reported a rate of 2 eggs during 4 days. Richards *et al.* (1963) recorded a rate of 0.42 eggs/female/day in *R. eglanteria* and *R. erinacea*, respectively. Holden *et al.* (1971) observed *R. brachyura* (77 days), *R. clavata* (43 days), and *R. montagui* (20 days) under aquarium conditions and recorded rates of 0.42, 0.74, and 2.0 eggs/day, respectively. These data cannot be translated into estimates of fecundity without further information. However, Holden *et al.* 1971) believe that when these data are combined with the data of Clark (1922), they indicate that the approximate upper limit of egg production in the three species is 90, 150, and 60 eggs/year, respectively,

Information on fecundity in viviparous species is both more plentiful and more reliable. Fecundity appears in most cases to be low. Capapé (1974) has reported on fecundity in 16 aplacental squaliform species from Tunisian waters. He has found that fecundity varies between 1 and 40 embryos/year. Eight of the species have a maximum of 10 embryos/year and five have a maximum of 20 embryos/year. Numerous studies of fecundity in *S. acanthias* have been completed. Ketchen (1972) tabulated fecundity of fish from various sea areas as follows: North Pacific of British Columbia, 2–17, average 6–7 embryos/year; Sea of Japan, 3–25, average 12 embryos/year; Northwest Atlantic, 1–9, average 4 embryos/year; Northeast Atlantic, 1–10, average 3–5 embryos/year. Templeman (1944) demonstrated that among *S. acanthias*, fecundity increases with the size of the fish; this is no doubt generally true. *S. acanthias* of 74–79 cm in length carry an average of 3.24 embryos, but fish of length 94–99 cm carry 5.34 embryos. Another aplacental species in which fecundity varies with size is the Japanese dogfish *Mustelus manazo*. The adults range from 60 to 90 cm in length, and the number of embryos varies from 2 to 8 (Teshima *et al.*, 1971). However, in the placental sumitsuki shark *Carcharhinus dussumieri*, only 2 embryos are found regardless of size of the mother (Teshima and Mizue, 1972). In *Mus-*

telus mediterraneus, which is also a placental species, ovarian fecundity is appreciably higher at 31.3 embryos, and uterine fecundity is 16.8 embryos (Capapé and Quignard, 1977). The highest level of fecundity recorded in an aplacental squaliform is for *Hexanchus* in which Breder and Rosen (1966) report 108 embryos per brood.

In viviparous rays, information is available on fecundity for several species. *Torpedo marmorata* from Italian waters had 5–36 embryos according to size (Lo Bianco, 1909); Ranzi (1932) reported only 3 embryos. In a French Atlantic location, Mellinger (1971) found fecundity in the same species ranging from 4 to 18 embryos with an average of 8 embryos. Capapé (1979) found 2–13 fetuses in *T. marmorata* from Tunisian waters.

The eggs of Holocephali are laid in pairs, but nothing is known with certainty of the reproductive cycle or fecundity.

C. The Testis and Spermatogenesis

The testes of elasmobranchs are paired and suspended from the dorsal body wall by mesorchia. They are usually large; at certain times of the year they may account for about 4% of the total body weight. In *S. canicula* the testes are elongated, subcylindrical, and extend throughout almost the full length of the body cavity.

Testis morphology differs from that of most other vertebrates in that the unit of structure is the spheroidal ampulla (or follicle) rather than the tubule. It is also unusual in the zonate arrangement of the ampullae (Figs. 3 and 4). Each concentric zone consists of ampullae at the same stage of spermatogenesis, and divisions of the germ cells are roughly synchronous within each ampulla. The latter are initially closed, but later acquire ducts which open into the efferent duct system. The testes, like the ovaries, in the few elasmobranchs so far examined, although not in holocephalans, are usually closely associated with large lymphomyeloid epigonal organs, which produce granulocytes and lymphocytes (Fänge and Mattisson, 1981). Testis structure has been described in some detail for the following elasmobranchs: *Cetorhinus maximus* (Matthews, 1950, who has also reviewed the earlier literature), *Scyliorhinus canicula* (Fratini, 1953; Dodd *et al.*, 1960; Collenot and Ozon, 1964; Mellinger, 1965; Stanley, 1966, 1971a,b; Dodd, 1972; Dobson and Dodd, 1977b), *Squalus acanthias* (Simpson and Wardle, 1967), and *Torpedo marmorata* (Stanley, 1966).

1. Testis of *S. canicula*

Ampullae originate in the ampullogenic zone (Figs. 3II and 4A,B,C,D), which occupies a ventrolateral site, and extends the full length of the testis.

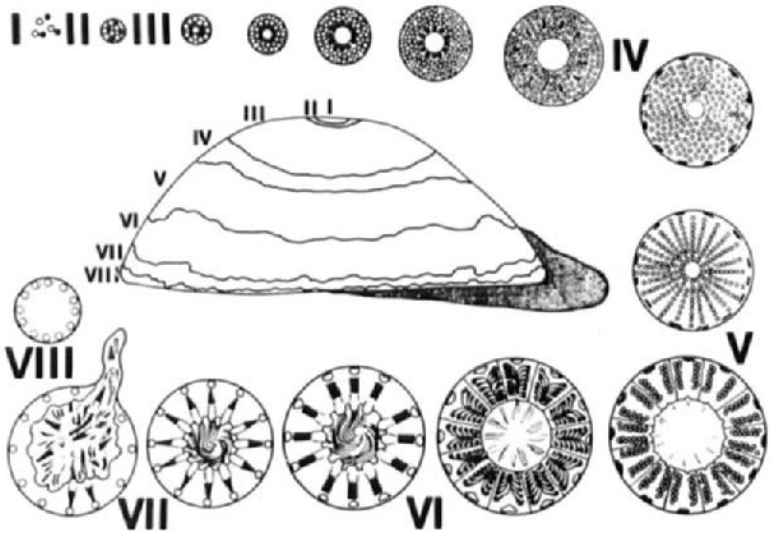


Fig. 3. Diagrammatic representation of zonate testis and spermatogenesis in *S. canicula* (transverse section): (I) aggregation of gonia and Sertoli cells; (II) ampullogenic zone; (III) spermatogonia; (IV) primary and secondary spermatocytes, (V) spermatids and spermiogenesis; (VI) spermatozoa; (VII) release of spermatozoa; (VIII) ampullae containing only Sertoli cells. (From Dobson, 1974.)

Cells of two main types are identifiable in the zone: large spherical, primary spermatogonia 18 μm in diameter, and smaller, fusiform, epithelial cells. The latter are considered by Stanley (1966) to be the homologues of mammalian Sertoli cells. Stanley's discussion is of particular importance *inter alia*, because it reviews the previous literature and exposes a major error committed by many of the earlier workers including Matthews (1950), who mistakenly identified the lumenar Sertoli cells as spermatogonia and believed that Steroli cells were derived from these during their migration to the periphery of the ampulla. Ampullogenesis begins when one or two gonia become associated with a number of Sertoli cells to form small spherical units (Fig. 4B,C,D). Both types of cell then undergo several mitotic divisions until there are approximately 500 of each in two concentric layers; the Sertoli cells surround a central lumen, and the spermatogonia lie peripherally. Phase-contrast microscopy has shown that at this stage each Sertoli cell engulfs a single spermatogonium to form a unit which Stanley termed a spermatocyst. All subsequent divisions, both mitotic and meiotic occur within the spermatocyst. Each primary spermatogonium undergoes four mitoses to give rise to 16 secondary spermatogonia. By this point the Sertoli cell nuclei have migrated from a central position in the ampullae to a peripheral one (Fig. 4G). The secondary spermatogonia then enter the pro-

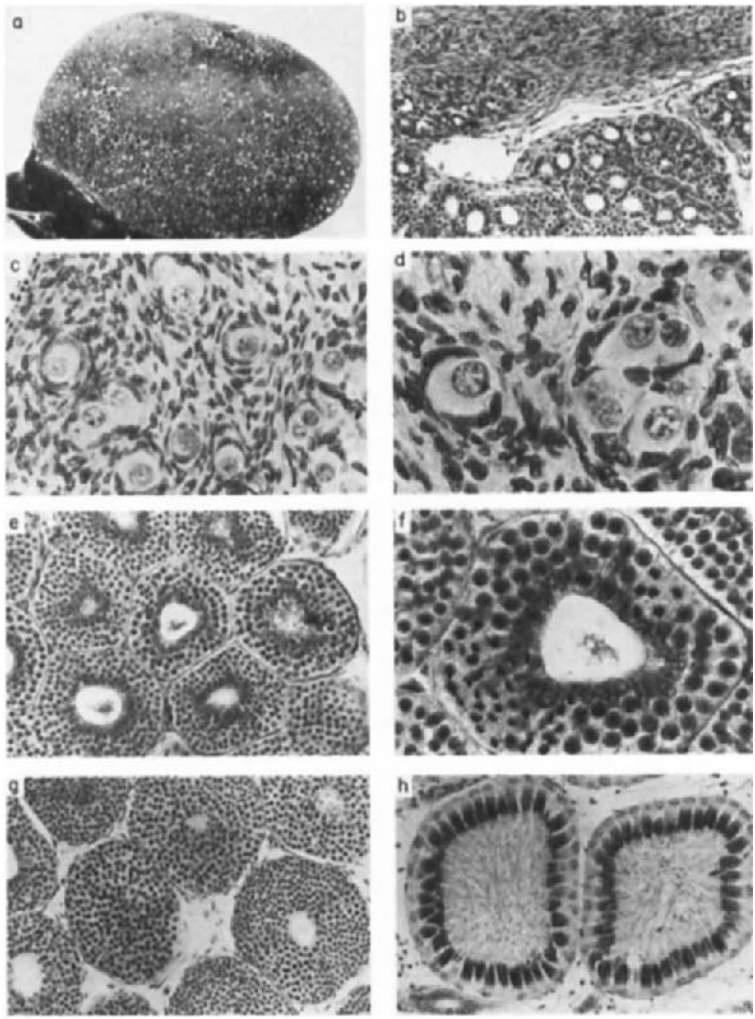


Fig. 4. The testis of S. *canicula* and stages in spermatogenesis. (a) Transverse section of testis. Note ampullar structure and zonation (from Dodd, 1960a). (b) Ampullogenic zone. Note aggregation of gonia and Sertoli cells, vascular sinus, and early ampullae. (c), (d) Ampullogenic zone. Note stages in the aggregation of gonia and Sertoli cells. (e) and (f) Spermatogonial ampullae. Note mitotic divisions in discrete spermatocysts (from Dobson and Dodd, 1977a). (g) Spermatogonial ampullae to the left, primary spermatocytal ampullae to the right showing first meiotic metaphases. (h) Spermatozoal ampullae. Note peripheral Sertoli cell nuclei, sperm heads embedded in Sertoli cell cytoplasm, and sperm tails projecting into the lumen of the ampulla.

phase of the first meiotic division, becoming primary spermatocytes (Fig. 4G), and during this phase the ampullae increase in diameter (in *S. canicula*, from about 125 μm to 225 μm). The secondary spermatocyte phase, which is of short duration, is follwed by the spermatid stage, and then by spermiogenesis (Fig. 4H) which has been described in detail for *Squalus suckleyi* by Stanley (1971a,b).

The mature testis is zonate. The zones radiate from the ampullogenic region and contain, respectively, primary and secondary spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids, and spermatozoa, the latter zone being the widest. Stanley (1966) has estimated that each ampulla in this zone in *S. canicula* contains 32,000 sperms. The peripheral region of the spermatozoal zone usually consists largely of ampullae which are empty, except for Sertoli cell remnants, and which are embedded in the epigonal organ.

The gametogenic ampullae are closed until the end of spermatid development. However, from an early stage, each ampulla is associated with a solid cord of cells which forms part of an incipient duct system that ramifies through the testes and ultimately becomes patent and makes contact with the vasa efferentia.

Dobson (1974) has shown that there is a pronounced cyclicity in testis weight, expressed as a percentage of body weight in the oviparous *S. canicula*. It varies from a low value in January (2.4%) to a peak in August (3.83%). Analysis of changes in ampullar constitution of the testis demonstrated that between April and August there is a significant increase in the abundance of ampullae which contain spermatocytes, spermatids, and immature spermatozoa. Between August and October testis weight decreases and the number of ampullae which contain spermatocytes and spermatids also decreases. From October to April there is a significant decline in the numbers of ampullae containing immature and mature sperm, respectively, and an increase in the number of empty ampullae. Mitotic division of spermatogonia occurs mainly between November and March.

2. TESTIS OF *SQUALUS ACANTHIAS*

Simpson and Wardle (1967) described the histology of the *Squalus acanthias* testis throughout a complete annual cycle and noted that it is very similar to that of *S. canicula*. In *S. acanthias* cyclicity is strict. This permitted Simpson and Wardle to demonstrate that all primary spermatocytes are transformed into spermatozoa within a 12-month period and that maximum sperm accumulation coincides with the annual mating congregation (i.e., November–January in the population they studied). A band of degenerating spermatogonia, identical to that found after removal of the ventral pituitary

lobe in S. *canicula,* appears each May. This lies between the spermatogonial ampullae and the ampullae which contain primary spermatocytes. Reinitiation of ampullogenesis, after a resting period, causes the zone to undergo an apparent movement through the testis until it reaches the epigonal region where it is resorbed.

Teshima (1981) has described a similar annual cycle of spermatogenesis in the seasonally breeding Japanese smooth dogfish *Mustelus griseus* and *M. manazo* (Fig. 5), which are viviparous aplacental species in which ovulation and copulation occur between June and August. Spermatogonial ampullae are most plentiful in July when sperm are virtually absent from the testes. Spermiogenesis starts in October. By May, the testis consists mainly of spermatozoal ampullae from which the mature sperm migrate into the male ducts and seminal vesicles prior to copulation.

3. Testis of Holocephali

Stanley (1963) and Vu Tan Tue (1972) reported detailed studies of testis structure in *Hydrolagus colliei* and spermatogenesis in *Chimaera monstrosa.* Further, they noted that testis structure and spermatogenesis in these species resemble those seen in elasmobranchs. There is an ampullogenic zone followed by concentric zones of ampullae containing spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids, spermatozoa, and empty ampullae. The relationship between Sertoli cells and gonia is the same as in elasmobranchs, and germ cell divisions are largely synchronous. The main difference is the complete absence of the hematopoietic epigonal organ.

4. Steroidogenesis

Whether or not Leydig cells (interstitial cells) are present in the elasmobranch testis has been the subject of much discussion. Most researchers (Stephan, 1902; Kolmer and Scheminzky, 1922; Battaglia, 1925; Matthews, 1950) found few, or no, Leydig cells, but Chieffi *et al.* (1961) identified such cells in four squaliform and three raiiform species of elasmobranchs. Considerable quantitative variations between species and between individuals of the same species were noted, but no seasonal changes were discovered. Chieffi *et al.* (1961) and Della Corte *et al.* (1961) have identified typical interstitial cells in *Torpedo marmorata* and *S. stellaris* lying in nests between neighboring ampullae and being particularly plentiful in the spermatozoal zone. The cells were sudanophilic and Schultz-positive and gave a positive histochemical reaction for 3β-HSD. That steroidogenic tissue is present in the elasmobranch testis, receives support from steroid analysis of

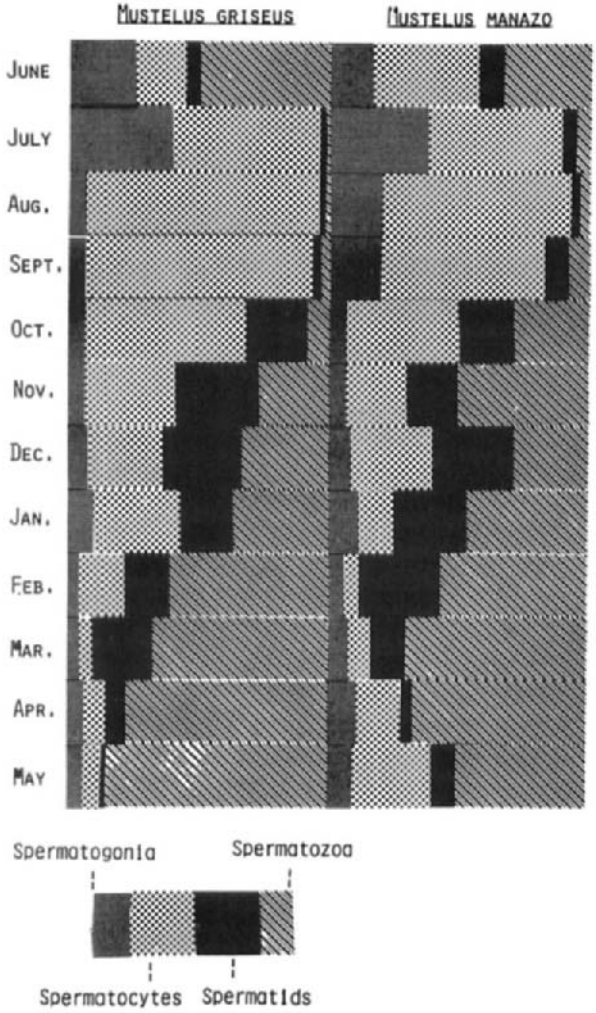


Fig. 5. Monthly changes in germ cell constitution in the testes of the cyclically breeding smooth dogfishes *Mustelus manazo* (viviparous, aplacental) and *M. griseus* (viviparous, placental). (From Teshima, 1981.)

testis extracts (Chieffi and Lupo di Prisco, 1961; *S. canicula*) in which testosterone, androstenedione, progesterone, and estradiol were identified. Further support comes from the demonstration by Simpson *et al.* (1964) that the testis of *S. acanthias* can synthesise testosterone from [^{14}C]progesterone and studies by Kime (1978) who showed that incubation of testicular tissue of *S. canicula* with radioactive progesterone or pregnenolone yields androstene-

dione and testosterone. Furthermore, Darrow and Fletcher (1972) have identified and quantified testosterone and its glucuronide in testicular and peripheral plasma of the skate, *Raja radiata*. However, these studies give no clue as to the identity of the steroid-secreting cells in the testis. Therefore, the possibility that the Sertoli cells are responsible cannot be ruled out. Dodd (1972) has suggested that these cells may have a role in the endocrine control of spermatogenesis in view of their special relationship with the dividing germ cells. This view gains some support from the work of Collenot and Ozon (1964) who demonstrated that the Sertoli cells of *S. canicula* yield a positive 3β-HSD reaction. Collenot and Ozon (1964) and Simpson and Wardle (1967) obtained a similar result using *S. acanthias*.

D. Secondary Sexual Characters and Behavior

In elasmobranchs, as in other vertebrates, secondary sexual characters may be classified as morphological, physiological, and behavioral. Morphological sex structures develop both internally and externally, the former being mainly the ducts that carry the gametes to the exterior. As in amphibians and amniotes, these orginate as urinary ducts; however, there is no evidence that in elasmobranchs they ever function in this capacity.

1. The Female

Oviducts differentiate from the embryonic pronephric (Müllerian) ducts and during development undergo regional specialization, the nature of which varies with species and mode of reproduction. In the majority of elasmobranchs and all holocephalans, two oviducts develop and are functional, but, according to Ranzi (1934), in *Centrophorus squamosus* only that of the right side is functional and in *Dasyatis* and *Myliobatis* only the left one develops. Metten (1939) has described the oviducts of the oviparous *S. canicula* (Fig. 6A,B). They are divided into a slender upper segment, which leads from the peritoneal cavity, by an osteum in the falciform ligament. The upper oviduct is narrow and short and it leads immediately into the highly differentiated oviducal or nidamentary gland, a structure which has three distinct zones, an anterior albumen secreting zone followed by a narrow mucus-secreting zone, and a much larger shell-secreting zone (Fig. 7C). The names are descriptive of the functions of the various regions. The shell-secreting zone acts also as a receptaculum seminis. Internal fertilization is universal in elasmobranchs. It appears that sperm from a single insemination is stored in a shell gland and is available for successive fertilizations; Metten (1939) and Richards *et al.* (1963) have reviewed earlier evidence on sperm survival. Clark (1922) reported that a female *Raia brachyura* kept in isolation

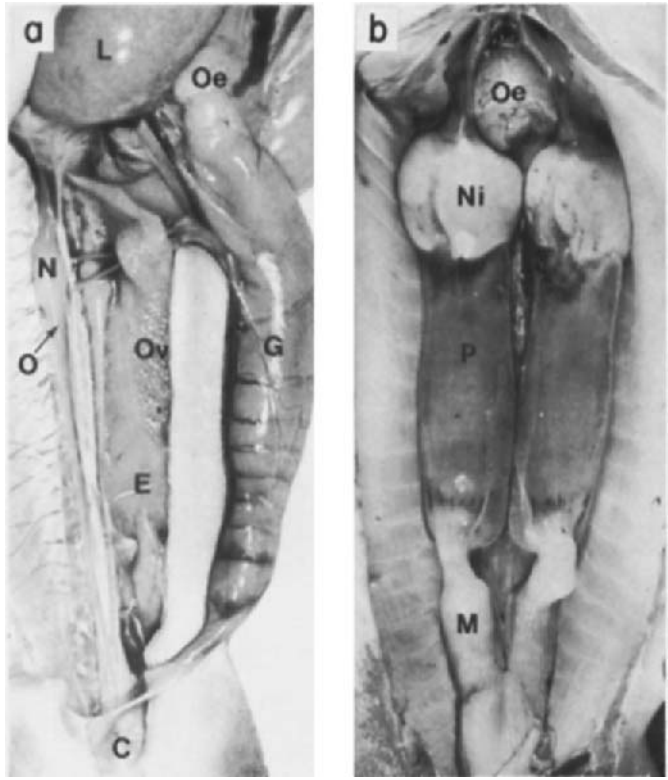


Fig. 6. (a) Ovary, epigonal organ, and oviducts of an immature female of *S. canicula*. (b) The gravid oviducts of a mature female of *S. canicula* (gut, liver, and ovary removed). (C, cloaca; E, epigonal organ; G, gut; L, liver; M, muscular region of oviduct; N, nidamentary gland of immature oviduct; Ni, nidamentary gland of mature oviduct; O, immature oviduct; Oe, oesophagus; Ov, ovary; P, mermaid's purse in oviduct).

for 5–6 weeks laid 30 eggs, all of which developed. Clark also mentioned similar findings in two other species of ray. In studies by Dodd and co-workers of *S. canicula*, sperm were found to possess striking longevity: several females isolated from contact with males laid fertile eggs up to 15 months after capture, and one specimen, caught on April 5, 1979, laid a pair of fertile eggs on April 23, 1981, that is, more than 2 years after separation from males.

The portion of the oviduct posterior to the oviducal gland is differentiated into a long thin-walled region and a shorter thicker more muscular segment which opens into the cloaca. In immature females each oviduct is closed by a hymen.

In viviparous species the oviducts are modified for gestation (Budker,

1958). The oviducal glands are reduced in size, but even in such species the shell-secreting zone develops, and the eggs are usually enclosed in a membrane which in some species takes part in placenta formation.

Oviducal modifications to accommodate gestation have been described in a number of species: *Carcharhinus falciformis* and *Sphyrna tiburo* (Gilbert and Schlernitzauer, 1966); *C. dussumieri* (Teshima and Mizue, 1972); *Mustelus manazo* (Teshima *et al.*, 1971) *Gymnura altavela* (Capapé *et al.*, 1979), and in the putatively viviparous *C. maximus* (Matthews, 1950) (Fig. 7). In all of these species the oviducal regions can be recognized but the most devel-

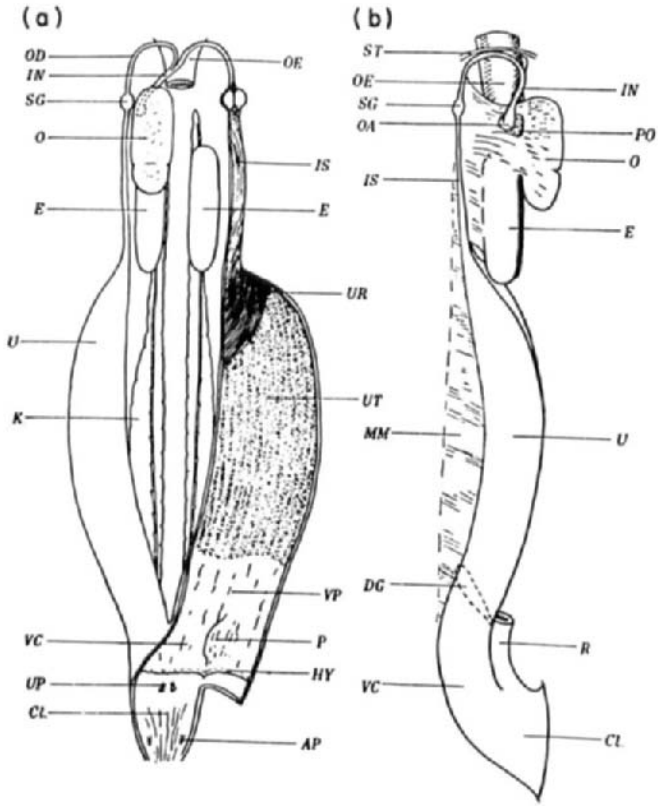
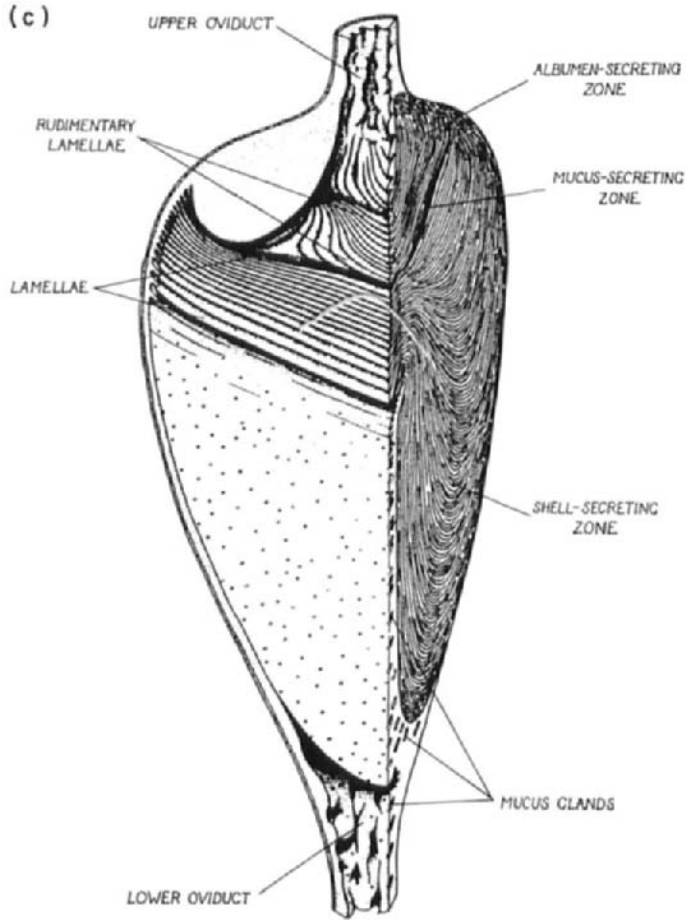


Fig. 7. Female reproductive organs and ducts. (a), (b). *Cetorhinus maximus*. General view of the female reproductive tract, (a) from the ventral surface, the oviduct on the right of the figure opened longitudinally; (b) lateral view from the right side. (AP, abdominal pores; Cl, cloaca; DG, digitiform gland; E, epigonal organ; HY, hymen; IN, unpaired oviduct; IS, isthmus; K, kidney; MM, mesometrium; O, ovary; OA, ostium abdominale; OD, paired oviduct; OE, oesophagus; P, pad in lateral wall of common vagina; PO, pocket in right side of ovary; R, rectum, SG, nidamentary gland; ST, septum transverum; U, uterus; UP, urinary papilla; UR, uterus lined with folds; UT, uterus lined with trophonemata; VC, common vagina; VP, paired

oped of them is the lower oviduct which forms a "uterus" variously modified to support gestation. In *C. dussumieri* the oviducal gland is small, heart shaped, and produces a shell homologue termed an "embryonic membrane" (Figs. 8 and 9). The uterus is unique in being divided, by a perforated partition, into two parts, one of which is designated the embryonic membrane store chamber (Fig. 8). The fertilized egg is enclosed in a membrane in the oviducal gland. It then passes through the store chamber and enters the uterus. Membrane, continuous with that surrounding the embryo, continues to be secreted by the gland, and the newly formed material is depos-



vagina of the left side.) (From Matthews, 1950.) (c) Diagrammatic stereoscopic longitudinal section through one-half of an oviducal gland of *Scyliorhinus canicula*. (From Metten, 1939.)

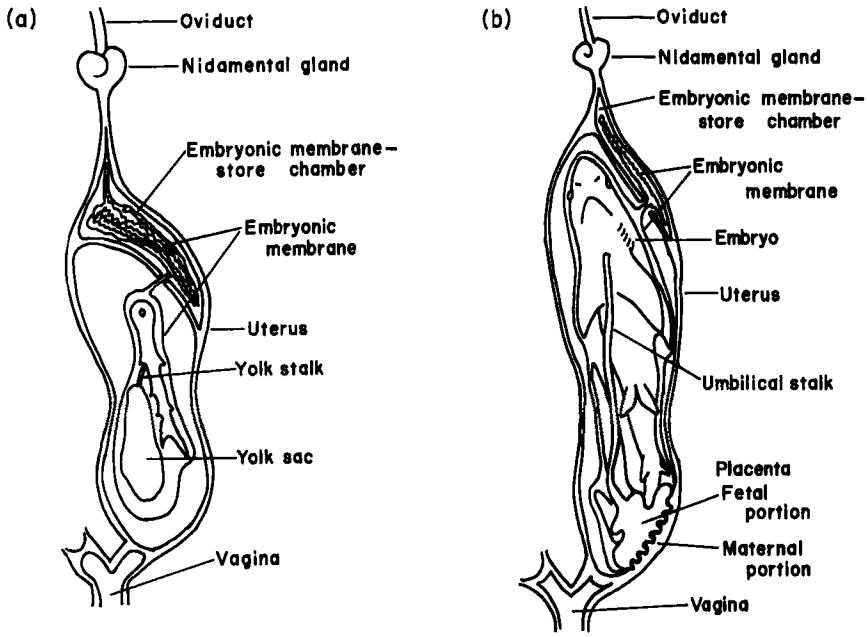


Fig. 8. *Carcharhinus dussumieri*, (viviparous, placental species) diagrams of gravid uteri. (a) Gravid uterus with embryonic membrane store chamber; placenta not yet established. (b) Gravid uterus; placenta established; embryo surrounded by membrane. (From Teshima and Mizue, 1972.)

ited in the store chamber in folds. This transparent brownish membrane extends to about 40 mm in length. During gestation, the membrane is gradually released, through a small hole in the uterine partition, to accomodate the growing embryo. The membrane remains intact until parturition, completely surrounding the embryo and is involved in placenta formation, as in *M canis* (Te Winkel, 1963). The uterine wall is folded and the process of folding accelerates especially in the ventral region just prior to the formation of the placenta. Later in gestation the nonplacental region of the uterus becomes expanded and thin walled and the folds disappear (Teshima and Mizue, 1972).

Matthews (1950) has provided a detailed description of the oviducts of *C. maximus* in nonpregnant specimens. The oviducal gland is relatively small, although not necessarily functionless, and is not divided into regions. Matthews (1950) has reviewed the literature on the modifications that are found in the uterine wall to subserve pregnancy and noted that they vary from rounded lobes on longitudinal folds in the uterine mucosa (*S. acanthias*) to long, histologically complex, strap-shaped growths, usually called tropho-

nemata, in some of the Raiiformes. These structures in *Cetorhinus* are intermediate in form; they lie in rows and their free ends are branched. Each consists of a connective tissue core, which carries the blood supply, and a covering of secretory epithelium. These epithelial cells become distended with secretion and slough off forming the so-called embryotroph; the epithelium between the bases of the trophonemata also hypertrophies and decomposes. In *Cetorhinus* the lower ends of the oviducts join to form a common vagina whose lateral walls bear thick fibrous pads believed to be important in providing an anchor for the clasper spine copulation.

The only fine-structural study on changes in the uterine mucosa during pregnancy is that of Jollie and Jollie (1967) who compared nonpregnant,

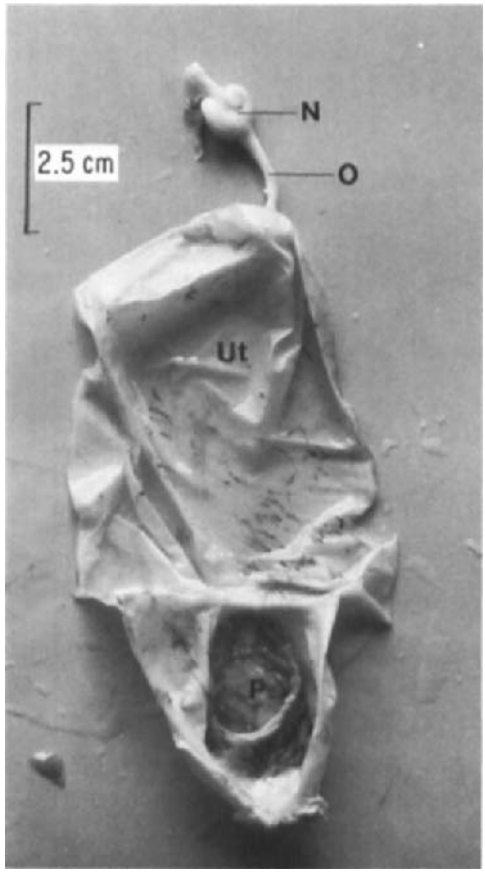


Fig. 9. *Carcharhinus dussumieri.* Dissected uterus showing maternal portion of placenta (P) (N, nidamentary gland; O, upper oviduct; Ut, uterus). (From Teshima and Mizue, 1972.)

pregnant, and postpartum uteri of S. *acanthias*. The main changes during pregnancy were a striking increase in surface area by folding and the development of an extensive ramifying system of blood capillaries, accompanied by a reduction in the tissues lying between the blood vessels and the lumen. Other observed differences were believed to be related to water and electrolyte transport.

The oviducts in holocephalans are derived, as in elasmobranchs, from the pronephric (Müllerian) ducts of the embryo, and they are, at maturity, structurally similar to the oviducts of oviparous elasmobranchs (Dean, 1906; Stanley, 1963). The osteum opens into the slender anterior oviduct. This in turn communicates with the shell gland which Stanley (1963) believes is similar to that of elasmobranchs with an anterior albumen and mucus-secreting region and middle and posterior shell-secreting regions. The shell gland varies greatly in size with the state of maturity of the animal and its transition with the lower oviduct is abrupt. This uterine region of the oviduct is somewhat more differentiated than in elasmobranchs. A deep middorsal groove assists in molding the egg case. The last 45 mm of the oviduct is highly muscular and is not ciliated. A small accessory genital gland is present in both sexes. In adult females the gland opens separately to the exterior between anus and vagina and produces a white semisolid secretion of unknown function. Unlike the elasmobranchs there is no cloaca; the oviducts open separately to the exterior.

External secondary sexual characters in female elasmobranchs are less striking than those in males. The mature female is larger than the mature male. The female body wall musculature is more flaccid. The female cloaca is larger and more distensible; and the female cloacal lining is thicker and more richly supplied with mucous- and sensory-cells. Plasma in the female contains vitellogenin, and the hepatosomatic and thyrosomatic indices vary markedly throughout the year, but in the male they show much smaller changes (Lewis and Dodd, 1974). Finally the peritoneal epithelium and several of the viscera show extensive areas of ciliation in the female (Metten, 1939).

2. The Male

In male elasmobranchs the mesonephric (Wolffian) ducts and a group of mesonephric tubules connect with the testes to form vas deferens and vasa efferentia (epididymis), respectively. The former usually become differentiated into one or more specialized regions; the latter consist of a number of coiled tubes (Fig. 10). The number of vasa efferentia in Squaliformes varies from two to six; in Raiiform species there is only one (Daniel, 1928; Babel, 1967). In *C. maximus*, the vas deferens takes the form of a large ampulla with

a highly complex internal structure in which the sperms are packaged into spermatophores. Each spermatophore is 2.0–3.0 cm in diameter and consists of a solid opaque core of spermatozoa surrounded by a translucent cortex. These float in a fluid believed to be secreted in part by Leydig's gland, a structure derived partly from the nonurinary portion of the mesonephros and partly from the epithelium of the upper vas deferens. The capacity of the ampulla is estimated by Matthews to be "several gallons" (1950). Matthews also observed a newly inseminated female, in which the vagina and uterus were reported to contain "several gallons" of seminal fluid. A small diverticulum arises from the posterior end of the ampulla. This appears to be the homologue of the seminal vesicle, a structure found in a number of other elasmobranchs as a functional differentiation leading off the posterior region of the vas deferens and used for storing sperm. The ampulla and the production of spermatophores are believed by Matthews to be unique to the basking shark although this is far from certain. However, the male ducts in this species illustrate all the basic regions found in other elasmobranchs although the degree to which each is developed shows considerable variation (Fig. 10).

Stanley (1963) described in detail the male reproductive duct system of *H. colliei*. Apart from a highly complex ampulla ductus deferentis of three distinct secretory regions, it is similar to that of elasmobranchs.

The only striking external secondary sexual characters of the male are the so-called claspers (Fig. 11A,B), structures associated with the intromission of sperm (Leigh-Sharpe, 1920, 1921, 1922; Matthews, 1950; Gilbert and Heath, 1972; Dodd and Sumpter, 1982). However, Mellinger (1966) lists a number of other characters which show sexual dimorphism in at least some male elasmobranchs including smaller size at maturity, earlier onset of sexual maturity, shorter life-span, modified teeth, stronger jaws, placoid spines on the wings of some skates which are clawlike and retractile, greater activity, and increased aggressiveness. The claspers represent modified margins of the pelvic fins and consist of a pair of scroll-like appendages which border the cloaca and which have an intricate jointed cartilaginous skeleton. In *S. canicula* and most other Squaliformes the claspers are rodlike and relatively short, but in batoids they are large bloated pendulous structures often heavily armed with spines (Fig. 11). In the former they open proximally into a specialized muscular sac, the siphon, and distally into the surrounding water. The siphon is lined by a secretory epithelium which in *Squalus acanthias* (Mann and Prosser, 1963) produces large quantities of 5-hydroxytryptamine. Whether, as has been suggested, this is introduced into the oviduct during copulation and induces contractions which facilitate sperm transfer is not known.

In *H. colliei* and *Chimaera monstrosa*, in addition to the prepelvic tenta-

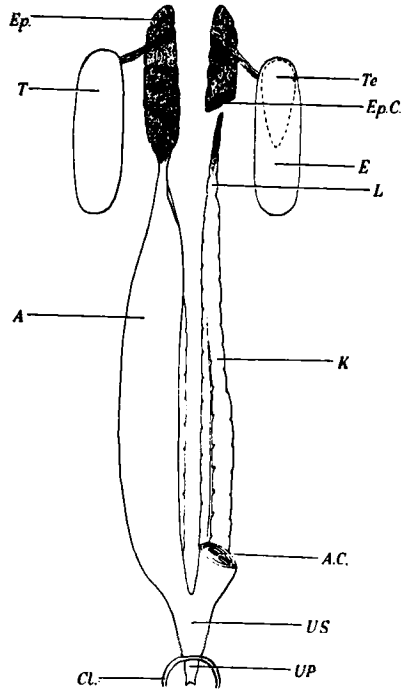


Fig. 10. *Cetorhinus maximus.* General view of the internal reproductive organs in the male. Most of the ampulla on the right side of the diagram has been removed to expose the kidney (A, ampulla ductus deferentis; AC, cut end of ampulla; Cl, cut wall of cloaca; E, epigonal organ; Ep, epididymis; EpC, cut end of epididymis; K, kidney; L, Leydig's gland; T, testis and epigonal organ; Te, position of testis tissue defined by dotted line; UP, urinogenital papilla; US, urinogenital sinus). (From Matthews, 1950.)

cula and the claspers, there is also a median erectile cephalic clasper which lies in a groove when at rest and ends in a spherical knob armed with large recurved teeth (Dean, 1906; Leigh-Sharpe, 1922; Raikow and Swierczewski, 1975).

3. Copulatory Behavior

Matthews (1950) has discussed clasper–siphon interrelations in *C. maximus.* Gilbert and Heath (1972) have investigated them in *Squalus* and *Mustelus* and have found that during copulation sperm passes from the urinogenital papilla into the clasper tubes. The sperm is then flushed into the oviduct by seawater ejected from the siphon. In batoids the siphon is replaced by a clasper gland which also is muscular and secretory, producing a viscous fluid believed to facilitate sperm transfer during copulation (La Mar-

ca, 1964; Babel, 1967, in *Urolophus* spp.). Wourms (1977) has reviewed the mechanics of copulation in the few species of elasmobranchs that have been observed (presumably in captivity). Dodd and Sumpter (1982) have recently recorded, as follows, detailed observations by A. C. Brooks of *S. canicula* copulating in the sea:

> the female lay straight and rigid but slightly tilted to the right. The male was coiled tightly around her pelvic region with his right flank in contact with the female's body and his ventral surface facing backwards. The left clasper lay across the right one, was curved through 90° and inserted into the female's cloaca. The right clasper was straight and occupied its normal position. The process is obviously protracted and the mating pair appeared oblivious to what was happening in their immediate vicinity. They were being harried by a group of about eight males which were swimming in tight circles around the copulating pair. One of these was seen to tug violently at the female's tail. It then moved round to face the female's head and carried out a similar assault whilst gripping her snout in its jaws. The female's only reaction was to close its eyes momentarily. After 20 minutes the pair were still motionless but had turned round, or been turned round, through 180°. One of the supernumerary males was lying on the bottom in contact with the female's head. Whilst still under observation, the copulating male, which had its eyes closed throughout the mating procedure, opened them and twitched its body slightly and several seconds later the female shot out of the loop formed by his body and swam off at high speed leaving the male writhing around, upside down, on the sea bed. The male was found to have the left clasper still bent and considerably frayed. These events took place in daylight at a depth of 50 feet in May.

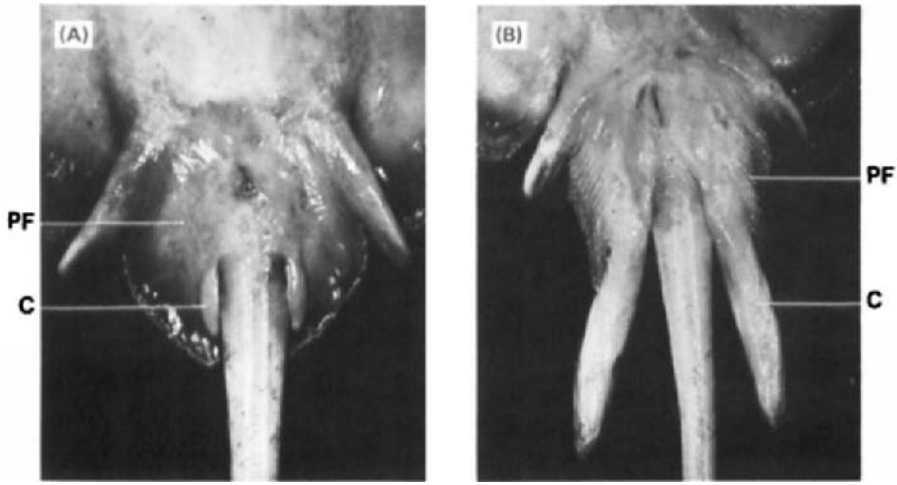


Fig. 11. Claspers of *Raia clavata*. (A) Sexually immature male; disk width 32 cm. (B) Sexually mature male; disk width 57 cm (C, clasper; PF, pelvic fin). (Photograph by courtesy of Dr. D. B. Carlisle, reprinted from Dodd, 1960a.)

Copulation in rays appears also to be a protracted process and associated with precopulatory biting behavior. Smaller species are reported to copulate with their ventral surfaces apposed; others rest on their ventral surfaces (Wourms, 1977).

Copulation in holocephalans has never been observed, but Dean (1906) has suggested that the cephalic clasper grasps the female in the region of the dorsal fin and that the erectile prepelvic claspers also are used for attachment during insemination.

4. Egg-laying Behavior

Dodd and co-workers have on several occasions witnessed egg-laying behavior in *S. canicula* in captivity. It is a highly motivated operation associated with active swimming. The fish selects the object to which the egg is to be attached (e.g., airstone, water inlet, or outlet pipes) at a time when the posterior tendrils of the egg case have just begun to protrude from the cloaca. The fish then swims rapidly around the chosen object in a tight circle, propelled only by the tail. Eventually, the tendrils become attached and further swimming assists in pulling the egg out of the oviduct. The anterior tendrils of the first egg of the pair become entwined in the posterior tendrils of the second. The process continues until both eggs are laid and firmly anchored to the substrate by the tendrils. The eggs are soft on first emergence, but in contact with the seawater they contract and harden.

Egg-laying behavior in captive *H. colliei* has been described by Sathyanesan (1966).

E. Endocrine Control of Reproduction

1. The Pituitary Complex in Elasmobranchs

The elasmobranch pituitary gland is unique because of the extent of its subdivision into largely separate lobes. This offers possibilities for partial hypophysectomy and the detailed investigation of the functions of the separate regions (Dodd *et al.*, 1960). Terminology of the lobes is confused and confusing; here they are referred to as rostral (RL), median (ML), ventral (VL), and neurointermediate lobes (NIL) (Fig. 12A). All, except the neural moiety of the NIL, are derived from Rathke's pouch and are homologous with pars distalis and pars tuberalis of the tetrapod pituitary and with the rostral and proximal pars distalis and pars intermedia of the teleost pituitary. All regions have been reported to be associated with reproduction, but there is now general agreement that the VL is the most important lobe in controlling reproductive processes (Dodd *et al.*, 1960; Mellinger, 1963; Mellinger,

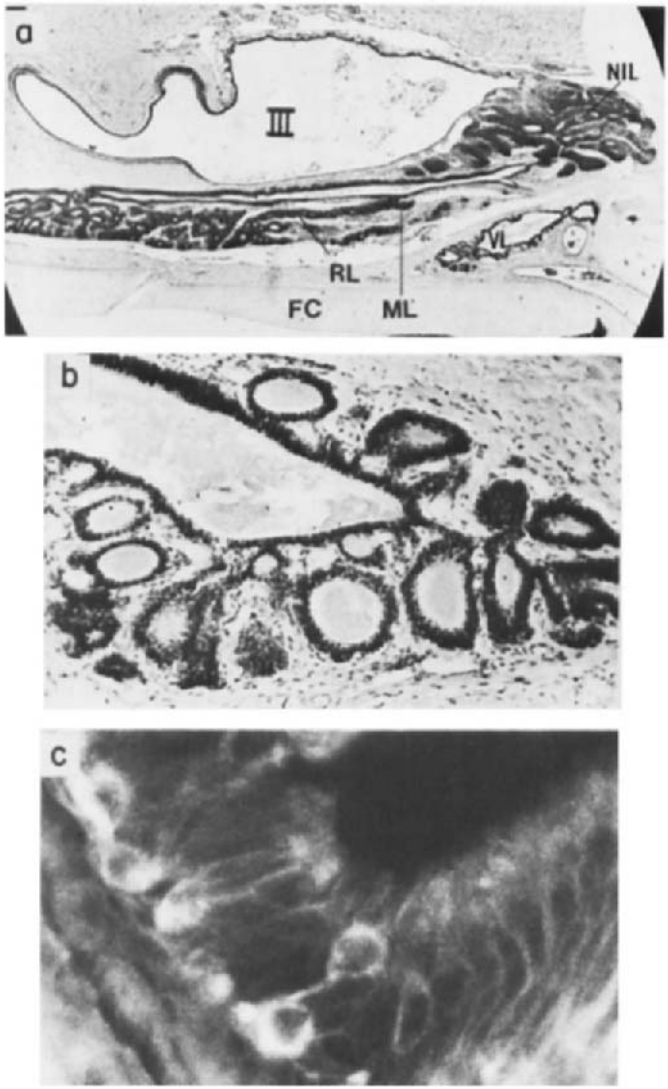


Fig. 12. Pituitary gland of *S. canicula*. (a) Parasagittal section of entire gland (FC, floor of cranium; NIL, neurointermediate lobe; RL, rostral lobe; ML, median lobe; VL, ventral lobe; III, third ventricle). (From Dodd, 1972.) (b) Parasagittal section through ventral lobe showing secretory epithelium and colloid. All the apparently closed follicles open into the central cavity. (From Dodd, 1972.) (c) Part of secretory epithelium of VL after treatment with fluorescein-labeled antibody to quail luteinizing hormone viewed in ultraviolet light. Note fluorescent gonadotrops.

1966; Holmes and Ball, 1974). Compared with the teleost pituitary, the complex in elasmobranchs has received rather little attention after its first description by Collins in 1685 (see Perks, 1959, for a detailed early history). Müller (1871, quoted by Perks, 1959), who studied the gland in *Raia clavata, Scymmus lichia,* and *Acanthias vulgaris* (= *Squalus acanthias*), was the first to name the separate lobes and to recognize the VL, which he termed the "inferior sac." Several more recent researchers of the pituitary of selachoid elasmobranchs have missed the VL because of its fossorial location. Baumgartner (1915) described the gland in *Squalus acanthias* and conducted the first detailed investigation of its embryology. He demonstrated that it originates from two lateral outgrowths of Rathke's pouch which grow downward and fuse beneath the main part of the pituitary. This indicates that the gland's homology, *sensu stricto,* is with the pars tuberalis of tetrapods (Wingstrand, 1966; Meurling, 1967), a point of special significance in view of the reproductive functions of both the VL and the pars tuberalis. More recent contributions to elasmobranch pituitary structure and function may be found in Holmes and Ball (1974), Knowles *et al.* (1975), and Dodd *et al.* (1982). In what follows, most attention is directed to the VL. The other lobes are briefly mentioned in the context of their putative roles in reproduction.

a. The Ventral Lobe and Its Blood Supply. In squaliform elasmobranchs, the VL is surrounded by a tough connective tissue capsule and lies embedded in the cartilage of the base of the skull (Fig. 12A,B). In contrast, the VL of Raiiformes has no capsule; the straplike lobe lies in a shallow transverse depression in the basal cartilage. In both groups, the lobe retains a connection with the ML via the interhypophysial stalk. In *S. canicula,* the stalk is a thin strand of tissue, 10–20 μm in diameter, which may be hollow or solid, or both in the same structure. The position of the VL, relative to the rest of the pituitary, varies but is typically posteroventral to the NIL. The morphology of the VL, and its relationship to the other lobes and to the internal carotid arteries, in 54 plagiostome (elasmobranch) genera, have been exhaustively treated in a monograph by Norris (1941). However, as Dodd *et al.* (1982) have noted, it is likely that much of his research material was sexually immature, and the small size of the lobe led him to the erroneous conclusion that it was functionless. However, in eight of the genera examined by Norris, the lobe was large, glandular, well vascularized, and had the structure of a functioning endocrine organ. Subsequent research, although restricted to two or three species, supports this opinion.

Unlike the RL, ML, and the pars distalis of all other vertebrates, the tinctorial reactions of the VL are disappointing; most of the cells are chromophobic (Dodd and Hendry, quoted by Dodd *et al.*, 1960; Knowles *et al.*, 1975). However, Te Winkel (1969) demonstrated that, in mature specimens

of both sexes of the viviparous dogfish *Mustelus canis*, the large central cavity in the VL and the saclike evaginations which surround it contain a granular colloid which is periodic acid–Schiff positive and aldehyde fuchsin positive. The granular colloid varies in amount and appearance. The significance of the colloid, a striking feature of VL histology, is unknown. The cells surrounding the colloid-filled cavities are mainly chromophobic, but there are scattered giant basophilic cells which show cycles of activity that may be related to reproduction; these are the putative gonadotrops. Knowles *et al.* (1975), in a fine-structural study of the pituitary of S. *canicula*, have identified cells in the ML and VL which contain granules (peak sizes 365 nm and 100–700 nm respectively), and they suggest that these are gonadotrops. It is difficult to evaluate this conclusion because granule size is now recognized as an unsatisfactory basis for the identification of cell function. A more acceptable basis for the identification of gonadotrops in the VL is the data of Mellinger and Dubois (1973) who demonstrated that certain cells in the VL of *Torpedo marmorata* react with a fluorescein-labeled antibody to ovine luteinizing hormone. This is further confirmed by unpublished studies of Dodd and co-workers in which specific cells in the VL of S. *canicula* were shown to react with a fluorescent antibody raised against avian (quail) luteinizing hormone (Fig. 12C). But, these were both heterologous antibodies and this raises problems of interpretation. However, the evidence from histology, histochemistry, and immunohistochemistry gives support, if qualified, to the presence in the VL of cells usually accepted as gonadotrops (or thyrotrops, see further discussion).

In most vertebrates, the activities of the pars distalis are under hypothalamic control, mediated via a portal blood supply, the hypothalamic portal system, that connects the two at the level of the median eminence. In elasmobranchs the situation is somewhat different. Although there is a well developed median eminence (Knowles *et al.*, 1975) (the anterior portion of which supplies RL and ML while the posterior region connects with the NIL), there is no direct vascular connection between the hypothalamus and the VL; the latter receives its supply from the internal carotid arteries (Mellinger, 1961, 1964; Meurling, 1960, 1967). This gives rise to a situation that is unique among the vertebrates, and raises the important, although unresolved, question as to how the reproductive functions of the VL are mediated. The same question arises also in the Holocephali.

b. Hormones of the Pituitary. The chemistry and biological activities of the reproductive hormones of elasmobranchs have been investigated in only one species, the dogfish, S. *canicula*. Partial purification of glycoprotein extracts of the various lobes, followed by bioassay, has indicated that all the lobes contain some gonadotropic activity (Sumpter *et al.*, 1978a,b,c). How-

ever, it must be noted that gonadotropin was measured by heterologous bioassay which can be misleading (Dodd and Sumpter, 1982). With this reservation, the amount present in the VL (98.8% of the total) is much greater than in either RL + ML (0.3%) or NIL (0.9%) (Sumpter *et al.*, 1978c). The functional significance of the small amounts of gonadotropin present in lobes other than the VL cannot yet be assessed. Purification and partial characterization indicate that only one gonadotropin was purified, but this does not mean that only one is present (Dodd and Sumpter, 1982). Antibodies have been raised to this gonadotropin which, when injected into dogfish, produce a significant decline in circulating steroids (Sumpter *et al.*, 1978d).

2. The Pituitary Complex in Holocephalans

The pituitary complex of the Holocephali has attracted a good deal of attention, *inter alia*, because, as in elasmobranchs, there is a lobe that like the VL is highly segregated from the rest of the pituitary. Indeed, in adult holocephalans it is completely isolated in a ventral-facing fossa in the roof of the pharynx. The lobe is termed the Rachendachhypophyse or buccal lobe (BL). Dodd *et al.* (1982) have reviewed the literature and provided a detailed description of the BL and shown that its blood supply like that of the VL is from the carotid artery. These close similarities between the two lobes have raised the question of whether the BL is the gonadotropic lobe of the holocephalan pituitary. Investigation of the BL of *H. colliei* in the context of gonadotropic properties by Dodd *et al.* (1982) has demonstrated that, in fact, extracts of buccal lobe of *H. colliei*, like those of the VL of *S. canicula* are highly steroidogenic in the quail bioassay. On the basis of the evidence, Dodd and co-workers have suggested that the BL of *H. colliei* may be the main source of gonadotropin in this fish also. However, it should be noted that a heterologous bioassay was used and until the effects of BL removal and replacement have been investigated the question must remain unresolved.

3. Pituitary–Gonad Interrelations

In all vertebrates, except cyclostomes, removal of the pituitary gland has a profound effect on gametogenesis and steroidogenesis in both sexes. The question of pituitary involvement in elasmobranch reproduction has been investigated by both total and partial hypophysectomy. Early research, involving total hypophysectomy, was reviewed by Dodd *et al.* (1960) who found results difficult to evaluate. Vivien (1941) found that pituitary removal, in *S. canicula*, blocked sexual development in young dogfish and caused slow involution of the gonads in mature fish. Implantation of pituitary glands

and injection of extracts restored gonadal activity. In the only other published study of the effects of hypophysectomy (still the only research of its type on a viviparous elasmobranch), Hisaw and Abramowitz (1938, 1939; quoted by Dodd, 1955) found: (1) that ovulation in *Mustelus canis* is suppressed by hypophysectomy, (2) that the corpora lutea of this species are not pituitary dependent, and (3) that the first 3 months of gestation are independent of the pituitary. However, it should be noted that there is no placenta up to this point.

Dodd *et al.* (1960) were the first to show that reproductive structures and processes are strikingly affected after removal of the VL, but not after removal of any of the other regions. In the male a precise "zone of breakdown" occurs in the testis which is already well developed 3 weeks after the operation provided the temperature is higher than ca. 13°C (Fig. 13A,B,C). Dobson and Dodd (1977a,b) have reported in detail the histological, ultrastructural, gravimetric, hormonal, and biochemical effects of ventral lobectomy (VLX) on testis function. They have found that the most sensitive stage is the ultimate mitotic division of the spermatogonia. In the absence of the ventral lobe, gonia about to enter this division lose their integrity, and are removed by the associated sertoli cells (Fig. 13C,D). Earlier spermatogonial divisions appear to be blocked, but the gonia do not break down. Cells that have become primary spermatocytes are apparently unaffected and can complete meiosis; this is followed by spermateliosis and spermiation. Therefore, several months after ventral lobectomy, the testis contains only early spermatogonial ampullae, the zone of breakdown, and a reduced zone of spermatozoal ampullae (Fig. 13A). These changes are reflected in a greatly reduced gonosomatic index (GSI). Mellinger (1963, 1965) also found that a zone of breakdown follows VLX, but he believed that the stage affected was earlier than that identified by Dodd *et al.* (1960). However, Dobson and Dodd (1977a) have argued that the difference between them was apparent rather than real and was attributable to difficulties in the precise identification of spermatogonial stages which have been largely resolved by the work of Stanley (1966). Simpson and Wardle (1967), as previously mentioned, have described a naturally occurring zone of breakdown in the testis of the seasonal breeder *S. acanthias*. This appears each May and affects precisely the same ampullar zone as that affected in *S. canicula* after VLX. By way of explanation, they have suggested that the degenerate zone is attributable to a resting phase in the production of a specific pituitary gonadotropin over a period of about 4 months, i.e., between approximately January and May.

Dobson and Dodd (1977b) also found that VLX reduces the uptake of [^{3}H]thymidine by the testis in winter but not in summer, and that total hypophysectomy, but not removal of individual pituitary lobes, produces a

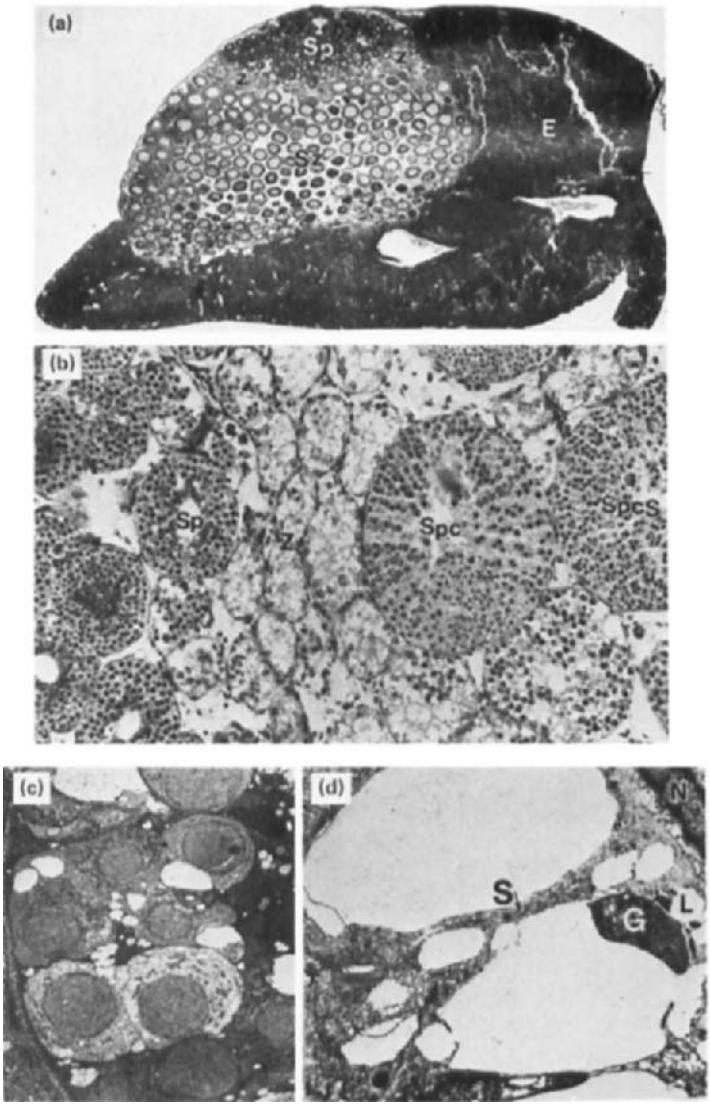


Fig. 13. *S. canicula*. (a) Transverse section (TS) of testis 22 months after removal of VL of the pituitary. Note zone of degeneration and absence of spermatocytes and spermatids. All large ampullae contain only spermatozoa (E, epigonal organ; Sp, spermatogonial ampullae; Z, zone of degeneration; Sz, spermatozoal ampullae). (From Dodd, 1960a.) (b) TS testis in the region of the zone of degeneration (Sp, spermatogonial ampullae; Z, degenerate ampullae; Spc, ampulla with primary spermatocytes undergoing meiosis; SpcS, part of an ampulla containing secondary spermatocytes). (From Dobson and Dodd, 1977a.) (c) Electron micrograph of a portion of a spermatogonial ampulla showing early degeneration of germ cells. (d) Electron micrograph of a portion of a spermatogonial ampulla showing final stages of degeneration (S, persistent Sertoli cytoplasm; N, Sertoli cell nucleus; G, remains of germ cell; L, lipid droplet). (From Dobson and Dodd, 1977a.)

small but significant effect on plasma testosterone levels (see also Sumpter *et al.*, 1978a). Therefore, ventral lobe removal affects gametogenesis in the testis of *S. canicula*. The role of the pituitary regions in controlling steroidogenesis is less well established, but Sumpter *et al.* (1978a) have shown that injection of an extract of ventral lobes into hypophysectomised male dogfish produces a rapid and highly significant rise in androgen levels.

In the female also, the VL appears to be the main source of gonadotropin and removal of the VL has a profound effect on the ovary, the speed of onset of effect being greater at higher temperatures. Ovulation ceases and all eggs which have begun vitellogenesis at the time of the operation become atretic. The large follicles become hyperaemic and flaccid (corpora atretica); they then become yellow in color and gradually shrink in size (Dodd, 1972, and Fig. 1D).

Additional evidence that the VL is a source of gonadotropin in elasmobranchs comes from the studies of Lance and Callard (1978b) who have shown that extracts of the VL of preovulatory *S. acanthias* can stimulate steroidogenesis in enzyme-dispersed cells of the turtle testis.

The roles of the other pituitary lobes in reproduction, if any, are as yet unresolved; their removal has not been shown to affect gonad function in any discernible fashion (Dodd *et al.*, 1960; Mellinger, 1963). However, in view of the occurrence of marked seasonal cycles in some elasmobranchs, there would appear to be a need for some type of central nervous control, such as might be mediated by hypothalamic control of the intracranial pituitary lobes. It has also been noted that possible effects of RL and ML on, for example, secondary sexual characters including sex behavior, have not as yet been investigated (Dodd, 1975). Chieffi (1967) has reviewed histological data on pituitary changes in relation to reproductive events in both oviparous and viviparous species, including the appearance of giant cells in the NIL of *T. marmorata* during pregestation suggesting that other pituitary lobes may be implicated in reproduction, but the evidence is difficult to evaluate. Clearly there is a need for more study in this important area of research.

4. Hypothalamus and Median Eminence

The morphology and histology of the chondrichthyan hypothalamus and median eminence have been described by Mellinger (1964), Meurling (1967), and by Munro and Dodd (1983) who have also reviewed earlier work. The hypothalamus is the region of the brain surrounding the lateral recesses of the third ventricle. It consists of dorsal and ventral regions and large lobi inferiores. Several nuclei of neurosecretory neurons have been identified, including large paired preoptic nuclei (PON) the axons from which are aldehyde fuchsin positive (peptidergic) and converge to form a tract which

ends mainly in the NIL. Further, three other nuclei have been identified in the hypothalamus of S. *canicula* by Mellinger (1964) and in several other elasmobranch species by Meurling (1967). These are the median anterior nucleus (nucleus medius hypothalamicus), the median posterior nucleus (nucleus tuberis), and a pair of nuclei in the lateral walls of the third ventricle (nuclei lobi inferiores). The destinations of the axonal pathways from nuclei other than the PON are largely unknown. None terminates in the VL, but Knowles *et al.* (1975) have suggested that aminergic fibers of unknown origin enter the ML and innervate the putative gonadotrops. Similarly, some peptidergic neurons from the PON terminate in the posterior median eminence; these may control the activities of the ML and, therefore, may have a function in reproduction. The meager evidence in favor of such a role is reviewed in further discussion.

In S. *canicula*, the median hypothalamic floor, between the lobi inferiores, is modified to form a median eminence which is structurally differentiated into anterior and posterior regions. The RL and ML are firmly attached to its ventral surface, and in it lie capillaries of the primary plexus. These unite to form the portal vessels which supply the NIL and which to a lesser extent flow ventralward to supply RL and ML (Mellinger, 1960, 1961; Meurling, 1960, 1967). Therefore a vascular route exists for the transport to RL and ML of hypothalamic releaser hormones. However, it is well established that the lobe mainly associated with reproduction, the VL, has no such supply, and that if it is in fact, under hypothalamic control then the releasers must reach it via a systemic route (see further discussion).

5. Hypothalamic–Hypophysial Interrelations

As noted, there are no direct vascular connections between the hypothalamus and VL in S. *canicula* and nerves between the two are also absent (Mellinger, 1963; Follénius, 1965; Meurling, 1967). However, in skates and rays, the entire pituitary is intracranial. The interhypophysial stalk is shorter, thicker and hollow. It may provide a direct route by which hypothalamic secretions could reach the VL, but this is not confirmed. However, it has been noted that in S. *canicula*, when the interhypophysial stalk is transected, or even when it is removed surgically together with ML and RL, the gonad appears to be unaffected; ovulation continues and there is no zone of breakdown in the testis. It appears that, in the dogfish, there is no direct functional intercommunication between hypothalamus and VL.

Although, as noted, several nuclei of neurosecretory neurones have been described in the dogfish hypothalamus, it is not known which, if any, secrete releasing hormones. The evidence that gonadotropin releasing hormone (GnRH) is present in the dogfish hypothalamus is equivocal although pre-

dominantly favorable. Deery (1974) tested acid extracts of dogfish hypothalami using a radioimmunoassay technique and an antibody to synthetic mammalian GnRH and found no immunoreactivity. By contrast, King and Millar (1979, 1980), using a similar technique obtained a positive reaction from hypothalamic extracts of the elasmobranch *Poroderma africanum* which belongs to the same family as *S. canicula*. Deery and Jones (1974) found that neutralized acid extracts of dogfish median eminence produced a dose-related activation of adenylyl cyclase in all lobes of the dogfish pituitary *in vitro*. They also tested synthetic GnRH and found that it activated the VL enzyme, but had no effect on the other three lobes. On the basis of this indirect, and not wholly satisfactory, evidence they suggested that all four lobes of the dogfish pituitary are under hypothalamic control. More recently, Jenkins and Dodd (1980) have shown that intravenous injection of synthetic mammalian GnRH into dogfish (5 μg/fish) causes a significant rise in testosterone in the plasma within 4 h. Similarly treated females (10 μg/fish) had significantly increased levels of 17β-estradiol 90 min after injection, and there was an effect on oviposition rate in November. In February, twice the dose was ineffective. Similar results were obtained in both sexes when acid extracts of dogfish hypothalamus were injected, and the latency was similar. It was concluded that the dogfish hypothalamus contains a factor which stimulates steroid secretion and is similar in its action to mammalian GnRH. In view of the suggestion that if, in fact, GnRH is functional on the VL it must reach the lobe via a systemic route, the recent tentative report of King and Millar (1980) that immunoreactive GnRH is present at a high concentration (0.6 ng/ml) in the plasma of the dogfish *Poroderma africanum* is of particular significance. However, as King and Millar state, the authenticity of the discovery awaits investigation. To summarize, although the evidence is somewhat meager and restricted to two species, it supports the suggestion that a substance like GnRH related to, though perhaps distinct from, mammalian Gn-RH, is produced by the elasmobranch hypothalamus and reaches the VL via a systemic route, although it may reach the RL and ML through the hypophysial portal system. Only selective lesioning in the hypothalamus will show whether the putative GnRH is implicated in the control of reproduction.

Other recent studies suggesting that the dogfish hypothalamus may play a role in reproduction have been reported by Jenkins *et al.* (1980). Using the techniques of autoradiography and radioreceptor assay of cytosol, Jenkins and co-workers have demonstrated that the cytosol of the dogfish hypothalamus contains a high concentration of estradiol-specific receptors. The main areas implicated were shown, by autoradiography, to be the preoptic, habenular, and tuberal nuclei and the ependyma of the third ventricle. These findings suggest that the estrogen receptors may be the site of es-

trogen feedback and that they may have a role in the control of gonadotropin secretion, but there is no direct evidence that this is so.

6. Role of the Thyroid Gland in Reproduction

The elasmobranch thyroid gland is single and as in all other vertebrates it consists of colloid-filled follicles. Unlike the gland in most teleosts, it is discrete and encapsulated, and it can be surgically removed. In *S. canicula*, the only species in which surgical removal has been conducted, the thyroid gland is pear shaped. The posterior broader region lies at the level of the anterior afferent branchial arteries. Anteriorly, the gland becomes a thread-like structure, one or two follicles wide, which terminates at the posterior margin of the lower jaw. As in all other vertebrates, the thyroid gland synthesizes the hormones thyroxine (T_4) and triiodothyronine (T_3) and their precursors mono- and di-iodotyrosine. The hormones have been characterised and measured in peripheral plasma (Gorbman, 1969; Lewis, 1975; Lewis and Dodd, 1974).

The identification of a functional role for thyroid hormones in poikilotherms has been difficult, with the striking exception of amphibian metamorphosis, and in elasmobranchs the evidence that the thyroid is implicated in reproduction is mainly, although not entirely, circumstantial. The evidence is (1) from demonstrations that there are changes in thyroid activity (albeit based on histological criteria which are difficult to evaluate) which appear to be related to sexual cycles, (2) from differences in gland size in the two sexes, (3) from the effects of thyroidectomy on the ovary and ovulation, and (4) from signs of strikingly high activity in the female at the onset of "puberty."

There is general agreement that histological studies indicate a low level of thyroid activity in immature specimens of both sexes and Leloup (1949, 1951) showed that ^{131}I is fixed less actively in young females than in mature and maturing specimens. Olivereau (1949b) found that in *S. canicula* (Mediterranean population) the thyroid, by histological criteria, is less active in males than in females, and that the thyroid reaches its peak when the testes first mature and their vascularity increases. In females, activity increases when oocytes of 1–5 mm begin to grow at the onset of sexual maturity. Later in the growth cycle, thyroid activity decreases, but there are smaller peaks of activity reported when the eggs are in the nidamentary gland. Sage and Jackson (1973) found that in juveniles of the ray, *Dasyatis sabina*, the activity of the thyroid is constant and low. Sage (1973) reports "we have recently shown that in *Dasyatis sabina* the cyclic activity in the thyroid is clearly related to reproductive development and to the reproductive cycle and not to seasonal environmental changes." However, Mellinger (1966), in a de-

tailed biometrical and histophysiological study of the interrelations between gonads, liver, and thyroid in S. *canicula*, concluded that the highly variable features of the thyroid did not show any relation to either a cycle or to reproductive activity. However, this assessment is attributable mainly to a failure to recognize that the large thyroids found in females approaching first maturity are exceptional and must be considered as a special case and not lumped with those from small immature, and large mature, fish. The individuals that gave rise to the confusion are clearly shown in Figure 7 of Mellinger's (1966) report; indeed, this figure provides powerful, if circumstantial, evidence that the thyroid in *S. canicula* is firmly implicated in reproduction. There is no recent report of thyroid physiology in viviparous elasmobranchs, but earlier researchers agree that gestation is accompanied, at least at certain times, by increased activity of the thyroid (Ranzi, 1936; Zezza, 1937; Olivereau, 1949a; Leloup, 1949). In *Torpedo ocellata*, which has a gestation period from May to September, Zezza (1937) found histological signs of high activity during the entire period. However, Woodhead (1966), studying S. *acanthias* which has a 22-month gestation period, found a cycle of activity associated with the seasons (including more or less complete breakdown in winter) rather than with gestation. Woodhead related the cycle to migration rather than to reproduction. There is clearly a need to study the role of the thyroid in viviparity by the use of RIA techniques.

Olivereau (1954), Matty (1960), and Lewis and Dodd (1974) have drawn attention to the fact that the thyrosomatic index (TSI: thyroid wt. $\times 10^3$/body wt.) of female S. *canicula*, although it shows significant changes through the phases of immaturity, puberty, and maturity, is always at least twice as great as in the male. Mellinger (1966) stated that of all the sexual differences observed, thyroid dimorphism was the most spectacular. Furthermore, as shown in Table I (a), there is a significant difference between both absolute thyroid weights and TSI of immature fish weighing less, and more, respectively, than 500 g. Table I (b) indicates that the difference between thyroid weights and TSI of fish identified as maturing for the first time and mature, egg-laying fish, is highly significant. These differences strongly suggest that the thyroid gland in the female dogfish has a specific function in reproduction.

Lewis and Dodd (1974) have provided the only direct evidence available that the thyroid gland is essential for reproductive success in the female dogfish. They thyroidectomized and sham-operated three groups of fish in May and autopsied them in October, November, and January, respectively. At autopsy, the ovaries of mature thyroidectomized fish contained only previtellogenic oocytes and corpora atretica (Fig. 1C); those of the mature sham-operated animals contained previtellogenic oocytes, corpora atretica, and yolky eggs up to the size at which they might be ovulated. Vitellogenesis was

Table I

Relationships between Thyroid Weight and Body Weight in Groups of Female *S. canicula* Classified by State of Sexual Maturity and Weight[a]

Group	Body weight (g)	Thyroid weight (g)	TSI
a. Immature fish of two weight ranges compared with mature fish			
immature fish (<500 g, $N = 5$)	437 ± 24	0.0233 ± .0026	0.0535 ± .0059
immature fish (>500 g, $N = 14$)	720 ± 29	0.0895 ± .0093*	0.1227 ± .0106*
mature fish ($N = 54$)	926 ± 18	0.0735 ± .0068	0.0818 ± .0081*
b. Fish identified as maturing for the first time compared with mature egg-laying fish			
female first maturity ($N = 11$)	—	0.1295 ± .0204	0.1621 ± .0184
female laying ($N = 54$)	—	0.0735 ± .0068	0.0818 ± .0081
		$P < .001$	$P < .001$

[a]Data from Lewis (1975).

not observed in any of the thyroidectomized fish. These experiments demonstrate that the thyroid is essential for the annual ovarian recrudescence noted in *S. canicula* each autumn. However, the studies do not provide any information as to the manner by which the thyroid hormones act in the vitellogenic process. Whether the thyroid hormones, like the estrogens, mobilize yolk constituents from the liver, or whether they act at the level of the follicle, or in some other manner, remains to be determined.

In summary, although the evidence is mostly circumstantial and based on histological criteria that are difficult to evaluate, there is some recent direct evidence, and no longer any reason to doubt, that the thyroid gland in female elasmobranchs has an important, indeed mandatory, role in reproduction. The precise nature of its role and the point at which it acts have not yet been established, although it appears to be associated with vitellogenesis. Whether or not the thyroid also has a role in the reproductive physiology of the male has not yet been established.

7. Role of the Pineal Organ in Reproduction

The epiphyseal complex in elasmobranchs consists of a well developed pineal and a parapineal which is usually transitory (Munro and Dodd, 1983). The pineal is overlain by a "window" of cartilage modified for transmission of light, and its outer segment has the ultrastructural characteristics of a light-sensitive organ. Whether or not the pineal is implicated in reproduction in these fish remains an open question. Dobson (1974), in preliminary experiments, found that removal of the pineal in the dogfish, *S. canicula*, resulted in an increase in the gonadotrophin content of the VL, whilst injection of pineal extracts into pinealectomized fish reduces the gonadotropin content

to nondetectable levels. Therefore, although temperature may be the main environmental variable controlling reproduction in the dogfish and some other elasmobranchs (see Section II,E), a role for light cannot be ruled out. Moreover, the relative importance of the two variables may differ in different species

F. Environmental Regulators of Reproduction

Unlike the teleosts, which have been studied with regard to environmental influences on annual and circadian rhythms associated with reproduction virtually nothing is known about the effects of such variables on elasmobranch reproduction. Dobson and Dodd (1977b) concluded that the main environmental trigger for the annual reproductive cycle in the dogfish *S. caricula* is temperature, but the evidence is slight and circumstantial. They showed, in studies using [^{3}H]thymidine, that an increase in temperature stimulates spermatogonial mitosis and that a zone of breakdown in the testis follows ventral lobectomy only when the ambient temperature is higher than about 13°C, i.e., at temperatures corresponding to those found in summer in the local sea area of this dogfish. Investigation of the effects of VL removal under a range of photoperiods showed no discernible effects of light on the testis; again breakdown occured above 13°C irrespective of light schedule. In female dogfish also, temperature seems to be the important variable. Peak breeding activity (ovarian growth and ovulation) is associated with low winter temperatures, but whether the effect is mediated directly, or through endogenous rhythms is not known. Furthermore, it is likely that high temperatures are associated with the metabolic preparations for ovarian growth. Reserves are laid down in the liver during the summer, and activity at low temperatures consists mainly of the transfer of these reserves into the oocytes under the influence of estrogens.

Nothing is known of environmental influences on reproduction in viviparous species; however, the precision of the timing of their annual and biennial cycles indicates that such influences may play an important role in reproduction.

III. MODES OF REPRODUCTION AND CYCLICITY

Internal fertilization is universal in chondrichthyan fishes. In oviparous species, horny shelled eggs (mermaid's purses) are laid soon after fertilization, but in the vast majority of species the fertilized eggs are retained in the

oviducts and the young are born alive. Such species have been characterized as either ovoviviparous or viviparous depending on the degree to which they are dependent on the mother for nutriment during development. However, the distinction is an artificial one and difficult to apply because the degree of dependence varies from almost nothing, although no species appears to have eggs that are completely without yolk, to almost complete (Ranzi, 1934). Even when an intimate nutritive connection develops between mother and developing young, this is usually preceded by a period which may in some species be of several months duration, during which development is entirely yolk dependent (e.g. *Mustelus canis;* Te Winkel, 1950).

Wourms (1977) has analyzed data from Breder and Rosen (1966) and Budker (1971) on the modes of reproduction of the extant chondrichthyan fishes that have been studied (relatively few of the 600 species available). His analysis shows that of the 16 families of Squaliformes, 12 are entirely viviparous, 2 are oviparous, and 2 are mixed. Of the 12 Raiiform families, 9 are entirely viviparous and 3 are oviparous. It is therefore clear that the vast majority are viviparous and these have been classified into placental and aplacental species (Budker, 1958); because this scheme begs fewest questions, it is followed here. Wourms (1977) has further subdivided aplacental species into those that: (1) are solely dependent on yolk reserves, (2) practice oophagy and, or, intrauterine cannibalism, and (3) develop placental analogues such as uterine villi or trophonemata which secrete "uterine milk." In placental species early dependence on yolk is replaced during ontogeny by nutrition via a placenta. Selected examples of oviparity and the various grades of viviparity are discussed further, partly in the context of the reproductive cycles associated with them.

A. Oviparity

Oviparity is associated in elasmobranchs with fish of relatively small size which occupy benthic littoral environments (Tortonese, 1950); however, it may be noted that contemporary holocephalans, which are all oviparous, usually live in deep water. Ovulation is followed by a period, usually of a few days, in which the egg is retained in the oviduct and during which time it is fertilized and invested with albumin and a horny capsule or purse (Fig. 6B). These egg cases vary considerably in size, the largest in the Squaliformes being that of *Rhineodon*, the whale shark, which measure 150 mm × 300 mm (Baughman, 1955); the egg case of *Scoliodon sorrakowah* is only 3 mm × 5 mm (Prasad, 1951). They also vary in shape. In sharklike species, the purse is usually quandrangular; the four corners are drawn out into long spiral contractile tendrils for attachment. In skates, which lay their eggs on

sand, the tendrils, if present, are short and stiff. The egg case in *Heterodontus francisci* is about 12 cm long and 6 cm wide; it is coneshaped and two broad flat flanges twist spirally around it (Dempster and Herald, 1961). De Lacy and Chapman (1935) state that the eggs of *R. binoculata* are probably the largest among skates being 265–305 mm in length and 110–140 mm in width at their narrowest point. Furthermore, this species may be unique in having 2–7 eggs per case. Similar observations on the egg cases of this species have been reported by Hitz (1964).

Wourms (1977) has comprehensively reviewed the literature on the physical and chemical structure of elasmobranch egg cases and Wourms and Sheldon (1971, 1972) have investigated the fine structure and shown that collagen, not keratin as was earlier thought, is the main protein constituent. Foulley and Mellinger (1980) have described the eggs and egg cases of *S. canicula* and shown that their rate of development is temperature dependent. Eggs kept in artificial seawater at 14°C hatched between 6.5 and 7.5 months. In research by Dodd and co-workers, 92 eggs of *S. canicula* collected from captive fish between December and June and kept in running seawater at ambient temperature (between 8°C and 18°C) hatched between 4.5 and 7.5 months of incubation with a modal period of 5.75 months. In skates, Clark (1922) observed incubation times as follows: *R. clavata*, 4.5–5.5 months; *R. naevus*, 8 months; *R. marginata*, 15 months. In contrast, a Florida population of *R. eglantaria* required only 9 weeks (Libby and Gilbert, 1960).

The holocephalan egg case (e.g., *Hydrolagus, Callorhynchus,* and *Harriotta*) is said by Wourms (1977), who quoted unpublished work by Wourms and Sheldon, to differ markedly in structure and probably also in chemical composition from that of elasmobranchs; it consists of three distinct structural layers. Dean (1906) described the egg cases of *H. colliei* and also fossil egg cases and stated that there were few differences. The egg case is spindle shaped, golden brown in color, and of a papery consistency. The lateral margins are drawn out into thin finlike vanes, and the inner layer of the posterior tip is continuous with a long filament which is anchored by muscles in the wall of the oviduct until the animal releases its eggs. Sathyanesan (1966) has described egg laying in *H. colliei* and Dean (1906) reports an incubation time of 9–12 months for this species.

Reproductive cycles in oviparous elasmobranchs are much less precise than in viviparous forms, and there are several records in the literature of fish that lay eggs all the year round. However, as Wourms (1977) has noted, available information is often incomplete and fragmentary. Furthermore, populations of elasmobranchs are known to migrate (Harris, 1952), and it is highly unlikely that an unchanging population is being sampled throughout the year in a particular sea area. Adding to the complexity of the situation,

Dodd and Duggan (1982) have found that the highly stressful operation of trawling causes some fish to ovulate. It may be noted that all records for egg production in S. *canicula* are to some extent suspect because of this finding (see Section II,B,6). Evidence that S. *canicula* is in fact a cyclical breeder, albeit with a long active phase, comes from the demonstration that it has marked annual cycles in a number of physiological parameters associated with reproduction. Sumpter and Dodd (1979) have demonstrated that gonosomatic index (Fig. 14), gonadotropin content of the ventral lobe of the pituitary (Fig. 15), levels of testosterone and estradiol in plasma (Fig. 16), and frequency of ovulation (Fig. 17) are all cyclical. Craik (1978c) has shown

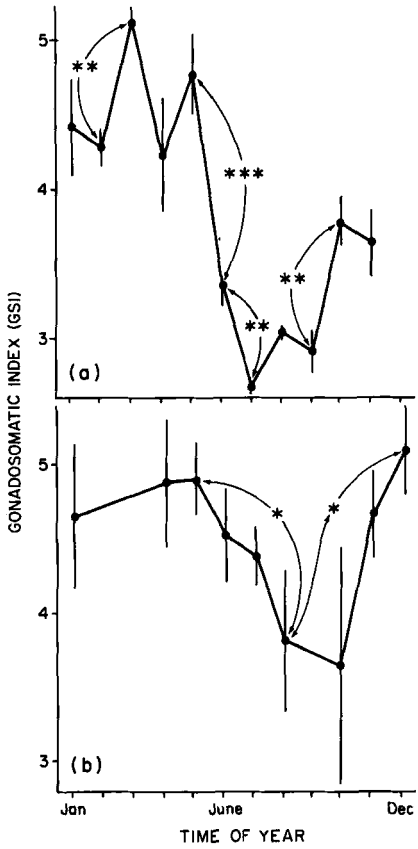


Fig. 14. Seasonal changes in the gonosomatic index of mature female dogfish. Each point represents the mean ± SEM. The number of fish examined each month was the same as that shown in Fig. 17 (a, fish obtained from Caernarvon Bay; b, fish obtained from Plymouth; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; Student's t-test). (From Sumpter and Dodd, 1979.)

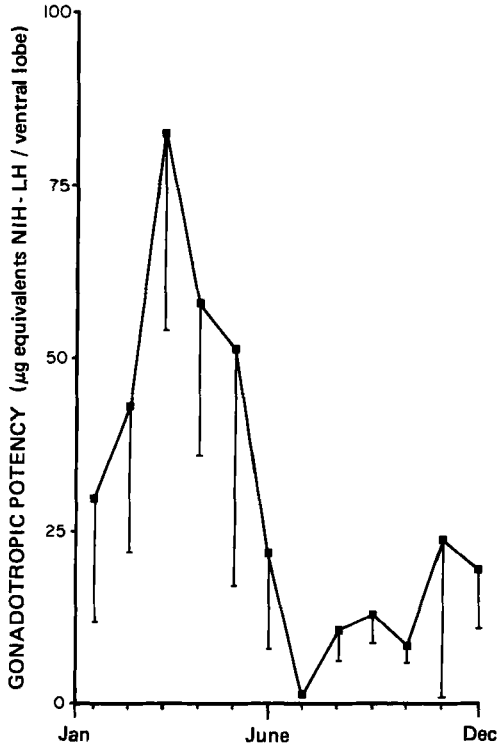


Fig. 15. Seasonal changes in the gonadotropic potency of the ventral lobe of the pituitary from mature female dogfish, assayed using the ^{32}P chick bioassay. The ventral lobes from between 3 and 5 pituitaries were pooled for each estimate. Potencies are expressed as μg equivalents ovine luteinizing hormone (NIH-LH-S19) per ventral lobe, with 95% confidence limits shown for each point. No gonadotrophic activity was detected in the July sample. The potency shown is the maximum possible level. (From Sumpter and Dodd, 1979.)

that vitellogenesis and hepatosomatic index are cyclical. In view of this evidence and the manifest sampling and other problems, it seems reasonable to assume that *S. canicula* is a cyclical breeder, and to suggest that it falls into Wourms' category 2 "those species with a partially defined annual cycle. Although reproductively active throughout the year, they tend to exhibit one or two peaks in activity." Indeed it is difficult to separate this from category 1 "species which are either reproductively active throughout the year or for the major part of the year" in which Wourms places *S. canicula*. A single category combining 1 and 2 would be more appropriate given the present state of knowledge.

Wourms (1977), citing Dean (1906) and Bigelow and Schroeder (1953), states that all holocephalans, both living and fossil, are probably oviparous. The portion of the statement referring to living species is undoubtedly true, but a recent report by Lund (1980) on a newly found fetal holocephalan, *Delphyodontos dacriformes*, from the lower carboniferous of Montana, casts doubt on the view that holocephalans have never developed viviparity. Indeed, Lund suggests that viviparity may have been a significant adaptive feature among Palaeozoic chondrichthyans in general. Lund (1980) describes two specimens of *D. dacriformes* (believed to be fetuses because of their abdominal swellings, body shape, curvature, and undifferentiated fins) which have a large well developed slashing and piercing dentition interpreted as being specialized for opening egg capsules during intrauterine feeding. Therefore, Lund suggests that this palaeozoic holocephalan was viviparous and that the developing young were oophagous.

The evidence for, or against, a cycle in *H. colliei* is fragmentary and difficult to evaluate, although Dean (1906), Stanley (1963), and Sathyanesan (1966) all state that eggs are produced throughout the year, ovarian activity being maximal in late summer and early fall.

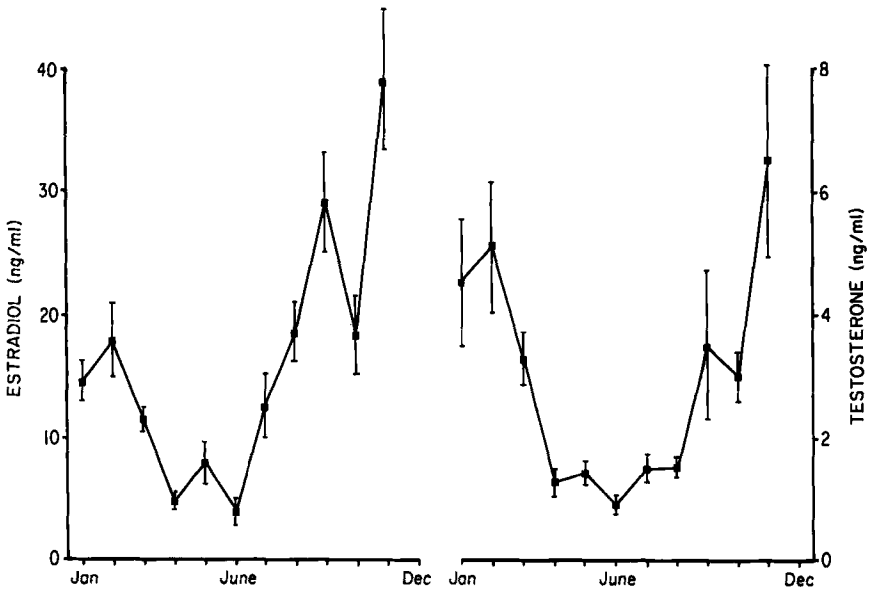


Fig. 16. Plasma estradiol (left) and testosterone (right) levels of mature female dogfish throughout the year. Each point represents the mean of between 4 and 7 fish ± SEM (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; Student's t-test) (From Sumpter and Dodd, 1979.)

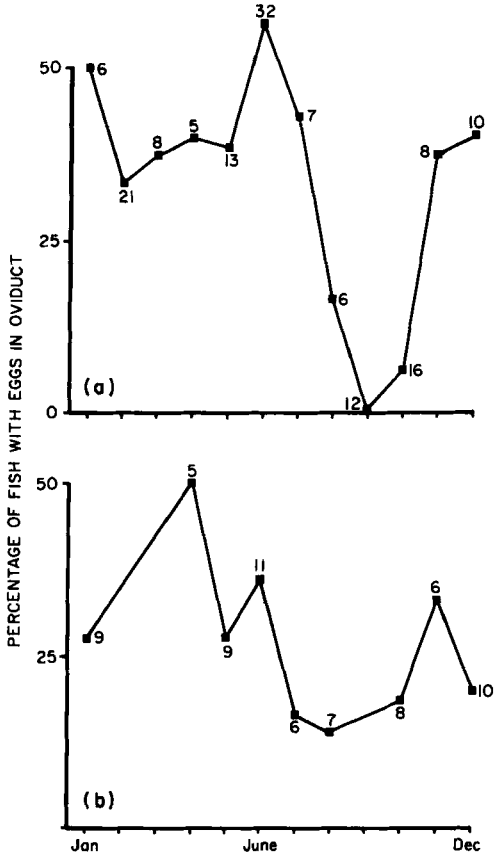


Fig. 17. Seasonal changes in the percentages of mature female dogfish having fully formed egg capsules in the oviducts. The number of fish examined each month is indicated beside each point (a, fish obtained from Caernarvon Bay; b, fish obtained from Plymouth). (From Sumpter and Dodd, 1979.)

B. Aplacental Viviparity

Some aplacental species rely mainly on yolk for development, the mother providing only protection and water (*e.g.*, *Squalus acanthias*, *Scymnus*, *Centrophorus*), but in most cases the uterine mucosa becomes modified, often strikingly so, to produce secretions, called embryotrophe or uterine milk, which are an important additional source of food (Ranzi, 1934; Needham, 1942; Amoroso, 1952). Uterine modifications, including compartmentalization (Fig. 18), vary in complexity in different elasmobranchs and their

Fig. 18. Embryos of the aplacental *Mustelus manazo* lying in uterine compartments. (Photograph by courtesy of Dr. K. Teshima.)

range has been discussed previously and comprehensively reviewed by the Ranzi, Needham, and Amoroso. Another device adopted by some viviparous aplacentals is oophagy (Lohberger, 1910; Shann, 1923; Ranzi, 1934; Springer, 1948). Shann (1923) has demonstrated that in porbeagle sharks (*Lamna* spp.), immature eggs and ovarian tissue are shed and taken up by the oviducts to be eaten *in utero* by the developing young which develop a distended "yolk stomach" for digesting this food. Another example of oophagy has been described in a sand-shark, *Odontaspis taurus*, by Springer (1948) in which the developing embryos show highly active feeding behavior *in utero*.

1. Reproductive Cycles in Aplacental Species

Most viviparous elasmobranchs have a well defined breeding season, insemination and parturition being precisely timed; however, the length of gestation varies considerably between species. Capapé (1979) states that the reproductive cycles of the aplacental elasmobranchs in Tunisian waters do not exceed 12 months and this appears to be generally true of such species. *Dasyatis violacea* has a cycle of only 2 months and in *D. centroura*, *Gym-*

nura altavela, *Rhinobatos rhinobatos*, and *R. cerriculus* it is between 4 and 5 months. However, in *Torpedo marmorata*, Capapé (1979) states that the duration of the cycle is "2 years at least" and Mellinger (1974) believes it to last for 3 years. Capapé emphasizes that in some cases (e.g., Dasyatidae and Rhinobatidae), the next crop of eggs is ready for ovulation at the time of parturition, whereas in *T. marmorata* vitellogenesis is totally inhibited during gestation and it recovers slowly after parturition taking nearly 2 years to complete. Since gestation lasts 1 year, the entire cycle covers a period of 3 years. This is certainly unusually long and may be unique.

Another aplacental viviparous species which is exceptional in having a reproductive cycle extending over more than 1 year is *S. acanthias* in which there is a gestation period of 20–22 months. Ford (1921), Te Winkel (1943), Templeman (1944), and Hisaw and Albert (1947) have described gestation in this species and related it to the reproductive cycle and migration. Hisaw and Albert studied the population of spiny dogfish that migrate northward along the eastern Atlantic seaboard in spring and southward in the fall. In May all females are pregnant, but some have recently ovulated and others have fetuses 12–20 cm in length. In early pregnancy each uterus contains an egg case called a "candle" with 1–4 embryos in it. Development continues inside the candle for about 6 months after which the embryos hatch to spend the remaining part of the 22-month gestational period free in the uterus. So far as the nutrition of the embryos is concerned, Hisaw and Albert (1947) state that the young obtain water from the parent, but whether they obtain inorganic and organic nutriment is uncertain. There is certainly a marked loss of both during development. Ovulation is believed to occur in February or March, soon after parturition in late fall, somewhere south of Woods Hole, the young being about 25–30 cm when born.

C. Placental Viviparity

Placental viviparity is confined to certain species of two families of sharks, the Carcharhinidae (requiem sharks) and the Sphyrnidae (hammerhead sharks). Even in these, the maternal contribution to the nourishment of the embryos varies from nearly nothing to almost complete. This variability is clearly a function of the amount of yolk in the eggs and the time at which a placenta develops. In some sharks (*Sphyrna tiburo* and *Mustelus canis*), a placenta is formed only after several months of an ovoviviparous existence. In all cases the placenta is of the yolk-sac type although the range of structure is considerable.

Teshima and Mizue (1972) have described the reproductive biology of *C. dussumieri* in which the persistenet right ovary produces a few yolky ova, only two of which exceed 20 mm in diameter and are ovulated. One egg enters each oviduct and is invested by a membrane in the nidamentary gland. Although each egg is only slightly greater than 20 mm in diameter, the membrane may exceed 40 cm in length, the excess being folded and stored in an anterior chamber of the uterus (Fig. 8A,B). During development, the membrane passes out into the main region of the uterus through a small hole to accommodate the growing embryo.

When the embryo reaches a length of 50 mm, the basal surface of the yolk sac attaches to the posterior region of the uterus. There is some folding and an increase in vascularity, but these are slight until the embryo reaches 100 mm and the yolk has almost gone. By the time the embryo reaches 150 mm, the placenta is fully established (Figs. 8B and 9). Epithelial folds on the posteroventral region of the uterus interdigitate deeply with similar folds on the basal and lateral regions of the yolk sac. The following tissue layers lie between fetal and maternal blood systems: maternal endothelium, maternal epithelium (largely unmodified), egg case membrane, greatly reduced fetal epithelium, and fetal endothelium. The placenta connects with the embryo via an umbilical stalk which contains a vitelline artery and vein and a flattened ciliated vitelline duct. Placental structure has been described in other species including: *Mustelus canis* (Ranzi, 1934, 1936; Te Winkel, 1963); *Prionace glauca* (Calzoni, 1936); *Carcharhinus falciformis* (Gilbert and Schlernitzauer, 1966); *Scoliodon sorrakowah* and *S. palasorrah* (Mahadevan, 1940); *Sphyrna tiburo* (Schlernitzauer and Gilbert, 1966).

In a number of species the embryos are separated from each other by uterine folds which form so-called gestation chambers (Fig. 18). These may lie transversely or, as in *C. falciformis*, be oriented longitudinally, one joining with the anterior oviduct lumen and one with its posterior region, just in front of the cloaca. The heads of the embryos point anteriorly.

The life-cycles of several placental species have been recorded and most are strictly seasonal and completed within a 12-month period. *Carcharhinus dussumieri* is unusual in that it breeds throughout the year; however, parturition in July and August is more common than at any other time of year (Teshima and Mizue, 1972). Ovulation and insemination occur immediately after parturition and the vitellogenic oocytes remaining in the ovary become atretic and are resorbed. When the embryos reach a length of 200 mm, a new crop of oocytes undergoes vitellogenesis. Unfortunately, nothing is known of possible endocrine involvements in gestation in either aplacental or placental elasmobranchs.

IV. SUMMARY AND DISCUSSION

The cartilaginous fishes are phylogenetically the oldest of the jawed vertebrates and therefore of special interest to comparative reproductive physiologists. However, because they are mostly of large size, and therefore difficult to accomodate, and not of great economic importance, their reproductive physiology has received little attention. Indeed, of the 800 extant species of elasmobranchs, only one *S. caricula,* has been investigated experimentally to any extent in the context of reproduction, although the reproductive biology and the histology and histochemistry of the reproductive organs have been described in a number of others.

All elasmobranchs studied except possibly the basking shark, have yolky eggs, but nothing is known about vitellogenesis except in *S. caricula* and there are no detailed fine structural studies. This is the only vertebrate group of which this is true. Corpora atretica are present in the ovary as in other vertebrate ovaries, and postovulatory follicles (corpora lutea), showing varying degrees and types of activity before they disappear, are also present, but whether or not these structures have any functional significance remains unknown. There is a case for believing that the situation with respect to these might be different in viviparous species, especially placentals, from that in oviparous species, but what little evidence there is for this is equivocal and nothing is known of any endocrine function the gonads, pituitary, and placenta may have in gestation and parturition.

The elasmobranch testis with its zonate structure and synchronous spermatogenesis is unique among vertebrates and ideally suited to investigations of regulating mechanisms, both endocrine and environmental, but these have received little attention.

The well developed and highly characteristic pituitary gland still poses major problems, including the significance of its subdivisions, in particular, of the ostentatiously segregated ventral lobe. This lobe, as noted, is believed to be the main reproductive lobe in both squaliform and raiiform elasmobranchs. In the former, it is almost completely separated from the rest of the pituitary, but the significance of this and the absence of a portal blood supply to it remain enigmatic as does the role in reproduction, if any, of the other pituitary lobes.

The elasmobranch hypothalamus contains a number of the neurosecretory nuclei characteristic of all vertebrates, and there is a well developed median eminence of two distinct regions, but the function of the hypothalamus and its relationship to the median eminence and pituitary remain virtually unknown. A hypothalamic portal system is present, but it does not appear to have any connection with the ventral lobe, at least in Squaliformes, and if releaser hormones are present and functional then it appears

that they must reach the ventral lobe via the systemic circulation. However, in batoids, the degree of segregation of the ventral lobe is much less marked; it is intracranial and connected to the median lobe by a broad stalk. It is possible that in these elasmobranchs the ventral lobe may receive a portal blood supply; this should certainly be investigated.

A thyroid gland, similar in structure to that of other vertebrates and producing the same two hormones is found in elasmobranchs. Recent research on the dogfish, (in which the gland can be surgically removed) has demonstrated unequivocally that it is essential for ovarian growth although the locus at which its hormones act remains unknown.

Of the other endocrine organs that may be involved in reproduction there is, as we have seen, some evidence that the pineal may have a role in controlling the gonadotrophic function of the pituitary ventral lobe, but this is tentative and restricted to the dogfish. However, it remains the only evidence that light may be an environmental cue controlling reproduction in these fish. What little evidence there is points to the importance of temperature, at least in the female dogfish. Ovarian activity, both gametogenic and steroidogenic, coincides with low winter temperatures; during the summer, the ovaries are atretic and circulating steroids are low. However, it would be interesting to have information on viviparous species in which the timing of ovulation, gestation, and parturition is usually precise because in these fish, as in those living at relatively constant temperatures, light would be a more appropriate environmental cue.

The foregoing summary exposes only some of the many gaps in our knowledge of the reproductive physiology of elasmobranchs and emphasizes the dangers in generalizing until a much greater range of species has been examined. Until this has been done, we shall lack anything approaching a comprehensive picture of reproduction in these important and interesting animals.

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3

THE BRAIN AND NEUROHORMONES IN TELEOST REPRODUCTION

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I. INTRODUCTION

Previous volumes of this series that dealt with endocrinology (Volume II) or reproduction (Volume III) did not contain chapters on neuroendocrine regulation of reproduction in teleosts. The birth of research in this field, based on appearance of published results, followed the appearance of these classic volumes in 1969, and can in part be attributed to the interest generated in this field by the *Fish Physiology* series. There has been an increasing

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number of reviews in this field (Peter, 1973, 1982; Holmes and Ball, 1974; Crim *et al.*, 1978; Peter and Fryer, 1983; Peter and Crim, 1979; Ball, 1981).

In this chapter, discussion is designed to develop an understanding of the input of information to the neurohormonal system, and how the brain might integrate neurohormone output to regulate reproductive activity. This requires analysis of both the elements of the system and the manner by which the neuroendocrine system functions as a unit to regulate the various stages of the reproductive cycle. However, the major problem of such a functional approach is that it leaves the matter unsettled because of the limited amount of information available, and because of the large number of questions that remain.

II. GONADOTROPIN RELEASING HORMONE

A. Evidence for Presence and Identity

Several studies have provided evidence for the presence of gonadotropin releasing hormone (GnRH) activity in the hypothalamus of teleosts. Breton *et al.* (1971) were the first to demonstrate GnRH activity in hypothalamic extracts (HE); addition of common carp (*Cyprinus carpio*) HE to carp pituitary cultures caused an increase in gonadotropin* (GtH) levels in the culture medium, as measured by radioimmunoassay (RIA). In other studies, carp HE was shown to be effective in increasing plasma GtH levels in carp (Breton and Weil, 1973; Weil *et al.*, 1975). Hypothalamic extracts from rainbow trout, *Salmo gairdneri*, were effective in increasing GtH release from carp pituitaries *in vitro* (Breton *et al.*, 1972).

Hypothalamic extracts and extracts of other brain regions contain materials immunoreactive in RIA's for GtH (e.g., see Crim *et al.*, 1976). Therefore, when one tests for GnRH activity in a certain HE, it is important that one determine whether the alterations in plasma GtH levels of the experimental animal, or in the medium of a pituitary culture, are caused by the release of GtH or caused by the GtH immunoactivity of the extract. This was not taken into consideration in the early studies of GnRH activity in HE, and may have influenced the results to some extent, although the essence of the findings still stand.

One should ask what the brain distribution of GnRH activity is in teleosts. Breton *et al.* (1972) stated that extracts of the "cortex" (telencephalon?)

*The gonadotropin measured by radioimmunoassay (RIA) in teleosts is equivalent to the "maturational" gonadotropin with high carbohydrate content (see Peter, 1981, for discussion of RIA measurement of gonadotropin in teleosts).

of carp are not active in stimulating GtH release from carp pituitaries *in vitro;* however, data were presented for only the acitivty of HE. Brain intraventricular injection of goldfish HE into goldfish was more effective than injection of extracts of cerebellum and medulla in increasing serum GtH levels (Crim *et al.*, 1976). Using the rainbow trout pituitary *in vitro* as the test tissue and luteinizing hormone releasing hormone (LHRH) as a reference, Crim and Evans (1980) found that extracts of both the hypothalamus and telencephalon, but not the cerebellum and pituitary, of winter flounder, *Pseudopleuronectes americanus,* contained GnRH activity. Crim and Evans (1980) also demonstrated GnRH activity in HE of American plaice (*Hippoglossoides platessoides*), precocious male parr Atlantic salmon (*Salmo salar*), and rainbow trout. King and Millar (1980) found that extract of extrahypothalamic brain of tilapia, *Sarotherodon mossambicus,* contained less LHRH-like immunoreactive material than HE. Therefore, several studies indicate that the hypothalamic region in teleosts contains GnRH biological activity and LHRH-like immunoactivity. The results of Crim and Evans (1980) on winter flounder indicate equivalent biological activity per brain segment in the hypothalamus and telencephalon. However, because none of the above-mentioned studies expresses results in terms of GnRH activity per unit weight of tissue extracted, it is not known whether the hypothalamus contains the highest activity per unit weight, although the results of several studies suggest this. In addition, none of the researchers cited did a complete survey of the brain to determine the relative distribution of GnRH activity.

There is relatively little information available on possible environmental effects or effects on gonadal condition on brain GnRH activity. To approach this problem, de Vlaming and Vodicnik (1975) used an *in vitro* ovulation bioassay with goldfish oocytes to test effects of HE from shiners, *Notemigonus crysoleucas,* on GtH release from goldfish pituitaries *in vitro.* They found greater GnRH activity in HE of shiners exposed to environmental conditions that normally stimulate gonadal activity (long photoperiod and warm tempratures) versus conditions that are inhibitory (short photoperiod and warm tempratures). Furthermore, there was less GnRH activity in HE from pinealectomized shiners undergoing gonadal regression (exposed to long photoperiod conditions) versus those in which pinealectomy caused acceleration of gonadal recrudescence (short photoperiod conditions; de Vlaming and Vodicnik, 1977). These results suggest that changes in hypothalamic GnRH content may portend changes in gonadal activity. However, it is difficult to evaluate results based on a double bioassay system that did not include any standard reference preparation. Greater GnRH immunological cross-reactivity was found in crude HE from common carp in February compared to October, which correlates with changes in pituitary GtH

content and the fact that the carp normally spawn in the spring (Peptide Hormone Group and Isotope Laboratory, 1977). However, these results are also difficult to evaluate because the antiserum used in this study was not characterized for parallelism between LHRH and carp HE, and cross-reactivity with other peptides. Idler and Crim (1982), using an antiserum which was well characterized and which had parallelism between winter flounder HE and LHRH, found there was an increase in LHRH-like immunoactivity in winter flounder HE as the fish progressed from the onset of gonadal growth, through recrudescence, to the postspawning period. Clearly there are changes in hypothalamic GnRH activity, on at least a seasonal basis, as gonadal condition changes.

Chromatography of carp HE on Sephadex G-25 suggested that GnRH activity, as measured by GtH release from carp pituitaries *in vitro*, is associated with a fraction having a molecular weight (MW) of less than 5,000 (Breton *et al.*, 1975b). Such a finding is appropriate considering that LHRH has a molecular weight of 1183 (Fig. 1). Studies of the immunological activity (Fig. 2) and behavior of immunoreactive LHRH fractions on cation-exchange columns (Fig. 3), and in affinity and high-pressure-liquid chromatography (HPLC) systems, indicate that teleost (*S. mossambicus*), elasmobranch, reptile, and bird GnRH are similar, if not identical, and that amphibian and mammal GnRH are similar (King and Millar, 1979, 1980). Interestingly, comparison of teleost and bird GnRH with mammalian and frog GnRH (GnRH as measured by RIA) for ability to cause luteinizing hormone (LH) release from sheep pituitary cells *in vitro* suggests that all these sources have about equivalent biological activity on a weight-specific basis. Although King and Millar (1979, 1980) could not provide the structural identity of tilapia GnRH from their studies, the various antisera they used indicated that the biologically active region of the molecule was probably similar in all vertebrates.

Chromatography studies by Jackson *et al.* (1980) and Barnett *et al.* (1982) on cod (*Gadus morhua*), and Idler and Crim (1982) on winter flounder GnRH confirm that it is similar, but not identical, to LHRH. Interestingly, the results of Jackson *et al.* (1980) and Barnett *et al.* (1982) on cod HE suggests that there are two molecules with GnRH activity that are similar in size to LH-RH, and a third with a larger MW that, they suggest, may be the

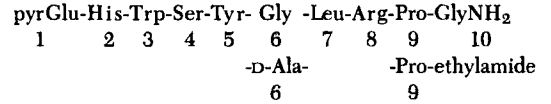


Fig. 1. The amino acid structure of luteinizing hormone releasing hormone and the structure of the superactive analogue des-Gly^{10} [D-Ala^{6}]LHRH ethylamide.

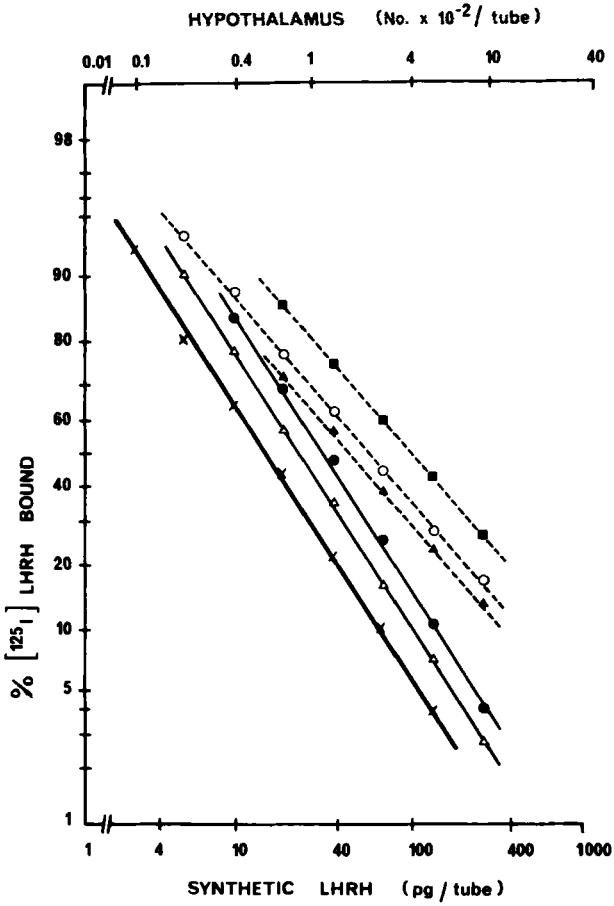


Fig. 2. Comparative displacement of [^{125}I]LHRH from antiserum 1076 by synthetic LHRH and hypothalamic extracts (X, synthetic LHRH; ●, rat; ○, chicken; ▲, tortoise; △, frog; ■, teleost). The displacement curves for synthetic LHRH and hypothalamic extract from rat and frog are parallel. The displacement curves for hypothalamic extract from chicken, tortoise, and teleost are parallel to each other, but are not parallel to synthetic LHRH. (From King and Millar, 1980. Reproduced from *Endocrinology* by permission.)

precursor of the authentic GnRH extended at the N-terminus. Idler and Crim (1982) also have evidence for a GnRH fraction from winter flounder that has a higher MW than LHRH, as well as one that is similar in size. On the basis of cross-reactivity of tilapia (King and Millar, 1980) and cod (Jackson *et al.* 1980; Barnett *et al.*, 1982) GnRH with different antisera to LHRH or fragments of LHRH, it is suggested that teleost GnRH may differ from LHRH by substitution at the seventh or eighth position of the peptide chain

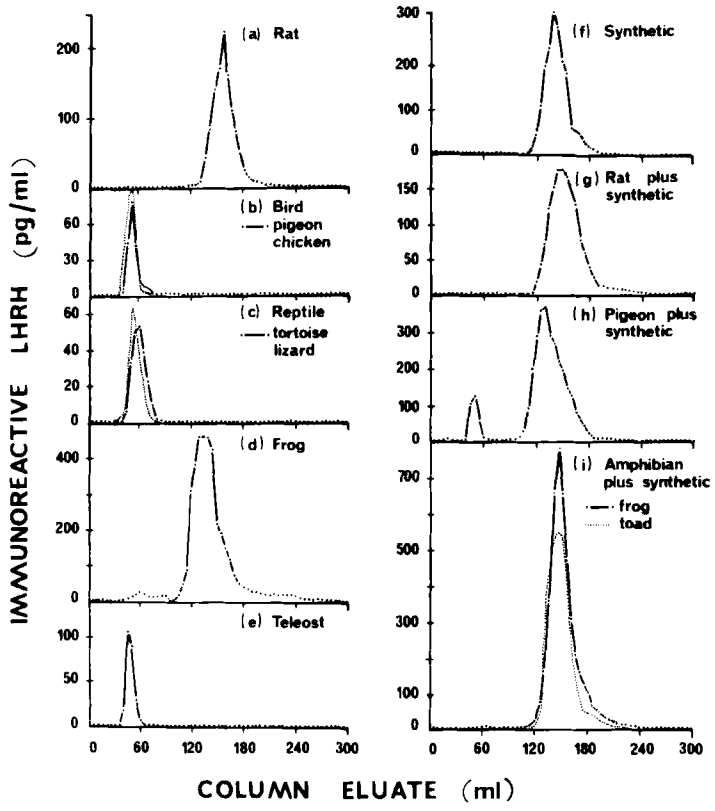


Fig. 3. Elution profiles of synthetic LHRH and hypothalamic immunoreactive LHRH from a variety of vertebrates on cellulose CM 32 cation exchange chromatography. Note the similar elution profiles of the immunoreactive LHRH from hypothalamic extracts from pigeon, chicken, tortoise, lizard, and teleost, and the similar elution profiles of synthetic LHRH and hypothalamic extracts from rat, frog, and toad. (From King and Millar, 1980. Reproduced from *Endocrinology* **106**, 707–717, by permission.).

(see Fig. 1). Therefore, although the structure of teleost GnRH is close to being identified, all that is certain is that it is similar to LHRH and that there is overlap in the biological activity of the molecules.

B. Brain Localization

1. Immunocytological Evidence

Immunocytochemical techniques have been used to localize material(s) reactive with antisera to LHRH in the brain and pituitary of several teleosts.

Immunoreactive material has been found in the neurohypophysial tissue in the proximal pars distalis (in the region of the GtH cells) of rainbow trout (Dubois *et al.*, 1979), platyfish (*Xiphophorus maculatus*) (Schreibman *et al.*, 1979; Münz *et al.*, 1981), Japanese eel (*Anguilla japonica*), and puffer (*Fugu niphobles*) (Nozaki and Kobayashi, 1979). However, there is lack of agreement on the location of immunoreactive perikarya (Fig. 4) and fibers, which may in part relate to differences in the reactivity of teleost GnRH, or other peptides, with the antisera used in the various studies. Goos and Murathanoglu (1977) and H. J. T. Goos (personal communication) reported scattered small immunoreactive perikarya in the area dorsalis pars medialis of the telencephalon of rainbow trout and in fibers scattered in the anterior hypo-

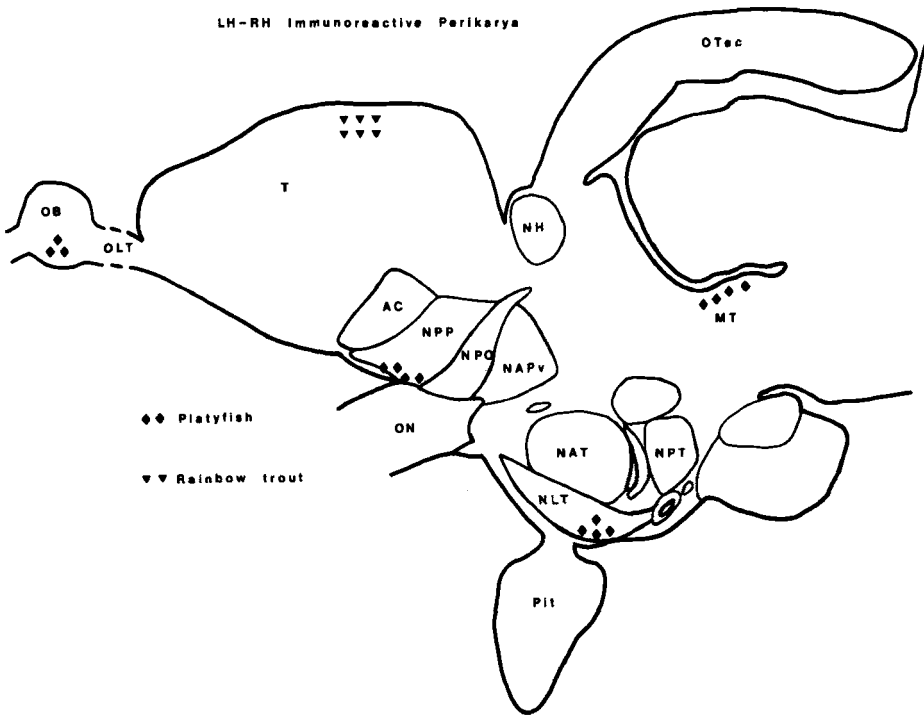


Fig. 4. The distribution of perikarya immunoreactive for LHRH in the brain of platyfish (Schreibman *et al.*, 1979; Münz *et al.*, (1981) and rainbow trout (Goos and Murthanoglu, 1977) plotted on a parasagittal outline drawing of the goldfish brain. AC, anterior commissure; MT, midbrain tegmentum; NAT, nucleus anterioris tuberis; NAPv, nucleus anterioris periventricularis; NH, nucleus habenularis; NLT, nucleus lateral tuberis; NPO, nucleus preopticus; NPP, nucleus preopticus periventricularis; NPT, nucleus posterioris tuberis; OB, olfactory bulb; OLT, olfactory tract; ON, optic nerve; OTec, optic tectum; pit, pituitary gland, T, telencephalon.

thalamus that formed a pathway oriented toward the pituitary stalk. However, Dubois *et al.* (1979) and Nozaki and Kobayashi (1979) could not detect reactive perikarya or fibers in the brain of rainbow trout. In carp, Pan *et al.* (1979) found immunoreactive fibers lateral and ventral to the preoptic recess, and in the lateral forebrain bundle, optic chiasma, and anterolateral hypothalamic region; perikarya originally reported by these investigators to be in the nucleus preopticus (NPO) were instead likely located in the meninges lateral to the preoptic region and ventral to the telencephalon (R. Peter, personal observations). In the Japanese eel, Nozaki and Kobayashi (1979) found immunoreactive fibers ventral to the preoptic region and in the infundibular nucleus (nucleus lateral tuberis), habenular nucleus, and optic tectum; however, no immunoreactive perikarya were found.

In the platyfish, Schreibman *et al.* (1979) described reactive perikarya in the nucleus lateral tuberis (NLT) pars posterioris and in the ventral nucleus preopticus periventricularis (NPP). This latter localization in the anteroventral preoptic region was mistakenly designated as being in the area ventralis pars ventralis and pars lateralis of the telencephalon (R. Peter and M. P. Schreibman, unpublished observations). Schreibman *et al.*, (1979) also found reactive fiber tracts dorsal to the optic chiasma through to a position caudoventral to the horizontal commissure in the anterior hypothalamus, dorsal to the pituitary stalk, and as a loose network in the NLT region. Münz *et al.* (1981) also examined the LHRH immunoreactivity of platyfish. They found small groups of reactive perikarya in the anteroventral telencephalon bordering the olfactory bulbs, with fibers originating from these cell groups coursing posteroventrally to enter the ipsilateral optic tracts, then decussating in the optic chiasma, and traveling in the optic nerve to the retina. Another set of fibers originating from these perikarya reach the olfactory bulbs by way of the olfactory tracts. A second population of reactive perikarya was found in the anterior ventrolateral preoptic region [designated by Münz *et al.* (1981) as nucleus preopticus basalis lateralis, part of nucleus preopticus periventricularis of Peter and Gill (1975)], in a similar location to reactive perikarya described by Schreibman *et al.* (1979). Reactive fibers were found periventricular in the preoptic region, and were found to cross in the anterior commissure and in a small subventricular preoptic commissure. Another group of fibers was found in a distinct tract bordering the optic tracts, that then continued ventral to the postoptic commissure (horizontal commissure), and ventromedially to and through the NLT region where a major part of the tract entered the pituitary stalk. Luteinizing hormone releasing hormone-reactive fibers were observed in most parts of the diencephalon. A third reactive perikarya grouping was found in the dorsal midbrain, posterior to the posterior commissure but anterior to the nucleus of the third cranial nerve. Parts of the midbrain, particularly the optic tectum,

contained an extensive network of LHRH reactive fibers; the cerebellum and medulla contained relatively few reactive fibers.

Viewing all of these results together, the anteroventral preoptic region is strongly implicated as a site for origination of GnRH in teleosts; two studies found LHRH immunoreactive perikarya in this location in platyfish and several studies described fibers in this brain region, including in one case a tract that was specifically traced to the pituitary stalk. The perikarya and fiber localizations in the NLT, telencephalon, and midbrain described in the various studies need to be independently confirmed. Figure 4 presents a summary diagram of perikarya localizations in teleosts. In future studies it would be useful if investigators used more than one LH-RH antiserum to confirm their observations. It is hoped that future research will reveal more information on the distribution of GnRH perikarya–fiber systems in teleost brain, and that these results can be combined with information from investigations on the functional neuroendocrine significance of these brain regions.

2. Functional Evidence

Because early researchers noted correlations between cytological signs of activity in the NLT and/or the NPO and reproductive cycles in several teleosts (see Peter, 1970), the hypothesis developed that one or both of these nuclei was involved in the regulation of reproductive activity. Peter (1970) found that destruction of the NLT in the pituitary stalk region (posterior NLT pars anterior) and the NLT posterior to the pituitary stalk (NLT pars posterior) caused a significant decrease in gonosomatic index (GSI) in both male and female goldfish; lesions in other regions of the diencephalon, including the NPO, had no such effects. In a later study, Peter and Crim (1978) reported that lesions of both the NLT and NPO caused a significant decrease in GSI in goldfish. However, in still other lesion studies, no effects on GSI or serum GtH levels were found following lesions of the NPO that did not also damage lateral tract regions or the NPP anteriorly (Peter and Paulencu, 1980; R. Peter, unpublished results); no explanation can be given for the effects of the NPO lesions reported by Peter and Crim (1978) except that the large size of the lesions must have had secondary affects leading to the decrease in GSI. A significant decrease in GSI and gonadal regression have been confirmed following destruction of the appropriate NLT area in goldfish by electrolytic of radiofrequency heat lesions (Peter and Paulencu, 1980; Peter, 1982; R. Peter, unpublished results). The results of these studies are interpreted to indicate that the NLT is a source of GnRH and that its destruction alters GtH secretion, leading to the gonadal effects.

A problem with the interpretation of the NLT lesioning studies is that a significant decrease in serum GtH levels was not found in goldfish in which

the lesions caused a decrease in GSI and gonadal regression (see Peter and Crim, 1978; Peter and Paulencu, 1980). However, such lesions abolished the daily cycle of serum GtH levels usually found in female goldfish exposed to environmental conditions that stimulate ovarian recrudescence (Peter, 1982). Hontela and Peter (1978, 1980a,b) and A. Hontela (personal communication) found that a significant daily cycle in serum GtH was usually present in female goldfish undergoing, or that had completed, ovarian recrudescence; however, the disappearance of a daily cycle correlated with the onset of regression of the oocytes most advanced in development. Apparently the NLT is involved in the expression of the daily cycle in blood GtH levels in goldfish, and lesions of the NLT alter or abolish the cycle, causing the effects on the ovary.

Lesioning the NLT, but not the NPO, of male Atlantic salmon parr blocked spermatogenic development of the testes, caused a significant decrease in GSI, and reduced the pituitary level of GtH (Dodd *et al.*, 1978). In the killifish, *Fundulus heteroclitus*, lesions in the NLT pars anterioris (NLT in the pituitary stalk region and anterior) consistently blocked testicular development, but lesions elsewhere had either no effects (e.g., NPO and NLT pars posterioris) or inconsistent effects (e.g., nucleus posterioris periventricularis) (Pickford *et al.*, 1981). These results are consistent with the findings from goldfish, except that a somewhat different region of the NLT appears to be involved in the two species.

A criticism of lesioning studies such as those previously reviewed is that the tracts traversing the area of the lesion are destroyed, as are the perikarya of the nucleus in question. However, axon-spacing lesions can be induced by monosodium L-glutamate and its analogues (Olney and Price, 1978). Peter *et al.* (1980) found that intraperitoneal injection of glutamate in goldfish causes a marked hypertrophy and edema (lasting about 2 days) of the NLT from the anterior margin of the pituitary stalk through to the posterior end of the nucleus; the edema was followed by necrosis of nearly all of the perikarya in the affected area, causing a major lesion in the NLT without disruption of pathways traversing through it. In addition, a smaller area of edema and necrosis occurred in the anteroventral preoptic region (the anteroventral NPP). Serum GtH levels were significantly increased for 2 days after glutamate injection; however, there were no significant differences at 5, 7, or 8 days postinjection, correlating with the periods of edema and necrosis, respectively. The increase in serum GtH levels during edema of the NLT presumably reflects an increase in GnRH release from the affected region; the return to normal levels may reflect basal release of GtH. Although there is no direct evidence in support of these interpretations, other data indirectly support this. In long-term experiments (31 days), glutamate caused a decrease in GSI on a dose-dependent basis, and, with the high dose of

glutamate used, there was also decreased pituitary and serum GtH levels (R. Peter and C. S. Nahorniak, unpublished results). This provides confirmation that the NLT is necessary for maintenance and stimulation of GtH secretion during gonadal development in goldfish.

Neurosecretory fibers directly invade the pars distalis in teleosts (for review, see Holmes and Ball, 1974; Peter and Crim, 1979). The proximal pars distalis, where GtH cells are located, receives innervation from two anatomically distinct types of neurosecretory fibers: (1) type-A peptidergic-like fingers thought to originate mainly from the NPO and (2) type-B aminergiclike fibers thought to originate mainly from the NLT (e.g., goby, *Gillichtys mirabilis*, Zambrano, 1970a,b, 1971; roach, *Leuciscus rutilus*, Ekengren, 1973; Båge *et al.*, 1974; Ekengren *et al.*, 1978; black molly, *Poecilia latipinna*, Peute *et al.*, 1976; Atlantic salmon and rainbow trout, Terlou and Ekengren, 1979). In goldfish the GtH cells are directly innervated by type-B endings, whereas type-A endings and other type-B endings are in close proximity (Leatherland, 1972; Kaul and Vollrath, 1974). Olivier Kah, R. Peter, and H. Cook (unpublished results) found that type-B fibers in the proximal pars distalis underwent degeneration following glutamate injection of goldfish; type-B fibers in the rostral pars distalis and all type-A fibers remained intact. These results indicate that the NLT in the pituitary stalk and posterior to it are the probable origins of the type-B fibers in the proximal pars distalis of goldfish. This provides confirming support for the results of lesioning studies on goldfish, which identified this same part of the NLT as being involved in regulation of gonadal activity, and strongly supports the idea that, in the goldfish, GnRH from the NLT is involved in regulation of GtH secretion. However, direct evidence for the presence of GnRH in perikarya of the NLT of goldfish is lacking, although LHRH immunoreactive perikarya have been identified in the NLT of platyfish (Schreibman *et al.*, 1979). In this regard, it is interesting to note that the functional evidence does not support a role of the preoptic region in regulation of GnRH secretion, although immunocytochemical data would strongly implicate it in such a role.

C. Actions

1. Gonadotropin Secretion

Early studies by Breton *et al.* (1972) indicated that carp and rainbow trout HE could stimulate LH release from sheep pituitaries *in vitro*. As indicated previously, results by King and Millar (1980) suggest that teleost, specifically tilapia, GnRH has about the same activity, on a weight-specific basis, as mammalian GnRH (LHRH) in stimulating LH release from sheep

pituitary cells *in vitro*. However, the relative potency of teleost GnRH compared to LHRH in mammalian systems, and the activity of LHRH compared to teleost GnRH in teleost systems, cannot be definitively determined until pure synthetic teleost GnRH is available.

It is well established that synthetic LHRH (Fig. 1) can stimulate GtH release from teleost pituitaries. Breton and Weil (1973) provided the first direct evidence; intravenous injection of LHRH in carp, *C. carpio*, caused a sharp increase in plasma GtH levels within 2–6 min postinjection. With a low dose of LHRH (250 ng/kg), an initial peak in plasma GtH levels occurred, followed by return to near normal levels within about 10 min; however, increased levels were sustained for at least 25 min in fish given the high dose (1 μg/kg). Two injections of LHRH (3 μg/kg) 3 hr apart caused an increase in plasma GtH lasting at least 12 hr (Weil *et al.*, 1980). Weil *et al.* (1975) found that the greatest responsiveness to LHRH during the reproductive cycle of carp was from the spring spawning season through the summer, and that the minimal responsiveness was in the winter when the fish were sexually inactive. However, Weil *et al.* (1980) were not successful at inducing ovulation in carp by injection of LHRH.

Treatment of sexually immature Atlantic salmon (Crim and Peter, 1978) or rainbow trout (Crim and Evans, 1979; Crim *et al.*, 1981b) with estrogenic steroids; or aromatizable androgenic steroids, causes *de novo* accumulation of GtH in the pituitary. Luteinizing hormone releasing hormone causes a dose-dependent release of GtH from the *in vitro* pituitary of steroid-treated immature rainbow trout; however, the pituitary from untreated fish does not release GtH in response to LHRH (Crim and Evans, 1980). In terms of the *in vivo* response to LHRH, Weil *et al.* (1978) found that sexually mature (prespawning) rainbow trout were more responsive to LHRH than fish at other stages of gonadal development. A single injection of LHRH caused a dose-dependent increase in GtH levels, persisting for at least 6 hr, in sexually mature (spermiating) male brown trout, *Salmo trutta* (Crim and Cluett, 1974). Crim *et al.* (1981a) demonstrated that synthetic analogues of LHRH that block LH release in mammals (inhibitory LHRH analogues, *i*-LRHa) also block GtH release induced *in vivo* by LHRH in mature male brown trout; however, superactive analogues of LHRH were not more active than LHRH in stimulating plasma GtH levels. Using the pituitaries from steroid-treated immature rainbow trout *in vitro*, Crim *et al.* (1981a) found that the only superactive LHRH analogue that was somewhat more active in stimulating GtH release than LHRH was des-Gly^{10} [D-Ala^{6}] LHRH ethylamide (LRHa, Fig. 1); *i*-LRHa was also effective *in vitro*.

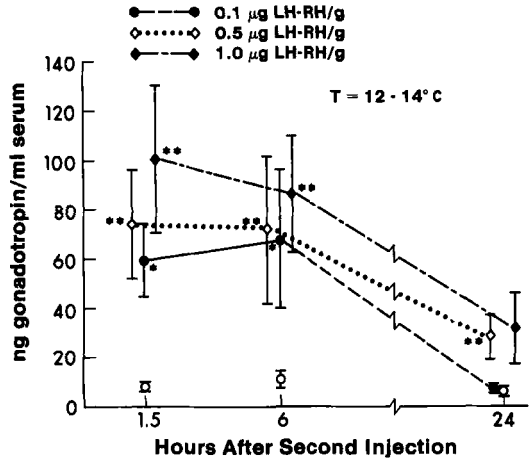
Although these studies indicate that LHRH and superactive analogues of LHRH are effective in stimulating GtH release in salmonids, they provide no clear evidence for distinction between the responses to the two. Howev-

er, in coho salmon, *Oncorhynchus kisutch*, a single injection of LRHa caused an increase in plasma GtH lasting at least 96 hr, whereas LHRH caused an increase lasting for only 24 hr (G. Van Der Kraak, H.-R. Lin, E. M. Donaldson, H. M. Dye, and G. A. Hunter, personal communication). The coho salmon were also much more sensitive to LRHa than to LHRH; 20 μg LRHa/kg caused the same magnitude increase in plasma GtH levels as 200 μg LHRH/kg. Although this requires testing in other salmonid species, these results clearly indicate that LRHa has a much more prolonged action than LHRH. However, there may be major species differences in the responsiveness to LHRH, LRHa, and other superactive analogues of LHRH.

Peter (1980) studied the effects of various dosages and injection combinations of LHRH and LRHa on serum GtH levels in goldfish held at 12°C. Two injections 12 hr apart of both LHRH and LRHa were much more effective than either a single injection or three injections 24 hr apart. Also, the results indicated that when two injections of a high dose of LRHa were given, the response was less than two injections of a lower dose (Fig. 5A). Therefore, in goldfish, the *in vivo* GtH-release response to LHRH and LRHa can be highly potentiated by a previous injection, but if the dosage of the previous injection is too high, there can be suppression or downgrading of the response. In this study there were no significant differences between the LHRH and LRHa-treated fish in terms of the peak levels of serum GtH induced, but LRHa did cause a more prolonged GtH-release response (Fig. 5A,B).

Lin *et al.* (1983) have extended the studies on the actions of LRHa on GtH release in goldfish. Goldfish have a marked seasonal variation in the GtH-release response to LRHa; the release response of male goldfish was greatest just prior to the spawning season in later winter, and in the spring in the early part of the spawning season. Late in the spawning season, the release response was less, even though the fish were still sexually mature. Sexually regressed fish in the summer had little or no release response. Temperature also influenced the responsiveness to LRHa. At a warm temperature (20°C), a short interval (3 hr) between a pair of injections of LRHa in recrudescing females induced higher serum GtH levels than a longer injection interval (9 hr). At a cold temperature (12–14°C), the low dose of LRHa (0.01 μg/g body weight) tested was as effective as the higher dosage (0.1 μg/g body weight) when the pair of injections were given with a 3 hr interval; however, there was no response to the low dose with a 9-hr injection interval. Although the results indicate that the shorter injection interval is advantageous at both high and low temperatures, it is apparent from these preliminary studies that there is no simple relationship between the level of serum GtH induced, the dosage of LRHa injected, the interval between injections, and temperature.

(A)



(B)

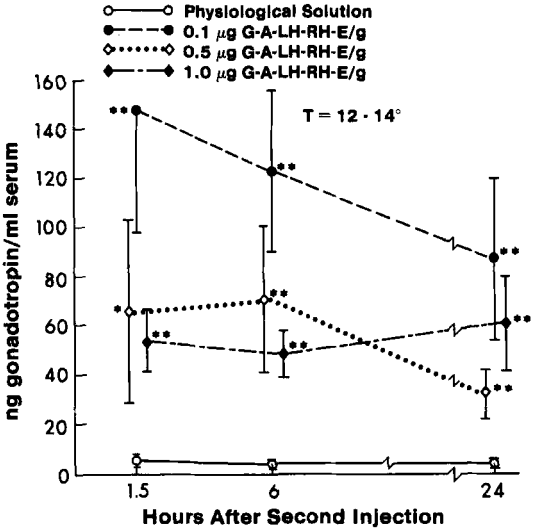


Fig. 5a,b. Serum gonadotropin levels (mean ± SE, $N = 6$ or 7) of male goldfish following two intraperitoneal injections 12 hr apart of (A) LHRH or (B) LRH-A (G-A-LH-RH-E). Significant differences from controls at the same sample time were determined by the U test (*, $p < 0.05$; **, $p < 0.01$). (From Peter, 1980. Reproduced from *Can. J. Zool.* **58**, 1100–1104, by permission.).

In carp, LRH-A has been demonstrated to stimulate GtH release *in vitro* (Fish Reproductive Physiology Research Group and Peptide Hormone Group, 1978), and has been used extensively in the People's Republic of China to induce ovulation in several species of domestic carp (see Section II,C,2). Pan *et al.* (1981) found that one injection of LRHa 10 min prior to an

injection of ^{125}I-labeled LRH-A ([^{125}I]LRHa) caused a greater uptake of ^{125}I into the pituitary gland of the mud carp, *Cirrhrinus molitorella*. This increased uptake of ^{125}I, presumably attributable to a more rapid uptake of [^{125}I]LRHa, probably reflects potentiation caused by the prior injection of unlabeled LRHa. In goldfish held at 12°C, administration of a potentiating dose of LRHa 12 hr earlier (based on results of Peter, 1980) caused a greater initial uptake of [^{125}I]LRHa into the pituitary at 20–30 min postinjection, followed by a more rapid and greater depletion of label at 24 hr (O. Bres, R. E. Peter, H.-R. Lin, and C. S. Nahorniak, unpublished results). This suggests that potentiation induces a greater uptake of GnRH, and that this is followed by a more rapid turnover of GnRH in the pituitary. Using ultrastructural autoradiography of pituitaries from mud carp injected with cold LRHa followed by [^{125}I]LRHa, Pan *et al.* (1981) found that the label appeared over the cell membrane, Golgi body, secretory granules, mitochondria, and nucleus of GtH cells, suggesting that multiple receptor sites exist. In the experiments reviewed, the greater uptake of [^{125}I]LRHa in the potentiated fish, is presumably attributable to an increase in receptors for GnRH. An increase in membrane LHRH receptors in the pituitary of the rat has been demonstrated during phases of the estrous cycle when the pituitary is more responsive to LH-RH, and following prior injection(s) of potentiating dosages of a superactive analogue (Clayton *et al.*, 1980; Loumaye and Catt, 1982); such changes in membrane receptors for peptide or polypeptide hormones are known from other systems as well (Catt *et al.*, 1979; Posner *et al.*, 1981). Internalization and incorporation of peptide and polypeptide hormones into secretory granules and subsequent release from the target cell, and binding of ligand to various cell organelles or structures is also known from other systems, and may be part of the normal actions of these hormones (Posner *et al.*, 1981). However, in general, relatively little is known about the dynamics of turnover or metabolism of the internalized hormone–receptor complex. Perhaps future studies on the dynamics of labeled GnRH in the pituitary of teleosts can contribute to the knowledge in this field.

2. Gonadal Responses, Including Ovulation

Given that LHRH and its superactive analogues can stimulate GtH secretion in teleosts, one may assume that administration of these synthetic compounds should provide a means of stimulation of gonadal activity by endogenous GtH. Indeed, responses have been found in the gonads; however, as indicated in several instances, the protocol for repetition of published results is not always clear, and in many cases very large dosages were used.

Chan (1977) induced ovarian development of the Japanese medaka, *Oryzias latipes*, from a sexually regressed to yolky oocyte condition by injec-

tions twice a week of large dosages of LHRH (0.1 μg and 1μg LHRH/g), increases in GSI also occured. Lin *et al.* (1983) found that 10 daily injections of LRHa at both low (0.01 μg/g) and high (1 μg/g) dosages caused a significant increase in GSI of sexually regressed female goldfish. Serum GtH levels were higher than in controls at all sampling times. In an experiment of similar design done with recrudescing fish, the high dose of LRHa tended to cause a decrease in GSI. However, there was no effect of the low dose; serum GtH levels were low relative to those found in the experiment starting with sexually regressed fish. On the basis of these results, although it may be possible to stimulate gonadal development by treatment with LHRH or its analogues, unexpected effects such as inhibition of gonadal activity can also occur. The mechanism for such inhibition is not known, but it could be the result of a direct effect on the gonads, similar to the situation in mammals. In the rat, it has been shown that there are receptors for LHRH and its agonistic analogues in the gonads (Harwood *et al.*, 1980; Pieper *et al.*, 1981) and that inhibition of various gonadal activities such as cyclic-AMP production (Harood *et al.*, 1980; Knecht and Catt, 1982), steroidogenesis (Hsueh and Erickson, 1979; Harwood *et al.*, 1980; Hsueh *et al.*, 1980), and induction of LH receptors (Harwood *et al.*, 1980; Hsueh *et al.*, 1980) can occur.

Ovulation has been induced in several teleost species by injection of LHRH or LRHa. Hirose and Ishida (1974) reported that a single injection of large doses of LHRH, approximately 2, 4, and 8 μg LHRH/g, were effective in inducing ovulation in 40%, 50%, and 83%, respectively, of mature ayu, *Plecoglossus altivelis*. Daily injections for 5 days of LHRH at 1, 2, or 10 μg/g were effective in inducing ovulation (≥80%) in goldfish (Lam *et al.*, 1975, 1976). Large dosages of LHRH (values not reported) were apparently necessary to induce ovulation of cultured carp (Cooperative Team for Hormonal Application in Pisciculture, 1977). Saline was the vehicle used in all of the aforementioned studies, but Aida *et al.* (1978) reported that a single injection of a relatively low dosage of LHRH (approximately 1 or 1.75 μg/g) in Freund's adjuvant was highly effective in inducing ovulation in plaice, *Limanda yokohamae*, and goby, *Acanthogobius flavimanus*, suggesting that a more prolonged release may be advantageous. However, the role that vehicle may play in the response to LHRH has not been systematically investigated.

Donaldson *et al.* (1981/1982a, 1982) have compared the relative effectiveness of LHRH, LRHa, and another superactive analogue, [D-Ser(Bu^t)^6]-LHRH ethylamide, in inducing ovulation in coho salmon. When a primer of partially purified salmon GtH was injected followed by single or multiple injections of LHRH or analogue, no differences in effectiveness of the compounds were evident. However, a single injection of LRHa or LHRH was effective in accelerating the time of ovulation, although the analogue was

more effective, requiring much less material and accelerating the time of ovulation more. The LRHa caused a prolonged increase in plasma GtH levels. Because there is a fairly gradual increase in blood GtH levels in rainbow trout prior to ovulation (Billard *et al.*, 1978; Jalabert and Breton, 1980), it may be that a relatively modest, but prolonged, increase in blood levels of GtH induced by LRHa is sufficient to induce ovulation in all salmonids.

Low dosages of the LRHa have been effective in inducing ovulation of cultured grass carp, *Ctenopharyngodon idellus*, black carp, *Mylopharyngodon picus*, silver carp, *Hypophthalmichthys molitrix*, and spotted silver carp, *Aristichthys noblis* (Cooperative Team for Hormonal Application in Pisciculture, 1977; Fukien-Kiangsu-Chekiang-Shanghai Cooperative Group, 1977; Jiang *et al.*, 1980). For grass carp a single injection ranging from 1 to 100μg/kg (0.001–0.1 μg/g) induced ovulation in 12–22 hr postinjection; two injections 7–10 hr apart reduced the time to ovulation following the last injection to 3–14 hr. Silver carp that had not previously been induced to ovulate were highly responsive to a single injection, usually ranging from 2 to 20 μg/kg, but "experienced" fish required two injections of a total dosage of 10 μg/kg. Black carp were the least responsive, requiring two or three injections of LRHa ranging from dosages of 1.5 to 400 μg/kg, and injection of pituitary extract in some cases.

On the basis of the foregoing information, LRHa is apparently much more effective in inducing ovulation in carp. However, the time to ovulation was highly variable (3–27 hr) within the carp species used in these studies in China. Other investigators have not been successful with LRHa (Weil *et al.*, 1980), although the dosages used in the study by Weil *et al.* were quite low. The environmental conditions under which the ovulation tests with LRHa were conducted in China are not clear. Stacey *et al.* (1979a,b) found that environmental conditions are very important for inducing ovulation in goldfish, and perhaps the successful induction of ovulation with LRHa in cyprinids may be dependent in part on environmental conditions, as suggested by Peter (1982). In addition, because at least some teleosts have been shown to have a seasonal variation in responsiveness to GnRH, selection of fish at the appropriate stage of gonadal development may also be important (Peter, 1982). Unfortunately, the information necessary to answer these questions is not yet available.

III. GONADOTROPIN RELEASE-INHIBITORY FACTOR

A. Evidence for Presence

In contrast to the studies in which small lesions in the NLT caused gonadal regression and alteration of the daily cycle of serum GtH levels in

goldfish (see above), Peter *et al.* (1978) found that large lesions in the NLT of mature female goldfish caused a dramatic increase in serum GtH levels at 2 days postlesioning; GtH levels continued to be elevated for at least 12 days, although the levels had decreased to near normal by this time. Nearly all of the lesioned females ovulated, although the fish were held at 12°C in running water without vegetation as a spawning substrate, conditions inappropriate for spontaneous ovulation (see Stacey *et al.*, 1979a,b); no ovulations occurred in control fish, or in fish lesioned in the dorsal telencephalon. The results were interpreted to indicate the presence of a GtH release-inhibitory factor (GRIF), and that abolition of GRIF allowed spontaneous release of GtH.

In subsequent studies, Peter and Paulencu (1980) found that it was not lesions of the NLT that caused the rise in serum GtH levels, but rather damage to the pituitary stalk. On the basis of a series of lesioning experiments in which lesions were placed in a variety of locations in the diencephalon, it was determined that the probable origin of GRIF was the anteroventral preoptic region (anteroventral NPP; Fig. 6). In addition, mature males gave the same response to preoptic or pituitary stalk lesions as mature females, indicating that GRIF is present in male as well as female goldfish. In mature male or female goldfish in which the pituitary stalk has been completely destroyed, effectively blocking transfer of neurohormones to the pituitary, there is a prolonged high rate of GtH release. Peter and Paulencu (1980) suggested, on the basis of these findings, that the normal ovulatory surge of GtH in goldfish could result from abatement of GRIF and spontaneous release of GtH, without the action of GnRH.

Gonadotropin release-inhibitory factor is of importance in goldfish throughout the reproductive cycle, but is relatively less important in sexually regressed females (Peter *et al.*, 1983). In a preliminary report, Peter *et al.* (1983) indicated that sexually regressed females have little or no increase in serum GtH levels following a preoptic lesion to destroy the GRIF area, but females undergoing ovarian recrudescence have an increase similar in magnitude and duration as the mature females. However, this may reflect changes in pituitary GtH content because regressed female goldfish have less pituitary GtH than recrudescing or mature females (Cook and Peter, 1980; R. Peter and C. S. Nahorniak, unpublished results), and therefore the regressed females may have less GtH to release spontaneously. Sexually regressed male goldfish release about as much GtH as a mature male following a preoptic lesion; however, there are no measurements available of the pituitary GtH levels in regressed males for comparison.

Peter *et al.* (1983) reported that transplantation of the pars distalis of a sexually mature female into another mature female either beside the brain (juxta implant) or into the third ventricle in the preoptic region results in a marked elevation in serum GtH levels of the recipient fish at 2 and 4 days,

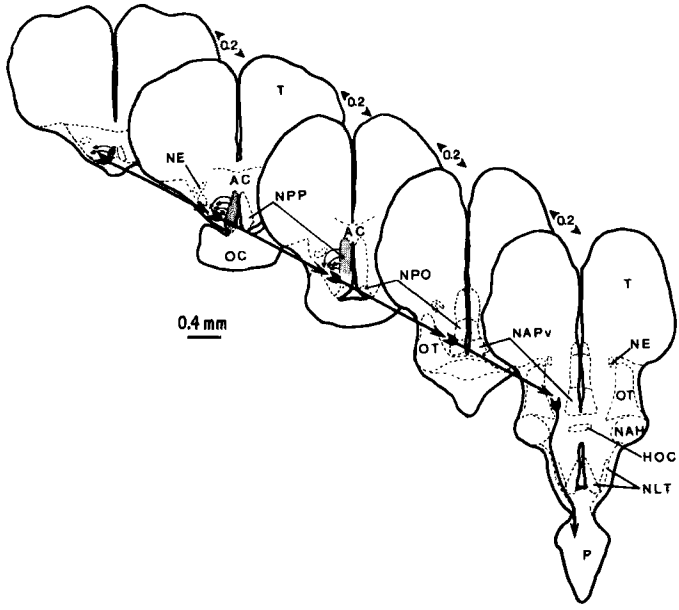


Fig. 6. Diagrammatic summary showing the proposed origin in goldfish of gonadotropin release-inhibitory factor in the anteroventral preoptic region (shaded area) and the pathway of the factor (arrows) in the lateral preoptic region, lateral anterior hypothalamic region, and the pituitary stalk. Distances between the cross-sections given in millimeters above the drawings. AC, anterior commissure; HOC, horizontal commissure; NAH, nucleus anterioris hypothalami; NAPv, nucleus antrioris periventricularis; NE, nucleus entopedunclaris; NLT, nucleus lateral tuberis; NPO, nucleus preopticus; NPP, nucleus preopticus periventricularis; OC, optic chiasma; OT, optic tract; P, pituitary; T, telencephalon. (From Peter and Paulencu, 1980. Reproduced from *Neuroendocrinology* **31**, 133–141, by permission of S. Karger AG, Basel).

respectively, postimplantation. Notably, the recipient fish with the juxta pars distalis implants had higher serum GtH levels than the fish given the preoptic implants. Similar results were obtained in experiments with mature and immature male goldfish and with immature female goldfish. These results indicate that there is some brain factor that suppresses the spontaneous release of GtH from the transplanted pars distalis, providing further support for the presence of GRIF in goldfish. Although these results suggest that GRIF is ubiquitous in the brain, because the transplants were bathed by cerebrospinal fluid, it is likely that GRIF reached the transplants by this route.

B. Dopamine as a Gonadotropin Release-Inhibitory Factor

In a preliminary report, Crim (1981) reported that some catecholamines inhibited GtH release from cultured pituitaries of rainbow trout. More spe-

cifically, dopamine not only inhibited the *in vitro* basal or spontaneous release of GtH, but it also diminished the release reponse to LHRH.

Chang *et al.* (1983) have determined the effects on serum GtH levels in goldfish of various drugs which alter synthesis of catecholamine neurotransmitters or the activity of aminergic neurons. 6-Hydroxydopamine, a catecholaminergic neurotoxin, caused an increase in serum GtH levels, suggesting that catecholaminergic neurons inhibit GtH release. Blocking synthesis of dihydroxyphenylalanine (L-DOPA), the precursor of dopamine, by treatment with α-methyl-*p*-tyrosine, and blocking conversion of L-DOPA to dopamine by treatment with carbidopa both caused an increase in serum GtH levels. However, treatment with diethyldithiocarbamate (DDC), which blocks conversion of dopamine to norepinephrine, had no effect on serum GtH; moreover, clonidine, an α-noradrenergic agonist, caused an increase in serum GtH. These results suggest that dopamine serves as an inhibitor of GtH release, and, because carbidopa does not cross the blood–brain barrier and the pituitary is outside the blood–brain barrier, it is likely that dopamine acts directly on the pituitary. Also, the results suggest that norepinephrine has a stimulatory effect on GtH release, but the level of action is unknown.

A direct inhibitory action of dopamine on GtH cells is supported by evidence cited in a preliminary report by Peter *et al.* (1983). The elevated serum GtH levels in preoptic lesioned goldfish were significantly reduced by treatment with dopamine or apomorphine, a dopamine agonist. In addition, apomorphine blocked or significantly reduced, depending on dosage, the stimulatory effects of LRHa on serum GtH levels. Therefore, although the results for rainbow trout are available from only one set of *in vitro* studies, dopamine clearly has GRIF activity in both goldfish and trout. Whether other factors also have GRIF activity is not known.

IV. INPUT OF ENVIRONMENTAL FACTORS

A. Photoperiod: The Role of the Pineal and Eyes

Photoperiod is an important environmental cue for regulation of the reproductive cycles of many teleosts (for review, see de Vlaming, 1974; Htun-Han, 1977; Peter and Crim, 1979; Peter, 1981). The pineal and/or eyes are the presumed route of input of photoperiod information (for review, see Peter, 1981).

In spring-spawning cyprinids and cyprinodonts undergoing the latter stages of gonadal recrudescence, the pineal and perhaps also the eyes have a progonadal effect when the fish are exposed to long photoperiod conditions.

Pinealectomy caused gonadal regression in recrudescing or mature goldfish (Fenwick, 1970b; de Vlaming and Vodicnik, 1978; Vodicnik *et al.*, 1978) and shiners, *N. crysoleucas* (de Vlaming, 1975), acclimated to long photoperiods and warm or cold temperatures. The effects of blinding were not investigated by these researchers. However, blinding and/or pinealectomy both caused gonadal regression in medaka, *O. latipes*, acclimated to a long photoperiod and warm temperature (Urasaki, 1972b, 1973, 1976). Therefore, the pineal and eyes of the cyprinodont, *O. latipes*, at least, are involved in the stimulatory effects of long photoperiods on gonadal activity.

In spring-spawning cyprinids and cyprinodonts, short photoperiods in late winter or spring cause onset of regression or block further gonadal development in fish during the latter stages of gonadal recrudescence. Pinealectomy of goldfish (Fenwick, 1970b; de Vlaming and Vodicnik, 1978), shiners (de Vlaming, 1975), and medaka (Urasaki, 1973, 1976) under these conditions causes stimulation of gonadal development. This indicates that the pineal has an antigonadal influence under these conditions.

Contrary to goldfish undergoing gonadal recrudescence, pinealectomy of sexually regressed goldfish, held under a variety of environmental conditions, had no effects on gonadal activity (Peter, 1968; Fenwick, 1970b; Vodicnik *et al.*, 1978).

Vodicnik *et al.* (1978) reported that pinealectomy of recrudescing female goldfish held on a long photoperiod and warm temperature for 22 days caused a decrease in plasma GtH levels at 4 hr but not at 10 hr after the onset of the photophase. These results are partly consistent with the significantly lower GSI in the pinealectomized fish. However, there was also a decrease in plasma GtH levels in pinealectomized goldfish held for 21 days on a short photoperiod and warm conditions, although the gonadal activity of the pinealectomized fish was apparently stimulated because 43% of the fish were postovulatory.

Hontela and Peter (1980a) investigated the effects of pinealectomy and blinding on serum GtH levels in female goldfish, and found that the effects of the operations were expressed, at least in part, by alterations in the daily cycle of serum GtH secretion. Pinealectomy and blinding both caused the daily cycle in serum GtH levels to disappear in fish held on a long photoperiod and warm temperature; a significant decrease in GSI also occurred in some experimental groups although the fish were killed at only 7–9 days after exposure to the environmental conditions, a relatively short time period for effects on GSI. Pinealectomy, but not blinding, of fish held on a short photoperiod and warm temperature caused a daily cycle in serum GtH levels; no effects on GSI were found. These results suggest that the progonadal effect of the pineal and eyes under long photoperiod conditions entails the promotion of a daily cycle in serum GtH levels; however, the antigonadal

effect of the pineal under short photoperiods entails the suppression of daily cycles in serum GtH levels. Although this is not consistent with the results of studies on goldfish found by Vodicnik *et al.* (1978), it is difficult to compare the two studies because Vodicnik *et al.* (1978) exposed the fish to warm conditions for 21 or 22 days, which in itself is enough to alter the gonadal condition and cycle of serum GtH levels in goldfish (Peter, 1981).

De Vlaming and Vodicnik (1977) found that pinealectomy of the shiner, *N. crysoleucas*, acclimated to a long photoperiod, caused the daily cycle in pituitary GtH, as measured by bioassay, to disappear; however, under a short photoperiod a daily cycle appeared. If the pituitary content of GtH somehow reflects the release of GtH into the blood, then these results fit the scheme suggested by Hontela and Peter (1980a) for goldfish. In addition, de Vlaming and Vodicnik (1977) found that HE from pinealectomized shiners held on a long photoperiod had less gonadotropin-releasing activity than in sham controls; however, there was greater activity in extract from pinealectomized shiners on a short photoperiod. These results, based on a double bioassay of GnRH activity (see foregoing discussion), suggest that the level of hypothalamic GnRH activity is influenced by input from the pineal.

Melatonin is a known pineal "hormone" in mammals, and, depending on the time of day of injection, it can have pro- or antigonadotropin effects in mammals (for review, see Reiter, 1980; Chen *et al.*, 1980). In teleosts, injection of melatonin for extended periods causes suppression of gonadal development, suggesting it is the antigonadal factor of the pineal (goldfish, Fenwick, 1970a; medaka, Urasaki, 1972a; killifish, *Fundulus similis*, de Vlaming *et al.*, 1974; Indian catfish, *Heteropneustes fossilis*, Sundararaj and Keshavanath, 1976; catfish, *Mystus tengara*, Saxena and Anand, 1977). De Vlaming *et al.* (1974) found no temporal variation in responsiveness of *F. similis* to melatonin injection; other investigations did not provide information in this regard. Further, most of these early studies were done before it was realized that the time of day of injection, length of photoperiod, and sexual condition might interplay in the response to the injections.

In a recent study on the three-spined stickleback, *Gasterosteus aculeatus* L., Borg and Ekström (1981) found that the antigonadal effect of daily melatonin injections occurred only with a high dosage (4 μg melatonin/g for 21 days) in fish in early gonadal recrudescence under long photoperiod conditions in the late autumn; injections at other times of year were ineffective in causing gonadal regression. In contrast, injection of a low dosage of melatonin (0.8 μg/g) caused acceleration of early stages of ovarian development under long photoperiod conditions, and stimulation of development in females with regressing oocytes held under short photoperiod conditions. This suggests that melatonin also has progonadal effects under certain conditions.

Medaka, *O. latipes,* have a circadian cycle of oocyte maturation such that ovulation occurs about 1 hr before the onset of the photophase and oviposition within 1 or 2 hr after the onset of the photophase (Egami, 1954; Iwamatsu, 1978a). Blinded females were capable of ovulation and oviposition (Egami and Nambu, 1961); however, the timing of these events was not recorded. Pinealectomized female medaka also were capable of ovulation and oviposition, but notably the 24-hr pinealectomized fish had some delay (about 45 min) in the timing of oviposition the following morning (Iwamatsu, 1978b). Melatonin injection 2 hr before the normal ovulation time did not alter the timing of ovulation (Iwamatsu, 1978b). Experiments were not conducted to determine if synchrony of ovulation and oviposition with photoperiod occurred in pinealectomized or blinded fish subjected to a shift in the timing of the photophase. Although the results suggest some slight effect of the pineal on the timing of oviposition, the data do not establish whether this is attributable to melatonin.

Plasma levels of melatonin vary on a daily basis in rainbow trout, with a reproducible and constant nocturnal rise (Gern *et al.*, 1978; Owens *et al.*, 1978). Assuming this also occurs in other teleosts, the time of day of administration of melatonin may be an important component of its action and should be thoroughly investigated. The retina in rainbow trout can synthesize melatonin (Gern and Ralph, 1979), and, considering the relative size of the pineal and retina in teleosts, the latter could be the major source of melatonin. Unfortunately, many investigations on pineal function have overlooked the possibility that the eyes may also be involved in the system under investigation. Finally, it is well established that the pineal in teleosts contains photoreceptors and that the pineal stalk contains nerve fibers that transmit action potentials to the rest of the brain reflecting the activity of the photoreceptors (Tamura and Hanyu, 1980). Unfortunately, none of the pinealectomy experiments to date have tested the possibility that the influences of the pineal on reproduction may be mediated by its neural input to the brain, rather than by its secretions.

B. Other Factors: Temperature and Feeding

Temperature and photoperiod affect the daily cycles of serum GtH in female goldfish (Hontela and Peter, 1978, 1980b). The secretion rate of GtH in goldfish, calculated from the metabolic clearance rate and the plasma GtH level, is also influenced by temperature (Cook and Peter, 1980); the secretion rate was approximately 6 and 2.5 times greater at 20°C versus 12°C in sexually regressed and recrudescing female goldfish, respectively. However, there is no information available to indicate the mechanism by which temperature infuences the secretion rate of GtH.

Time of feedings relative to the timing of the photophase influences the daily cycle of serum GtH in goldfish (Hontela and Peter, 1980b). How time of feeding influences GtH secretion is not known, but is presumably influences some aspect of the physiology of the animal, which in turn influences the temporal variations of the reproductive system (Peter, 1981).

V. INPUT OF PHYSIOLOGICAL FACTORS

A. Steroid Feedback

Steroid binding sites in the brain have been investigated by autoradiography in green sunfish, *Lepomis cyanellus* (Morrell *et al.*, 1975), paradise fish, *Macropodus opercularis* (Davis *et al.*, 1977), platyfish, and goldfish (Kim *et al.*, 1978a,b). In these species, labeled estradiol and testosterone were bound in a similar distribution in the brain to perikarya in the NLT, nucleus recessus lateralis, ventral NPO, ventral NPP, the area ventralis pars ventralis in the telencephalon, and in the pituitary. In goldfish, labeled estradiol was also bound in the nucleus posterioris periventricularis (NPPv) in the posterior hypothalamus and in the thalamic periventricular region dorsal to the NPPv.

A negative-feedback effect of sex steroids on GtH secretion was demonstrated by castration of male rainbow trout (Fig. 7; Billard *et al.*, 1976, 1977; Billard, 1978). A large, approximately fivefold, increase in plasma GtH was found following castration of mature trout at spawning time; however, there was only a slight increase in GtH following castration just prior to onset of testicular recrudescence, about a twofold increase in early stages of recrudescence, and a small increase following castration of fish in the latter stages of recrudescence. These results indicate that the negative-feedback effect is most prominent in mature fish at spawning time. The high plasma GtH levels following castration of spermiating trout were reduced by pituitary inplantation of 11-ketotestosterone (Billard, 1978), which confirms the negative-feedback effect.

Ovariectomy of rainbow trout near the end of vitellogenesis (end of ovarian recrudescence) caused an increase in plasma GtH levels (Bommelaer *et al.*, 1981). Ovariectomy of trout undergoing germinal vesicle migration (oocyte maturation) also caused increased plasma GtH levels, but examination of the changes in individual fish showed that about one-half of the fish had no change in plasma GtH levels; no significant effects were found in postovulatory females. Estrogen treatment of the ovariectomized trout was not effective in suppressing the plasma GtH levels in fish near the end of

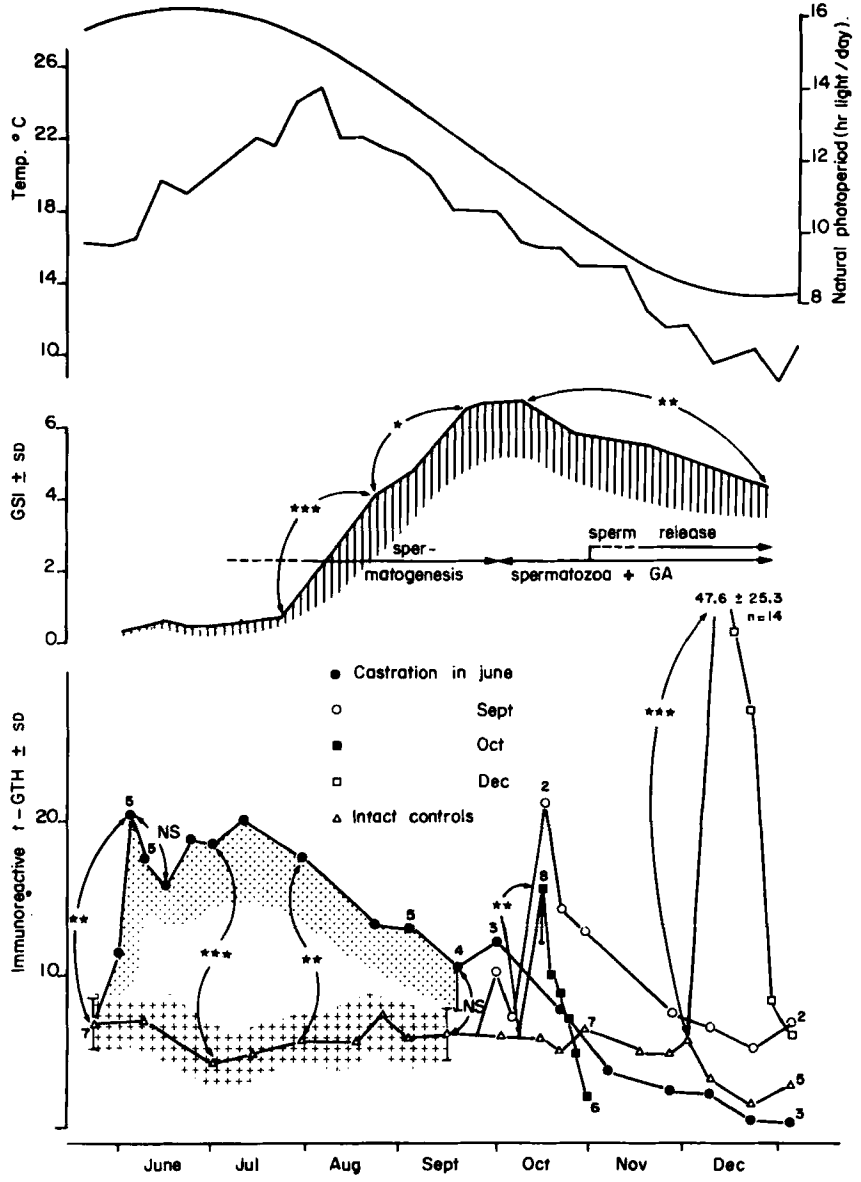


Fig. 7. Plasma gonadotropin levels (bottom graph) of control and castrated male rainbow trout. Castrations were done at four different times of year, and the fish were sampled for variable lengths of time postoperatively. The gonosomatic index and testicular condition are given in the middle graph. The data are given with standard deviations (shaded areas). Significant differences by analysis of variance and t tests, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. The changes in photoperiod and temperature during the study are shown in the top graph. (From Billard *et al.*, 1977. Reproduced from *Gen. Comp. Endocrinol.* **33**, 163–165, by permission.).

vitellogenesis; however, it was effective in females ovariectomized at the time of germinal vesicle migration. This suggests that the relative importance of estradiol in negative feedback changes during ovarian development. The finding that there was no negative-feedback effect in the postovulatory trout, although estradiol was effective in suppressing plasma levels of GtH is contradictory, and this contradiction cannot be explained.

As further evidence of a negative-feedback effects of sex steroids, it has been found that treatment with the antiestrogen clomiphene citrate caused ovulation in sexually mature goldfish (Pandey and Hoar, 1972; Pandey *et al.*, 1973), catfish *H. fossilis* (Singh and Singh, 1976), loach, *Misgurnus anguillicaudatus* (Ueda and Takahashi, 1976, 1977), and common carp in which ovulation had been blocked by indomethacin (Kapur and Toor, 1979). Further, treatment with the antiestrogen tamoxifen accelerated the time of ovulation of coho salmon, *Oncorhynchus kisutch,* primed by a previous injection of partially purified salmon GtH (Donaldson *et al.*, 1981/1982b).

Assuming that an antiestrogen would gradually and continuously reduce the effect of negative feedback, the resulting increase in blood GtH levels should also be gradual and steady. However, Breton *et al.* (1975a) found that in common carp there was a surge in plasma GtH levels of 8–20 hr duration occurring within 3 days after injection of clomiphene. This is inexplicable on the basis of the idea that the results are only a result of changes in negative feedback, indicating that other factors such as GnRH and GRIF may also be changing as a result of the treatment.

Billard and Peter (1977) found that pellet implants of clomiphene citrate and tamoxifen (ICI46474) in the pituitary of goldfish were both highly effective in increasing serum GtH; the clomiphene implants also caused ovulation. Implants of pellets containing tamoxifen in the NLT caused an increase in serum GtH, but of lower magnitude than the pituitary implants, suggesting that the pituitary is the most important location for negative feedback.

In contrast to the strong negative feedback of sex steroids in teleosts at spawning time, sexually immature salmonids show a positive feedback effect to sex steroids. Implantation of testosterone pellets in the NLT and pituitary, but not in the NPO or optic tectum, in male and female Atlantic salmon parr caused a marked increase in the pituitary content of GtH (Crim and Peter, 1978). Crim and Peter suggested that this positive-feeding effect may be a part of the mechanism causing onset of puberty in the precocious male parr. This was supported by the finding that males with the pituitary and NLT testosterone implants had advanced spermatogenesis. Crim and Evans (1979) found that immature rainbow trout also have an increase in pituitary GtH from very low or nondetectable levels to high levels following systemic treatment with testosterone. More recently Crim *et al.* (1981b) found that the positive-feedback effect in immature trout could be induced by es-

trogenic steroids (e.g., estradiol, estrone, estriol) and androgenic steroids that can be aromatized to estrogens (e.g., testosterone, testosterone propionate, 17α-methyltestosterone, androstenedione); however, nonaromatizable androgens (e.g., 5α-dihydrotestosterone, 11-ketotestosterone, 11β-hydroxytestosterone) were ineffective. In support of the importance of aromatase in the action of androgens, the positive-feedback effect of testosterone was blocked by the aromatase inhibitor 1,4,6-androstatrien-3,17-dione.

Olivereau and Chambolle (1978) and Olivereau and Olivereau (1979a,b) found that estradiol treatment of immature male and female eels, *Anguilla anguilla,* caused a marked stimulation of the cytological activity of GtH cells. Perhaps a positive-feedback effect of sex steroids may be a general phenomenon in immature teleosts.

The distribution of aromatase in the brain of the marine sculpin, *Myoxocephalus octadecimspinosus,* has been investigated by Callard *et al.* (1981a). They reported that the highest aromatase activity (conversion of [^{3}H]androstenedione to estrone and estradiol) per unit weight of tissue was in the combined preoptic area–anterior hypothalamus, accounting for 40% of the total estrogen produced by the brain. This brain area, along with the posterior hypothalamus and the medial and lateral telencephalon, accounted for 85% of the total brain activity. The aromatase activity in the combined preoptic area–anterior hypothalamus also changed seasonally (Callard *et al.*, 1981a), although the significance of this is unknown. Further, the pituitary of this marine sculpin was shown to contain aromatase activity, contrary to what was thought to be the case on the basis of investigations of mammals (Callard *et al.*, 1981b). From these investigations it is clear that aromatase activity is present in the appropriate parts of the brain, and in the pituitary, for it to play a role in steroid positive feedback effects and the neuroendocrine regulation of GtH synthesis and release. Whether aromatization of certain androgens to estrogens is involved in negative-feedback effects is unknown; however, Billard (1978) found that the nonaromatizable steroid 11-kerotestosterone was active in negative feedback in rainbow trout, suggesting that aromatization is not necessary for a negative-feedback effect.

B. Other Factors

The postcastration increase in plasma GtH in rainbow trout was partially inhibited by injection with seminal fluid that had previously been centrifuged so as to be free of spermatozoa and adsorbed with activated charcoal (Breton and Billard, 1980). A protein extract of the testis did not have the same acitvity. These results suggest that there is some factor in the seminal fluid that can inhibit GtH secretion in trout.

Prostaglandin (PG) E_1, PGE_2 and $PGF_2\alpha$ were injected into the third ventricle of the brain in goldfish, and the effects on serum GtH were determined (Peter and Billard, 1976). The highest dosages of PGE_2 and $PGF_2\alpha$ suppressed serum GtH levels, suggesting that PG's may have some inhibitory effects on GtH release. With simultaneous intraventricular injection of $PGF_2\alpha$ and LHRH in goldfish, there is an increase in serum GtH levels similar to LHRH alone (R. Peter, unpublished results). This indicates that the inhibitory effects of PG's are not at the level of the pituitary, and are probably not part of the GRIF mechanism.

VI. FUNCTIONAL NEUROENDOCRINOLOGY

A. Regulation of Onset of Puberty

The positive-feedback effect of estrogens, stimulating synthesis and accumulation of GtH in the pituitary, is probably an important part of the regulation of onset of puberty. In brown trout, *Salmo trutta*, Billard *et al.* (1978) reported that there was an increase in plasma levels of estradiol at the onset of spermatogenesis, and that the pituitary GtH level increased at the same time. Although a similar increase in plasma estradiol was found in male rainbow trout, there was no coincident increase in pituitary GtH (Billard *et al.*, 1978). Perhaps more intensive investigations of the hormonal changes around the time of puberty may clarify the situation.

Crim and Evans (1980) have shown that once GtH has accumulated in the pituitary as a result of the positive-feedback effect of steroids, its release can be stimulated *in vitro* by GnRH. However, *in vivo* experiments indicate that there is little or no response to GnRH in sexually regressed carp (Weil *et al.*, 1975), goldfish (Lin *et al.*, 1983), or rainbow trout (Weil *et al.*, 1978). Also, GRIF has relatively little effect in sexually regressed goldfish (Peter *et al.*, 1983), which does not explain the lack of response to GnRH.

Perhaps the initial step in the onset of puberty is some stimulation of GnRH release attributable to environmental input. This could in turn cause GtH release, which stimulates steroid release from the gonad, causing the positive-feedback effect on GtH levels in the pituitary. Following these initial steps, inertia would be inherent in the system, causing further development.

3. Regulation of Gonadal Recrudescence

Blood levels of GtH are low during gonadal recrudescence, relative to the levels found during ovulation and spawning (for review, see Peter, 1981).

Nevertheless, the daily cycles in blood levels of GtH in goldfish (Hontela and Peter, 1978, 1980a,b) and rainbow trout (Zohar, 1980) require precise neurohormonal regulation of GtH secretion, presumably because of the interactions of GnRH, GRIF, steroid negative feedback, and other potential factors such as temperature. Because there is inadequate information to evaluate the relative importance of these factors in any one species, it is necessary to speculate that the main factors regulating GtH secretion are GRIF and GnRH.

C. Regulation of Ovulation

The ovulatory surge of GtH in goldfish is synchronized with the photoperiod, and is primarily cued by the presence of a spawning substrate (Stacey *et al.*, 1979a,b). Carp have a similar ovulatory surge, although there is only limited information available on its timing and duration (Jiang *et al.*, 1980). Rainbow trout, in contrast, have only a relatively small, gradual increase in plasma GtH preceding ovulation, and a prolonged postovulatory rise in plasma GtH (Billard *et al.*, 1978; Jalabert and Breton, 1980). Because the patterns of GtH secretion at the time of ovulation are so different in cyprinids and salmonids, it may also be that the neuroendocrine regulation of GtH is also different.

Peter and Paulencu (1980) suggested that the ovulatory surge in goldfish could be induced by abatement of GRIF, allowing a surge of spontaneous release of GtH. However, a marked increase in blood GtH and ovulation in cyprinids can be induced by LHRH or LRHa (see Section II,C). In addition, treatment of sexually mature female goldfish with glutamate to induce a major lesion in the NLT blocks spontaneous ovulation, but not ovulation induced by lesioning the preoptic region to destroy GRIF (R. Peter and C. S. Nahorniak, unpublished results). Together these data indicate that the ovulatory surge in a cyprinid, such as the goldfish, entails abatement of GRIF and the action by GnRH, to provide for the combination of spontaneous and stimulated release of GtH.

In salmonids, the small prolonged increase in blood GtH leading to ovulation could be attributed to a gradual increase in GtH secretion caused by stimulation by GnRH. This would fit with the changes in plasma levels in GtH leading to ovulation following injection of LRHa in coho salmon (Donaldson *et al.*, 1981/1982a, 1983; G. Van der Kraak, H.-R. Lin, E. M. Donaldson, H. M. Dye, and G. A. Hunter, personal communication). The role that GRIF might play in this system is unknown; there is some *in vitro* evidence for GRIF activity by dopamine on rainbow trout pituitaries (Crim, 1982), but no *in vivo* evidence. In addition, plasma levels of estradiol de-

crease prior to oocyte maturation and ovulation in rainbow trout (Fostier *et al.*, 1978), providing the possibility for a decrease in the negative-feedback effect resulting in an increase in GtH release.

D. Regulation of Spermiation

Spermiating salmonids have relatively high blood levels of GtH compared to earlier phases of the reproductive cycle (for review, see Billard *et*

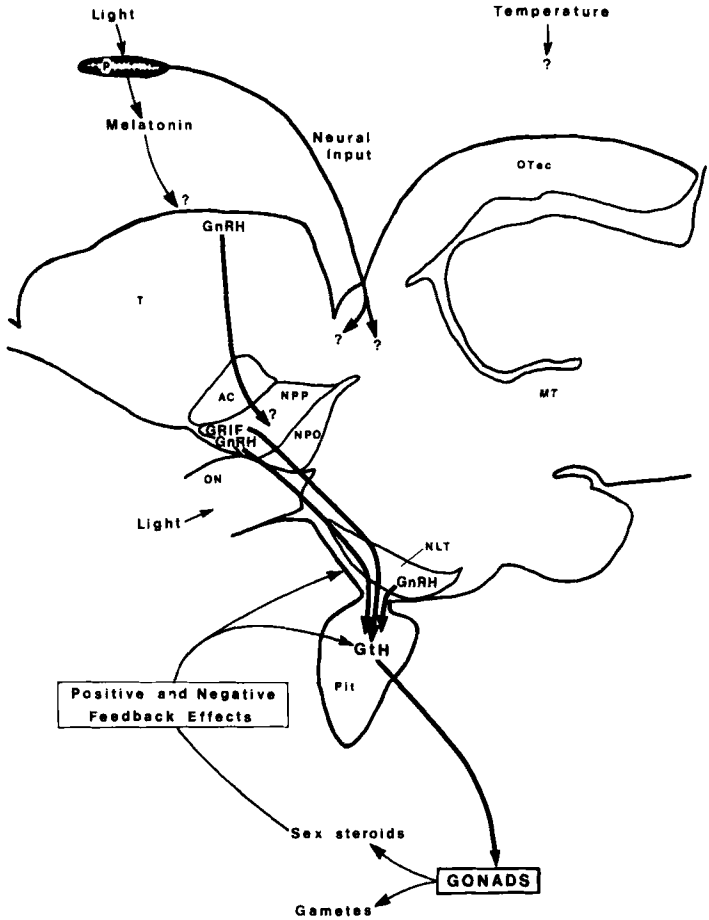


Fig. 8. A summary diagram of the neuroendocrine regulation of gonadotropin in teleosts. AC, anterior commissure; GnRH, gonadotropin releasing hormone; GRIF, gonadotropin release-inhibitory factor; GtH, gonadotropin; MT, midbrain tegmentum; NLT, nucleus lateral tuberis; NPO, nucleus preopticus; NPP, nucleus preopticus periventricularis; ON, optic nerve; OTec, optic tectum; P, pineal; Pit, pituitary gland.

al., 1978; Peter, 1981). Male goldfish (Kyle *et al.*, 1979) and carp (Fish Reproductive Physiology Group and Peptide Hormone Group, 1978) have an increase in blood GtH levels during participation in spawning; this coincides with an increase in the expressable sperm volume in goldfish (Kyle *et al.*, 1979). Although these changes in blood GtH are not as dramatic as those found in the females of these species, various evidence indicates that the same neurohormones are present and have similar actions in both sexes of a given species.

VII. CONCLUSION

Figure 8 presents a summary diagram of the neuroendocrine regulation of GtH in teleosts. This diagram does not purport to show all the neuroendocrine regulatory mechanisms, or their relative importance, but rather serves to illustrate important elements in the system.

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4

THE CELLULAR ORIGIN OF PITUITARY GONADOTROPINS IN TELEOSTS

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I. INTRODUCTION

Teleosts are no exception to the rule that in vertebrates gonadal functions are developed and maintained by gonadotropic hormones produced by the pituitary gland. In accordance with this general concept, the gonadotropic activity of the teleost pituitary is regulated by gonadal hormones and by neurohormones, mainly of hypothalamic origin. Therefore, in teleosts, as in almost all other vertebrates, the cells producing gonadotropic hormone have an important position in the chain of structures that serves to ensure reproduction and thereby survival of the species.

II. STRUCTURE OF THE PITUITARY

In general, the pituitary consists of the neurohypophysis and the adenohypophysis (Fig. 1). In teleosts, the neurohypophysis is derived from the floor of the infundibulum, immediately rostral of the saccus infundibuli,

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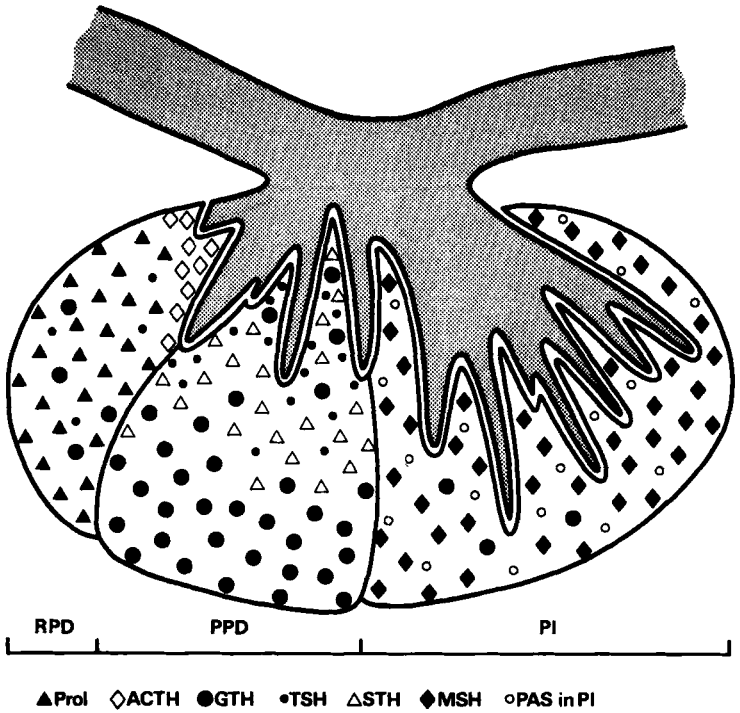


Fig. 1. Schematic representation of a sagittal section of a teleost pituitary showing the distribution of the functional cell types in the adenohypophysis (ACTH, corticotropic cell; GTH, gonadotropic cell; MSH, melanotropic cell; PAS in PI, periodic acids Schiff-positive cell in pars intermedia; PPD, proximal pars distalis; Prol, prolactin producing cell; RPD, rostral pars distalis; STH, somatotropic cell; TSH, thyrotropic cell; stippled area, neurohypophysis).

which develops into the saccus vasculosus. During embryonic development, bundles of neurosecretory nerve fibres enter the floor of the infundibulum, which becomes a neurohypophysis, consisting of somewhat irregular processes of nervous tissue penetrating the surrounding adenohypophysis. The adenohypophysis develops from a placode in the roof of the ectodermal stomodaeum, underlying the floor of the infundibulum. In primitive teleosts, such as *Elops saurus* and *Chanos chanos*, this placode has a lumen similar to Rathke's pouch of most other groups of vertebrates (Wingstrand, 1966). However, in virtually all other teleosts the placode is a solid structure, and, therefore, the adenohypophysis of most species of teleosts does not contain a lumen. Exceptions occur in the pituitaries of salmonids, clupeids, and apodes, where the rostral portion of the adenohypophysis is composed of follicles. These follicles arise from a schizocoel, formed in the rostral part of the placode during its development into an adenohypophysis.

Although the pituitaries of relatively few of the more than 20,000 species of teleosts have been studied, a wide variation in shape and composition of the gland has been observed. However, it is not the differences, but rather the similarities in structure that are most evident. There are two main types of pituitaries, the platybasic (e.g., found in eels), and the leptobasic (e.g., found in adult salmonids). In the platybasic pituitary, the neurohypophysis consists of the flat floor of the caudal infundibulum which sends nerve processes into the disc-shaped adenohypophysis. This adenohypophysis is generally divided into three parts, i.e.,the rostral pars distalis (RPD), the proximal pars distalis (PPD) and the pars intermedia (PI), situated one behind the other. Bundles of neurosecretory axons penetrate all three parts of the adenohypophysis, but those in the PI are coarser and more numerous than those in the RPD and the PPD. In the second type of teleost pituitary, the leptobasic, the neurohypophysis has a fairly well-developed infundibular stalk and the adenohypophysis is globular or egg shaped with its three parts situated in a rostrocaudal or in a dorsoventral position. There are many intermediates between the two types; in young teleosts the developing pituitary usually is of the platybasic type, but this may change into the leptobasic form within the lifetime of individual fish.

The main reason for dividing the adenohypophysis into three parts is the uneven distribution of the morphological, hormone-producing cell types. In light microscopy, the morphological criteria for distinguishing the various cell types are the size and shape of the cells and their nuclei and especially the stainability of their secretory granules. It has become common practice to stain sections of Bouin or Bouin–Hollande fixed pituitaries with Herlant's (1960) Alcian blue (AB)–periodic acid Schiff (PAS)–orange G (OG). Cells, traditionally labeled basophils, contain glycoproteins. These react with PAS, and therefore the basophils stain reddish purple. That color may be somewhat suppressed by the blue of AB which at low pH reacts with strongly acid groups; acid groups are present in some of the basophils after oxidation (cf. Val-Sella *et al.*, 1978).

The traditional acidophils contain neither glycoproteins nor substances with strongly acid groups. Therefore, they do not react with PAS and AB, but do stain with OG. Differentiation between two types of acidophils is possible by carefully treating sections of pituitaries with a polychrome staining technique, which includes OG and erythrosin, such as Cleveland and Wolfe's (1932) trichrome method. If well applied, a portion of the acidophils stains yellowish orange with OG, and a portion stains red with erythrosin.

In teleosts, these two types of acidophils show such a characteristic distribution that this forms the basis for dividing the pars distalis into the RPD and the PPD. The erythrosinophilic cells comprise most of the RPD; and in salmonids, clupeids, and apodes they form the major component of

the follicles in the RPD. The orangeophilic cells are concentrated in the PPD, commonly in its dorsal zone, along the neurohypophysial processes. Bordering such processes in the RPD are faintly basophilic cells that characteristically stain brownish black with McConaill's (1947) lead hematoxylin (PbH). Large, strongly PAS-positive cells are often concentrated in a ventral rim of the PPD, and smaller, strongly AB-positive basophils have a tendency to accumulate in the rostrodorsal part of the PPD near the processes of the neurohypophysis. However, both types of basophils can have a wider distribution in the PPD, and may even be found in the RPD. The PI has a majority of usually PbH-positive and weakly acidophilic cells, but it also contains cells which tend to be weakly PAS positive.

Each of the cell types has its own characteristics when viewed by the electron microscope (EM). First, the basophils can be recognized by the fact that their secretory granules contain glycoproteins, which react with periodic acid in the Thiéry (1967) technique. Moreover, the contents of the secretory granules in the acidophils have a more pronounced electron density than in the basophils. A usually reliable characteristic by which acidophils and basophils can be recognized is the shape of the granular endoplasmic reticulum (GER). In acidophils, the GER consists of flattened parallel cisternae; in basophils, the GER consists of irregularly dilated cisternae. The sizes and shapes of the secretory granules are much less reliable characteristics. The usually round, sometimes oval granules range in diameter between 100 and 500 nm. In the lead hematoxylin-positive cells of the RPD and the AB-positive basophils of the PPD the granules tend to be relatively small; in the acidophils and in the strongly PAS-positive basophils of the ventral PPD they are usually larger. These basophils in the ventral PPD typically contain not only secretory granules but also globules with a diameter varying between 0.5 and 3.0 μm, and contents of relatively low-electron density. In electron micrographs so-called stellate cells may be observed in between the hormone-producing cells. Moreover, in most teleosts studied to date, neurosecretory axons piercing the laminae that separate the neurohypophysis from the adenohypophysis penetrate the parenchyma of the adenohypophysis and form synaptoid endings on the endocrine cells. These nerve fibers along with centrifugal capillaries, running in the perivascular space between the interdigitating neuro–enadenohypophysis are the essential means of transport of information from the central nervous system to the adenohypophysial cells.

Seven different morphological cell types can be recognized with both the light and electron microscope. It is generally accepted that each of these cell types secretes one specific hormone. Admittedly, this concept will not be proven until it has been possible to culture the cell types separately and to identify their secretory products under culture conditions. However, assum-

ing that the "one cell type–one hormone" concept is correct, attempts have been made to demonstrate the respective functions of the cell types by way of histophysiological research. In these studies natural or experimentally induced changes in target organs were compared with changes (1) in the size and shape of the pituitary cells and their nuclei, (2) in the storage of secretory granules, and (3) in the abundance of certain organelles such as the Golgi system, the GER, and the mitochondria. Moreover, recently, the double-antibody immunocytochemical technique has been applied for the identification of the functional cell types. However, in most cases, pure and homologous antigens are not available, and heterologous systems are never completely satisfactory, because they leave the possibility of cross reactions with some unknown component of the pituitary cells. Therefore, the results of immunocytochemical studies have limited value and can at best only verify histophysiological data.

Such histophysiological data indicate that, in the teleost pituitary, the PbH-positive cells of the RPD produce corticotropin (ACTH) and the PbH-positive cells of the PI produce melanotropin (MSH). The acidophils of the RPD are presumed to secrete prolactin (PRL), and those of the PPD secrete somatotropin (STH). The AB-positive basophils of the PPD have been identified as the source of thyrotropin (TSH), and the PAS-positive cells of the ventral PPD have been identified as the producers of gonadotropin (GTH). The function of the weakly PAS-positive cells in the PI is uncertain; they may be involved in the regulation of calcium metabolism (Olivereau *et al.*, 1980a,b, 1981), or in the regulation of background color adaptation (Ball and Batten, 1981), or reproduction (Schreibman *et al.*, 1982).

This brief description of the teleost pituitary provides a background for a discussion of the literature concerning the gonadotropic cells. The excellent chapter on the teleost pituitary by Ball and Baker in "Fish Physiology," Volume II (1969) forms a good starting point for that discussion. Other current reviews include the following: van Oordt (1968, 1979), Sage and Bern (1971), Schreibman *et al.* (1973), Holmes and Ball (1974), Fontaine and Olivereau (1975), Doerr-Schott (1976a), Follénius *et al.* (1978), van Oordt and Ekengren (1978), and Ball (1981). The following are the most important points considered in these reviews and in the reports of original experimental data:

1. Correlative changes in gonadal functions and the structure of pituitary basophils.

2. The identification of cells producing glycoprotein–gonadotropin by means of immunocytochemical techniques, sometimes applied to pituitaries of fish kept under various physiological conditions.

3. The number of gonadotropic cell types (some researchers have identified two different types, others only one).

4. The function of the cell organelles, more especially of the secretory granules and globules in gonadotropin secretion.

5. The innervation of the gonadotropic cells as a morphological basis for the central regulation of the gonadotropic activity of the pituitary.

These points are discussed further in this chapter.

III. THE GONADS AND PITUITARY BASOPHILS

Ball and Baker (1969) emphasize that "any pars distalis basophils that are quiescent or absent before sexual maturity, and which show pronounced secretory changes in correlation with the gonadal cycle, are gonadotrops." They demonstrate this in a detailed account of the changes in the basophils of the ventral PPD that coincide with changes in the ovarian condition of the green sailfin molly (the green form of *Poecilia latipinna*). Moreover, Ball and Baker refer to many published studies on other teleosts and conclude that gonadotropin is formed in PAS-positive cells, which are usually concentrated in a ventral rim of the PPD, but also have been found in the RPD, e.g., in the eel and salmonids. Very often the gonadotrops not only contain PAS- and AB-positive granules, but also much larger PAS- and AB-positive globules. The granules stain not only with PAS and AB, but also with aldehyde fuchsin (AF) after permanganic oxidation. As a rule, the granules in the thyrotropic basophils show the same staining characteristics. This makes it difficult to differentiate between the gonadotropic and the thyrotropic basophils on the basis of the stainability of their respective secretory granules.

Because of that difficulty, and because of the observations of Baker *et al.* (1974) that in the Indian catfish (*Heteropneustes fossilis*) the gonadotropic basophils outnumber the thyrotropic ones, Sundararaj and co-workers (Sundararaj and Sehgal, 1970; Viswanathan and Sundararaj, 1974a,b; Anand and Sundararaj, 1974, 1975) did not believe it was necessary to bother with the thyrotrops. They accepted the number and histological condition of all basophils in paraffin sections of the PPD (i.e., gonadotrops and thyrotrops) as sufficient for measuring the gonadotropic activity of the pituitary under natural and experimental circumstances. Likewise, Singh (1970) used the number and size of the basophils in the pituitary of the catfish, *Mystus vittatus*, as an indication of its gonadotropic potency.

However, as Ball and Baker (1969) noted, it is often possible to differentiate between the gonadotrops and the thyrotrops under light microscopy on the basis of their topographical separation. Thyrotrops may be concentrated in the RPD, as in the eel, or in the dorsal or rostrodorsal PPD, as in cyprinodonts and in some cyclids and mullets, respectively. This led Tsuneki and Ichikawa (1973) to label the ventral basophils in the PPD of *Chasmich-*

thys doligognathus as gonadotrops and more dorsally situated basophils as thyrotrops, without any histophysiological study.

More commonly, in recent publications on the light microscopical identification of gonadotropic cells in teleosts, researchers have followed the classical approach of studying pituitaries during the cyclic development of the gonads. Examples are the studies of Khana and Pant (1969) with *Glyptothorax pectionopterus;* of Javaid and Gul-i-Nargis (1974) with *Channa punctatus;* of Bisht (1975) with *Schizothorax richardsonii;* of Prakash and Paliwal (1976) with *Entropiichthys vacha;* of Itoda and Honma (1977) with the Japanese dace (*Leuciscus hakonensis*); of Chiba *et al.* (1978b) with the ironfish (*Carassius hybr.*); of Christoforov (1978) with the polar cod (*Boreogadus saida*); of Yoshi and Honma (1978) with the fresh water goby, *Rhinogobius brunneus;* and of Jafri and Ensor (1980) with the roach (*Rutilus rutilus*).

Prasado Rao (1972), Baker *et al.* (1974), and Haider (1978) have studied the pituitary of the Indian catfish (*Heteropneustes fossilis*) under various natural and experimental conditions in search of the differentiation between gonadotrops and thyrotrops at the light microscopical level. The researchers appear to agree that in the Indian catfish the gonadotrops are large basophils, and together with acidophilic cells they comprise most of the PPD. However, the topography of the thyrotrops appears to be a matter of some dispute. Prasado Rao (1972) localizes these cells in the ventral and latero-ventral areas of the PPD. Baker and co-authors (1974) find the thyrotrops in the centrally situated neurohypophysis and in the PI. Haider (1978) ascribes to the thyrotrops a tendency to migrate into all parts of the pituitary, including the stalk region. All three observations may to some degree be correct. Joy and Sathyanesan (1979) find thyrotrops in the ventral part of the PPD, the caudal neurohypophysis, and the PI of another catfish, *Clarias batrachus.* In this species the gonadotrops are large basophils, which together with acidophils form the major part of the PPD, especially during the spawning season.

Gonadotropic basophils have also been identified in the PPD by combined studies of changes that correlate with the gonadal cycle and with the development of the gonads. Recent examples of this combined approach are the work of Fiodorov (1971) with the Greenland halibut (*Reinhardtius hippoglossoides*); of van den Hurk (1974) with the black molly (the black form of *Mollienisia* (*Poecilia*) *latipinna*); and of Moiseyeva and Zolotnitsky (1978) with the Black sea turbot (*Scophthalmus maeoticus*). Similarly, Sage and Bromage (1970b), Honma and Yoshie (1974), Sasayama and Takahashi (1975), and Honma *et al.* (1976), studying the guppy (*Poecilia reticulata*), the salmonid *Plecoglossus altivelis, Tilapia mossambica* (= *Sarotherodon mossambicus*), and the three-spined stickleback (*Gasterosteus aculeatus*),

respectively, have described the differentiation and development into fully active gonadotrops of basophils in the ventral area of the PPD. In these species the thyrotrops take the usual, more dorsal position in the PPD.

However, in the blind Mexican cave fish (*Anoptichthys jordani*) Mattheij (1968, 1970) found that both the gonadotrops and the thyrotrops show a more or less random distribution in the PPD. The thyrotrops could be identified as relatively small, angular, strongly AB-positive cells that differentiate at an early stage simultaneously with the development of the thyroid follicles, and degranulate in goitrogen treated fish. The gonadotrops are larger, globular cells with secretory granules staining purple with AB–PAS. Their cytoplasm may contain numerous small vacuoles that give the cells a foamy appearance, especially in the spawning period. During the spawning period, degranulation and hypertrophy are more pronounced in gonadotrops bordering the processes of the neurohypophysis than in cells situated at some distance from these processes. The gonadotrops in the pituitaries of young cave fish do not store stainable material until, at a fairly late stage, the gonads develop. Such a situation has also been described for the sailfish *Istiophorus platypterus* by Chiba and Honma (1980).

According to Chiba and Honma (1973) the topographies of the gonadotrops and the thyrotrops are very unusual in the puffer *Fugu niphobles*. They noticed a differentiation of basophils with PAS- and AF-positive granules and globules in the RPD which was concominant with the development of the gonads. Further, the size and granulation of these cells followed the gonadal cycle. However, basophils in the ventral PPD with one type of PAS- and AF-positive granules did not change during the sexual cycle. Therefore, it was concluded that the basophils in the RPD were gonadotrops, and that those in the PPD are thyrotrops. However, in another puffer, *Fugu stictonotus*, Chiba and Honma (1974) found that basophils of the RPD secreted thyrotropin and those of the PPD secreted gonadotropin. The latter conclusion is based on the treatment of juvenile fish with thiouracil, and on following the cell types during gonadal maturation. Interestingly, in both species the gonadotrops were the cells which, apart from secretory granules, contained large, PAS-positive globules.

With regard to the differentiation of the gonadotrops, Kallman and Schreibman (1973; see also Kallman *et al.*, 1973; Schreibman and Kallman, 1977, 1978) discovered a sex-linked gene that controls sexual maturation in male platyfish (*Xiphophorus maculatus*). Males homozygous for early differentiation were observed to mature before males homozygous for late differentiation. Heterozygotes were intermediate. In all groups the differentiation of ventral basophils in the PPD coincided with that of the testes, indicating that these basophils are the source of pituitary gonadotropin. A similar observation was made by Chestnut (1970) and by Olivereau (1976) and Murza

(1978) in precocious coho salmon (*Oncorhynchus kitsutch*) and Atlantic salmon (*Salmo salar*), respectively. The researchers found large, granulated basophils in the pituitaries of these precocious males which were absent in juveniles of the same age.

Fiodorov (1971), Moiseyeva and Zolotnitsky (1978), and Chiba *et al.* (1979) described a hyperfunction of the gonadotropic basophils of the PPD in sterile specimens of the Greenland halibut (*Reinhardtius hippoglossoides*), in the Black sea turbot (*Scopthalmus maeoticus*), and in a hybrid of the carp (*Cyprinus carpio*) and the crucian carp (*Carassius auratus*), respectively. Similarly, in mature and nearly ripe sockeye salmon (*Oncorhynchus nerka*), McBride and van Overbeeke (1969) observed a rapid degranulation of the gonadotropic basophils in the PPD following gonadectomy. In contrast, the administration of sex steroids led to the appearance of many granulated PAS-positive gonadotrops, both in the sockeye salmon (van Overbeeke and McBride, 1971) and in the guppy (*Poecilia reticulata;* Sage and Bromage, 1970a). However, van den Hurk and Testerink (1975), found that the gonadotropic cells in the ventral PPD of adult male black mollies [*Mollienisia (Poecilia) latipinna*] showed a marked regression and partial or complete loss of basophilic material when the fish were treated with methyltestosterone.

Several researchers have suppressed gonadotropic activity by treating fish with the dithiocarbamoylhydrazine derivative methallibure (I.C.I. 33, 828), and have thereafter studied paraffin sections of the gonads and pituitaries with the light microscope. They invariably found a decrease in cell size and a loss of secretory granules in the gondadotrops of the PPD, coinciding with a marked regression in gonadal functions. Such results were obtained by Leatherland (1969) in *Cymatogaster aggregata*, by Pandey and Leatherland (1970) in the guppy (*Poecilia reticulata*), by Mackay (1971) in *Plectroplitus ambiguus*, by Dadzie (1972) in several *Tilapia* species, by Hyder (1972) and Chiba *et al.* (1978a) in *Tilapia mossambica (Sarotherodon mossambicus)*, by van den Hurk and van de Kant (1975) in juvenile black mollies *[Mollienisia (Poecilia) latipinna]*, by van den Hurk and Testerink (1975) in adult male black mollies, and by van Ree (1976) in the zebrafish (*Brachydanio rerio*). However, the disadvantage of methallibure is that it not only causes regression of the gonadotrops, but also of other cell types, including thyrotrops and somatotrops, in the adenohypophysis, and therefore cannot safely be used for the identification of the gonadotrops.

In general, the major disadvantage of the histophysiological approach to the identification of functional cell types in the adenohypophysis is that changes in one morphological cell type, brought about by natural or experimental alterations in target organs such as the gonads, seldom appear alone. Further, when in the course of an experiment two or more morphological

cell types show simultaneous reactions, there is very often some doubt concerning their functional significance. Therefore, it is no wonder that soon after the introduction of immunocytochemical methods, students of the teleost pituitary resorted to this approach to identify the functional cell types of the adenohypophysis.

IV. IMMUNOCYTOCHEMICAL IDENTIFICATION OF GONADOTROPS

The first to use immunocytochemistry for identifying gonadotropic cells in the pituitary of a teleost were McKeown and van Overbeeke (1971), who treated frozen sections of sockeye salmon (*Oncorhynchus nerka*) pituitaries with anti-ovine-luteinizing hormone (LH) and anti-ovine-follicle stimulating hormone (FSH). Fluoresceine-labeled anti-LH produced fluorescence in basophils of the rostroventral PPD, but not in other basophils of the pars distalis. Anti-FSH showed no reaction at all. Further, a labeled antibody against ovine thyroid stimulating hormone (TSH), which is chemically related to the two gonadotropins, failed to produce fluorescence in any cells of the pituitary gland. Similarly, in paraffin sections of fixed carp (*Cyprinus carpio*) pituitaries, Billard *et al.* (1971) found fluorescence in basophils of the PPD after applying the double-antibody fluorescence technique with anti-ovine-LH as first antibody, and they found no reaction at all with anti-bovine-TSH. However, in the carp, contrary to the sockeye salmon, all basophils in the PPD without exception reacted to anti-ovine-LH; those forming a ventral rim of relatively large cells as well as smaller, polygonal cells among the acidophils in the central and dorsal PPD. All basophils in the PPD also reacted with anti-carp-GTH and anti-carp-TSH, but only the small, polygonal cells reacted with anti-carp-TSH when that antibody had been saturated with anti-carp-GTH. This indicates that, in the carp, the central and dorsal basophils of the PPD produce thyrotropin, and the more ventral basophils produce gonatotropin.

Very similar results were obtained by Follénius and Dubois (1975) in the goldfish (*Carassius auratus*) and the stickleback (*Gasterosteus aculeatus*), where these two cell types become fluorescent after application of anti-ovine-LH. Further, Peute *et al.* (1982a,b) could distinguish between large and small basophils in the PPD of the African catfish (*Clarias lazera*) by applying the double-antibody immunoenzyme and immunoferritin techniques to electron microscope sections of fixed pituitaries. Both cell types reacted with anti-carp-GTH as first antibody. However, an antibody to the β-subunit of carp-GTH only caused an immunocytochemical reaction in the secretory granules (diameter, 100–1000 nm) and in large, often fusing

globules (2 μm in cross section) of large basophils. The smaller (diameter, 60–160 nm) and larger (diameter, 160–480 nm) granules of the small basophils were the only ones to react with anti-human-TSH.

The question remains, does this suffice for identifying the gonadotropic and the thyrotropic cells in the teleost pituitary. Goos and van Oordt (1975) and Goos *et al.* (1976) have disputed the evidence. Using anti-carp-GTH, they applied the indirect-immunofluorescence technique to sections of 12 different teleost species, belonging to the family of the Poeciliidae, the Characidae, and the Cyprinidae. In the representatives of the Characidae and the Cyprinidae, their specific antibody reacted with constituents of all cell types. It was only in the Poeciliidae, i.e., the black molly [*Mollienisia (Poecilia) latipinna*], the guppy (*Poecilia reticulata*), and the swordtail (*Xiphophorus helleri*) that fluorescence showed a discrete distribution, and was restricted to two types of basophils of the PPD: larger ones forming a ventral rim, and smaller ones among the acidophils of the central and dorsal PPD. This means that when heterologous antibodies are used, one must be aware of unspecific reactions and of immunoreactive determinants which are present in the molecules of chemically related hormones.

In a heterologous system much depends on the first antibody. This is illustrated by the results of Ekengren *et al.* (1978a), who applied the double-immunofluorescent method to sections of fixed roach (*Rutilus rutilus*) pituitaries. When anti-carp-GTH was used as the first antibody, basophils in the PPD containing large secretory granules and globules (diameter, 500–2000 nm) reacted more strongly than basophils containing small granules (diameter, 500 nm). When anti-salmon-GTH was used the opposite results were obtained. Presumed thyrotrops of the RPD reacted to both antibodies. In another example, Peute *et al.* (1982a,b) and J. Peute (unpublished results), working with the African catfish (*Clarias lazera*), found that three cell types cross-reacted with anti-carp-GTH. These were large, strongly PAS-positive cells with granules and globules, 100–1000 nm and up to 2 μm wide, respectively, and small mainly AB-positive basophils with granules measuring 60–480 nm in diameter, both situated in the PPD, and some erythrosinophilic cells in the PI. Anti-salmon-GTH and anti-carp-β-GTH reacted only with constituents of the globular basophils, and anti-human-TSH exclusively stained the small basophils of the PPD. Obviously, the antiserum to carp-GTH contained more, and less specific antigen determinants than did the other antisera.

Other important factors in immunocytochemical studies are the techniques of processing the pituitaries and the dilution of the first antibody used in the double-immunocytochemical technique. This has been demonstrated by Leunissen *et al.* (1980, 1982) in their work with rainbow trout (*Salmo gairdneri*) pituitaries, which were fixed in glutaraldehyde and parafor-

maldehyde for the electron microscope. In plastic-embedded material, at high concentrations an anti-carp-GTH and an anti-salmon-GTH reacted indiscriminately with constituents of acidophils as well as basophils; at lower concentrations anti-carp-GTH and anti-salmon-GTH did not react at all. In cryoultramicrotome sections of similarly fixed material, dilutions of 1:8000 of the two antibodies also caused a diffuse reaction in most pituitary cells, but higher dilutions of anti-carp-GTH and anti-salmon-GTH exclusively labeled the contents of granules and globules in presumed gonadotrops and the granules in presumed thyrotrops. An antibody against the β-subunit of carp GTH at a dilution between 1:1000 and 1:4000, selectively reacted with the hormone-storing vesicles of the presumed gonadotrops. Further, according to van Putten *et al.* (1982, 1983), in the pituitary of the rainbow trout granules of the presumed thyrotrops selectively bind anti-human-β-TSH.

The aforementioned examples indicate that immunocytochemical techniques, when conducted with great care, show binding of antibody to constituents of one or at least a limited number of cell types. It is correct to say that such constituents must be immunochemically related to the original antigen. However, if, for example, the antigen is a fish gonadotropin, the substance reacting with its antibody may not necessarily be either fish gonadotropin or the chemically related thyrotropin. Gielen *et al.* (1982c) found that in the rainbow trout, prolactin cells selectively react with an antiserum to glucagon, but they did not interpret that as an indication of the presence of glucagon in those prolactin cells. Hormone producing cells in the pituitary contain many substances, most of which are unidentified. Therefore, it will remain necessary to combine immunocytochemical studies with histophysiological research, even when the immunocytochemical techniques are perfected, for example, by introducing monoclonal antibodies to absolutely pure hormones. Fortunately, researchers studying gonadotropin-producing cells in the teleost pituitary have been aware of this.

McKeown and van Overbeeke (1971) compared their results with those of McBride and van Overbeeke (1969) who studied the effects of gonadectomy on the pituitary of the sockeye salmon (*Oncorhynchus nerka*). Further, Peute and de Bruyn (1976) and Peute *et al.* (1976) treated male black mollies [*Mollienisia* (*Poecilia*) *latipinna*] with methyltestosterone. This led to a decrease in the number of secretory granules filled with an electron-dense substance, and a loss of immunoreactivity to anti-carp-GTH in the basophils forming the ventral rim of the PPD. For a related species, the platyfish (*Xiphophorus maculatus*), Margolis-Kazan *et al.* (1979, 1981), Schreibman and Margolis-Kazan (1979), and Margolis-Kazan and Schreibman (1981) used immunocytochemical techniques to verify the results of earlier studies concerning the differentiation of pituitary basophils along with gonad development (Kallman and Schreibman, 1973; Kallman *et al.*, 1973; Schreibman and

Kallman, 1977, 1978). Both at the light and the electron microscopical level, antibodies to trout-GTH and to carp-GTH labeled the thyrotrops and the gonadotrops. However, anti-carp-β-GTH exhibited immunoreactivity with the gonadotrops only, and anti-human-TSH reacted with the thyrotrops only.

In the zebrafish (*Brachydanio rerio*) the results of immunocytochemical studies support histophysiological data. Lambert and van Oordt (1974) demonstrated the hypertrophy of basophils in the ventral PPD during nuptial behavior in the female zebrafish, and believed that this indicated gonadotropin secretion by these cells. Accordingly, G. E. Fåhraeus-van Ree (unpublished results) observed a decrease in immunofluorescence in the same type of basophils of zebrafish pituitaries cultured in a medium containing LH-RH. Van den Hurk *et al.* (1982b) noted a similar disappearance of material reacting with anti-carp-GTH in the basophils of the PPD of male zebrafish showing prespawning agonistic and courtship behavior.

Using anti-carp-GTH as first antibody, Ekengren *et al.* (1978b,c) compared changes in basophils with secretory granules (diameter, 230 nm) and globules (diameter, 500–600nm) during the nuptial period of the Atlantic salmon (*Salmo salar*) with the results of a double-immunocytochemical reaction. Both approaches indicated that the globular basophils are the source of gonadotropin. This was confirmed by Lindahl (1980) who used anti-salmon-GTH. In the rainbow trout (*Salmo gairdneri*), seasonal changes in PPD basophils (characterized by granules of 100–300 nm diameter, globules of 400–800 nm diameter, and irregular cisternae of the GER) corroborate the positive reaction of these cells to anti-carp-GTH and anti-salmon-GTH. These results by Peute *et al.* (1978) are in agreement with those obtained by van den Hurk *et al.* (1982a), and van den Hurk (1982), who demonstrated an immunofluorescence reaction with anti-salmon-GTH and anti-carp-GTH in the PPD basophils of 45- to 100-day-old rainbow trout. Unlike the ventral basophils, those located between the acidophils in the central and dorsal PPD also reacted with anti-human-β-TSH. Accordingly, the basophils between the acidophils in the central and dorsal PPD developed earlier, together with the thyroid follicles, and the ventral basophils developed later, along with the gonads. Moreover, the ventral basophils could be stimulated by androgen treatment. Therefore, the central and dorsal basophils of the PPD of juvenile rainbow trout are considered to produce thyrotropin, and the ventral ones produce gonadotropin. In this respect, it is of interest that, in the hands of van Putten *et al.* (1982, 1983), a goitrogen treatment of immature rainbow trout led to degranulation and vacuolization of basophils containing small granules of 160 nm diameter. The small cells reacted selectively with anti-human-β-TSH. The experimental fish were somewhat older than those used by van den Hurk and co-workers, and concomitantly showed

a different distribution of the thyrotrops; the thyrotrops were no longer in the central and dorsal PPD, but were found in the RPD and the rostrodorsal PPD.

In combination with histophysiological results, the outcome of immunocytochemical studies allows for the identification of gonadotropin-producing cells in teleost pituitaries. However, immunocytochemical techniques have a marked restriction; that is, they can only be used to ascertain the cellular source of gonadotropins that cross-react with the antibodies that are applied. To date, with respect to teleost gonadotropins, with one exception that is discussed later, the only antibodies that have been used are those raised against carp (*Cyprinus carpio*) gonadotropin and its subunits (Burzawa-Gérard, 1971, 1974; see also Burzawa-Gérard and Kerdelhué, 1978), antibodies raised against the Sephadex G-100 fraction of Pacific salmon (*Oncorhynchus tschawytscha*) gonadotropin (Donaldson *et al.*, 1972), and antibodies against rainbow trout (*Salmo gairdneri*) gonadotropin (Breton *et al.*, 1976). These gonadotropins are mucoprotein hormones, considered by some researchers to maintain all gonadal functions in the carp, the Pacific salmon, and the rainbow trout, respectively. However, as explained by Idler and Ng (chapter 5, this volume), other researchers consider this mucoprotein gonadotropin as representative of only one of two different gonadotropins in female teleosts, this gonadotropin being particularly involved in the production of ovarian hormones, in maturation of the oocytes, and in ovulation. This means that the pituitaries of a few or possibly numerous teleost species may produce two different gonadotropins.

In fact, long before two separate fractions with gonadotropic potency were extracted from teleost pituitaries, the existence of two different teleost gonadotropins was postulated. The main basis for this hypothesis was the belief that the production of two gonadotropic hormones would be a common feature of all gnathostome pituitaries. Further, it was supposed that each of these gonadotropins would originate in a separate cell type. Therefore, from the beginning of interest in the histology of the teleost pituitary, attempts have been made to identify two types of gonadotropic cells. It is obvious that two different types of gonadotrops cannot be distinguished with certainty by immunocytochemistry as long as antibodies against only one type of mucoprotein gonadotropin are available. Indeed, the main approach to the problem of one or two types of gonadotrops in the teleost pituitary has to date been that of histophysiological research.

V. ONE OR TWO TYPES OF GONADOTROPS

When a histologist concludes that the adenohypophysis of some fish contains two different types of gonadotropin-producing cells, it is (or should

be) because among the basophils two morphologically different types with each showing changes in size, in granulation, and in vacuolization correlating with certain specific aspects of the gonadal cycle can be discerned. In presenting results, histologists usually describe clearly the two types of gonadotropic cells and the changes in these cell types during gametogenesis and the production of gonadal hormones.

As Ball and Baker (1969) emphasized, M. Olivereau is foremost among the advocates of two types of gonadotrops in the teleost pituitary. She has given an accurate light microscopical description of two types of gonadotropic cells, observed in the pituitaries of various species, including eel (*Anguilla anguilla*), mullet (*Mugil auratus, Mugil cephalus*), goldfish (*Carassius auratus*), and Pacific salmon (*Oncorhynchus spec.*). One of these types has granules that are not only PAS positive, but also stainable with AB, AF, and especially PbH. This cell type strongly degranulates and vacuolates during spawning. The other type of cell has PAS-positive, but AB-, AF-, and PbH-negative granules.

In contrast, Ball and Baker (1969), giving a detailed light microscopical description of the gonadotrops in the green sailfin molly (*Poecilia latipinna*), found that all of these cells have secretory granules that stain with PAS and AB and globules that are PAS positive and AB negative. Further, it appeared that none of the basophils had granules or globules staining with PbH. Most gonadotropic cells were seen to undergo changes in size, granulation, and vacuolization during the ovarian cycle. Therefore, as Ball and Baker conclude, the behavior and the staining properties of the gonadotrops make it impossible to say that *Poecilia latipinna* possesses two distinct types of gonadotrops.

In other words, what is found in one species may not be observed in another. This makes it impossible to reach a firm conclusion regarding the number of gonadotropic cell types. Since 1969, the situation has not changed; on the contrary, in 1981, there were few species about which histologists could agree regarding the number of gonadotropic cell types.

For the roach [*Leuciscus (Rutilis) rutilus*], Olivereau (1969) described two types of gonadotrops, both located in the PPD of the pituitary, on the basis of the stainability of their secretory granules. In the same species, Båge *et al.* (1974) and Ekengren *et al.* (1978a) also recognized two types of gonadotrops in electron micrographs of the PPD. One type consisted of large cells, containing granules and globules (diameter 500–2000 nm) and cisternae of an often dilated GER. The other type was smaller cells, containing granules and globules (never exceeding 500 nm in diameter) and small cisternae of the GER. The contents of both cell types cross-reacted with anti-carp-GTH, but only those of the first type reacted with anti-salmon-GTH. Itoda and Honma (1977), studying the pituitary of a related species, the Japanese dace (*Leuciscus hakonensis*), also found two types of basophils in

the PPD, but concluded, from a comparison of the cyclical changes in these cells with those in the thyroid and the gonads, that the larger basophils are gonadotrops and the smaller ones are thyrotrops. Possibly, the anti-carp-glycoprotein GTH in the experiment of Ekengren and co-workers reacted with thyrotropin as well as gonadotropin, and the anti-salmon-glycoprotein GTH reacted with gonadotropin only. At present, this appears to be more likely than the assumption that the pituitary of the roach produced two different glycoprotein gonadotropins.

Light microscopical studies of the pituitary of the perch (*Perca fluviatilis macedonica*) revealed two types of gonadotrops (Dimovska, 1970, 1977); one type in a ventral position in the PPD has coarse granules, which stain deeper with PAS, AB, and AF than the fine granules of the other, dispersed among acidophils in the central and dorsal PPD. Changes in the former correlate with vitellogenesis in the oocytes, and both cell types show secretory activity during the nuptial period. Malo-Michèle (1978) described two types of gonadotrops for the PPD of *Boops salpa,* with the same distribution and the same staining reactions of their granules as the two types of basophils in the perch. Ventral gonadotrops developed together with the gonads. More dorsally located gonadotrops became more prominent at maturity. Both cell types changed during the spawning season. Likewise, Simon and Reinboth (1974) could distinguish between dorsal and ventral gonadotrops in the PPD of the sunperch (*Lepomis cyanellis*); the dorsal gonadotrops reacted to castration and the ventral gonadotrops reacted to an increase in daily photoperiod. Further, O and Chan (1974) described stronger and weaker PAS-, AB-, and AF-positive gonadotrops in the PPD of the ricefield eel (*Monopterus albus*). However, the results of Mattheij (1970) and of Chiba and Honma (1980) with the Mexical cave fish (*Anoptichthys jordani*) and the sailfish (*Istiophorus platypterus*), respectively, lead to a different interpretation; that is, dorsal and ventral gonadotrops belong to one type. In the Mexican cave fish these cells develop simultaneously with the gonads and become hyperactive toward the spawning period. In both species, gonadotrops located near protrusions of the neurohypophysis tend to degranulate and vacuolate more markedly than others. Moreover, Abraham (1974) could not corroborate the older observations of Olivereau, of Leray, and of others (see review by Ball and Baker, 1969) that the pituitary of the mullet (*Mugil cephalus*) contains two types of gonadotrops. Both at the light and the electron microscopical level the cells displayed a vast array of cytological differences, and it was not possible to decide whether these were related to differences in secretory activity or to differences in secretory product.

With regard to the salmonids, it is appropriate to refer first to the light microscopical work of Olivereau (1976, 1977, 1978) with the Atlantic salmon (*Salmo salar*) and two species of trout, *Salmo gairdneri* and *Salmo fario.* In salmon, captured from the sea and possessing a low GSI, the PPD contains

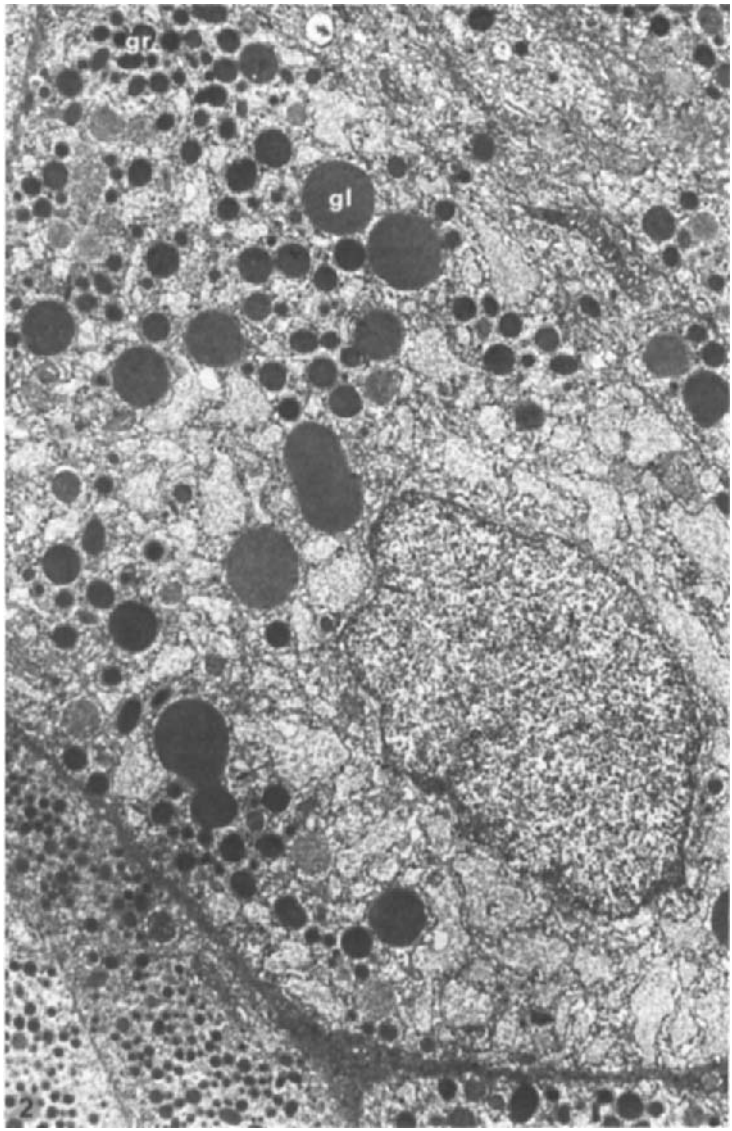


Fig. 2. Electron micrograph of a typical globular gonadotropic cell in the pituitary of an adult, sexually mature rainbow trout (*Salmo gairdneri*) with granules (gr), globules (gl), and cisternae (c) of the GER. The arrow indicates coalescing globules. Fixation was in 4% glutaraldehyde in 0.1 *M* cacodylic buffer, pH 7.3, at 0°C. Postfixation was in 2% OsO_4 in 0.2 *M* cacodylic buffer, pH 7.3, at 0°C. Dehydration was in graded ethanol and propyleneoxide. Material was embedded in Epon (Ladd) and stained with uranylacetate and lead citrate (magnification × 13,300). The technical assistance of M. G. A. de Mol Moncourt-de Bruyn and L. W. van Veenendaal is acknowledged.

numerous active cells with few glycoprotein granules. These cells show a progressive vacuolization and contain a few acidophilic granules when the fish enter the spawning grounds. In sexually mature salmon, glycoprotidic cells predominate in the rostral PPD. These are maximally developed shortly before spawning, and slowly degranulate during that period. In mature trout, Olivereau also found two gonadotropic cell types. One type which stained with PAS, AB, and AF, was abundant in the PPD and could also be found among the follicles of the RPD. Its contents showed a cross-reaction with anti-HCG, with anti-bovine-TSH, and, according to Olivereau and Nagahama (1982), also with anti-salmon-GTH. The other type did not react with these antibodies, was almost completely PAS-, AB-, and AF-negative, and was mainly restricted to the periphery of the dorsal glandular strands of the PPD. Cells of this latter type predominated during vitellogenesis and appeared active mainly in spring. They showed hyperplasia and hypertrophy in 3-year-old females with immature gonads.

In their immunocytochemical and electron microscopical studies of adult Atlantic salmon, Ekengren *et al.* (1978b,c) recognized only Olivereau's glycoprotidic cells as gonadotrops. Toward spawning, these cells did not show a gradual degranulation but did show a very strong and rapid release of secretory products and the appearance of numerous dilated cisternae of the GER. Likewise, a treatment with LHRH led to a strong vacuolization of the gonadotrops. Similar studies of immature and adult Atlantic salmon by Lindahl (1980) show that in parr and smolt, undifferentiated cells develop into gonadotrops which store electron-dense secretory granules (diameter about 200 nm) and less electron-dense globules (averaging 600 nm in diameter). In adult, sexually mature fish and in precocious male parr these granules and globules may be replaced by dilated cisternae of the GER. A second type of gonadotrop was not observed.

Boddingius (1975) could not demonstrate a second type of gonadotrop in her light and electron microscopical studies of the pituitary of the rainbow trout (*Salmo irideus* = *S. gairdneri*). Boddingius did observe Olivereau's second, largely chromophobic cell type, but did not recognize its possible gonadotropic function. Peute *et al.* (1978) described globular and cisternal gonadotrops in the pituitary of the adult rainbow trout and intermediates between the two (Figs. 2–6). These cells are characterized by the presence of secretory granules (75–500 nm in diameter and of varying electron density) and by relatively electron-lucent globules (500–1200 nm in diameter). According to J. Peute (unpublished results) another characteristic is the presence of bundles of microfibrils. These are not observed in any other cell type of the adenohypophysis except the MSH cells in the PI. The microfibrils are identical to the argyrophylic fibers demonstrated by Boddingius (1975) in the gonadotrops of the rainbow trout, and, as suggested by Boddingius, may function in the amitotic division of the cells (Fig. 5). The

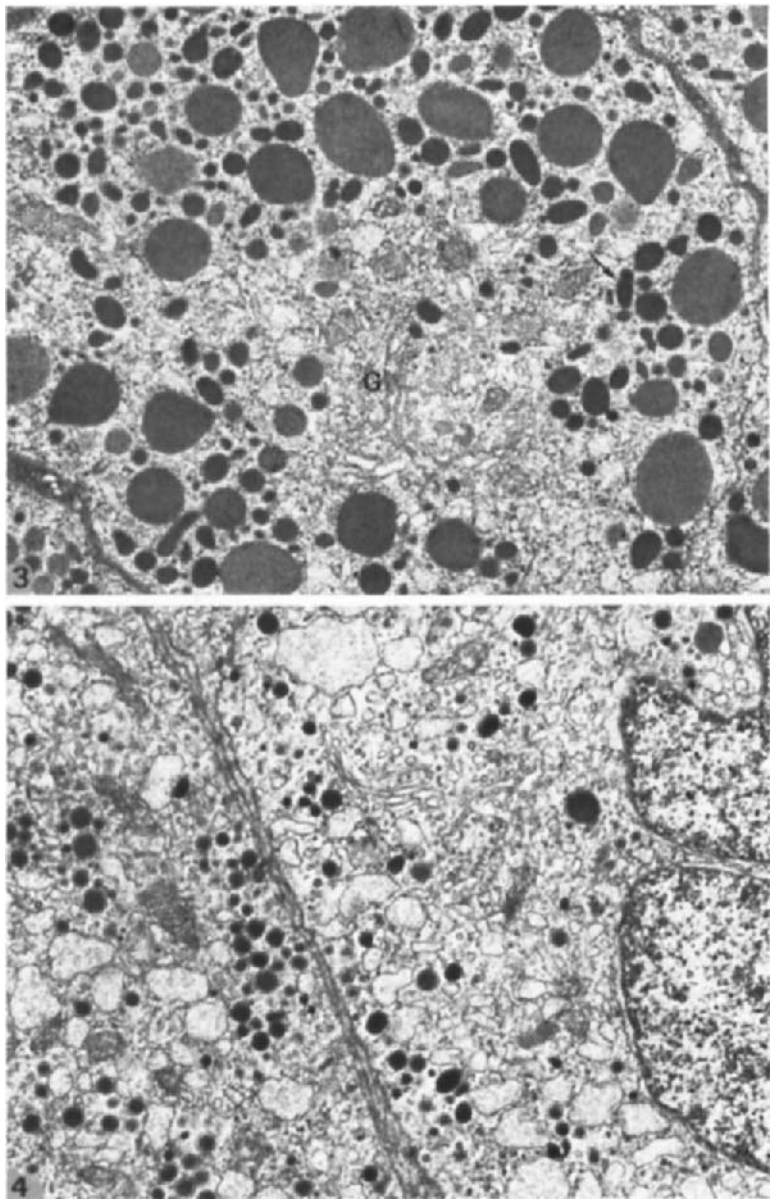


Fig. 3. Electron micrograph of a portion of a globular gonadotropic cell from a rainbow trout (*Salmo gairdneri*) pituitary, showing the Golgi system (G), granules, and globules. The somewhat pointed globules possibly result from the fusion of globules with granules. The arrow indicates coalescing granules. For technical details please refer to Fig. 2.

Fig. 4. Electron micrograph of portions of two gonadotropic cells from a rainbow trout (*Salmo gairdneri*) pituitary. The cells contain numerous granules and small cisternae of the GER, and are thus intermediate between the globular and cisternal cells. Technical details are as in Fig. 2.

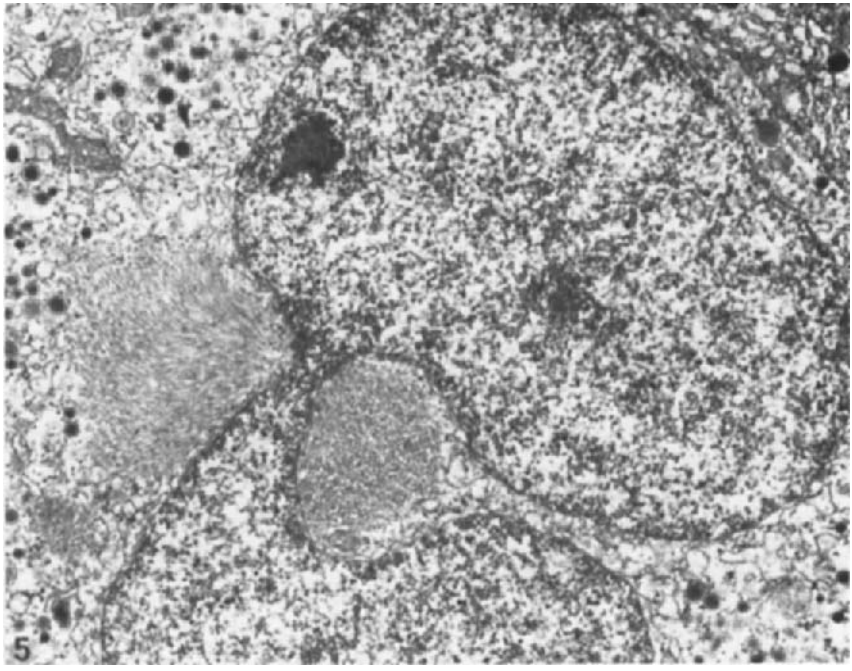


Fig. 5. Electron micrograph of a portion of a gonadotropic cell from a rainbow trout (*Salmo gairdneri*) pituitary. In this cell, the bundle of microfibrils, observed in the cytoplasm of most gonadotrops encircles a constriction in the nucleus, suggesting a possible role in its amitotic division. Technical details are as in Fig. 2.

globular forms of these gonadotrops were observed to degranulate and to develop dilated cisternae of the GER during the period of vitellogenesis in the ovary and accelerated spermatogenesis in the testis. A regranulation and a loss of dilated cisternae was observed in the gonadotrops at the end of gametogenesis. The storage of granules and globules appeared to reflect the amount of glycoprotein GTH in the pituitary. The conclusion that the pituitary of the rainbow trout probably has only one type of gonadotrop, which may be present under several forms, was confirmed by Peute *et al.* (1980) and van Putten *et al.* (1981) in their studies on the effects of ovariectomy on the ultrastructure of the pituitary. Concomitant with a strong release of GTH, the globular basophils (Figs. 2–3) lost their granules and globules and, by intermediate stages (Fig. 4), were transformed into cells with few if any small granules and numerous dilated cisternae of the GER (Fig. 6). However, it appeared that in addition to the globular and cisternal forms there were cells with numerous granules and GER cisternae of small dimensions, and without large globules. The sizes of these granules were intermediate to

those of the globular and cisternal gonadotrops, but unlike these gonadotrops they did not react with anti-salmon-GTH (Leunissen *et al.*, 1980, 1982). Van Putten *et al.* (1982, 1983) identified these cells as thyrotrops, making it clear that they do not represent a second type of gonadotrop. Further, a second type of gonadotrop could not be demonstrated in immature rainbow trout by Gielen *et al.* (1982a,b). In these fish, gonadotropin and testosterone administration both led exclusively to an accumulation of secretory granules and globules in initially undifferentiated cells, transforming them into the well-known globular gonadotrops.

Olivereau and Olivereau (1979a,b) observed a storage of secretory material in the gonadotrops of female silver eels (*Anguilla anguilla*) treated with 17β-estradiol, and the gradual disappearance of this effect as soon as the treatment was interrupted. Olivereau and Chambolle (1978, 1979; see also Olivereau *et al.*, 1979) demonstrated that at the ultrastructural level, estradiol causes the appearance of an increasing number of secretory granules, of occasional globules, and of dilated cisternae of the GER. Olivereau and Nagahama (1982) demonstrated the appearance of material cross-reacting with anti-salmon-GTH in the newly formed secretory granules of these cells. Similar observations were made by Yamamoto and Nagahama (1973) in Synahorin-treated Japanese eel (*Anguilla anguilla*) and by Ueda and Takahasi (1978) in silver females of the Japanese eel which had been injected with dissolved salmon pituitary powder.

Returning to the salmonids, the first thing to note is that Honma and Yoshie (1974) observed large, AF-positive gonadotrops in the ventral region of the PPD in the ayu (*Plecoglossus altivelis*). These cells increase in size and granulation during vitellogenesis and spermiogenesis, and degranulate after spawning. According to Hirose and Ishida (1974) it is this cell type that in the ayu reacts to LHRH treatment. Likewise, Nagahama and Yamamoto (1969a, 1970) and Nagahama (1973) found only one type of gonadotrop in the pituitary of the kokanee salmon, the landlocked form of *Oncorhynchus nerka*, and the chum salmon (*Oncorhynchus keta*). These cells appeared first in the ventral PPD and RPD during gonad maturation. In spent fish, they are strongly degranulated and may contain little more than some large, acidophilic granules. At the ultrastructural level the gonadotrops show the usual small granules, large globules, and irregular cisternae of the GER. In immature sockeye salmon (*Oncorhynchus nerka*) and coho salmon (*Oncorhynchus kisutch*), one type of gonadotrops was observed by McKeown and Leatherland (1973) and by Nagahama *et al.* (1977), respectively. Previously, Chestnut (1970) found one type of gonadotrop in the pituitary of the coho salmon; he termed this cell type the *granulated basophil.* From the beginning of sexual maturation a portion of the granulated basophils degranulate and become almost completely chromophobic. However, when the second-

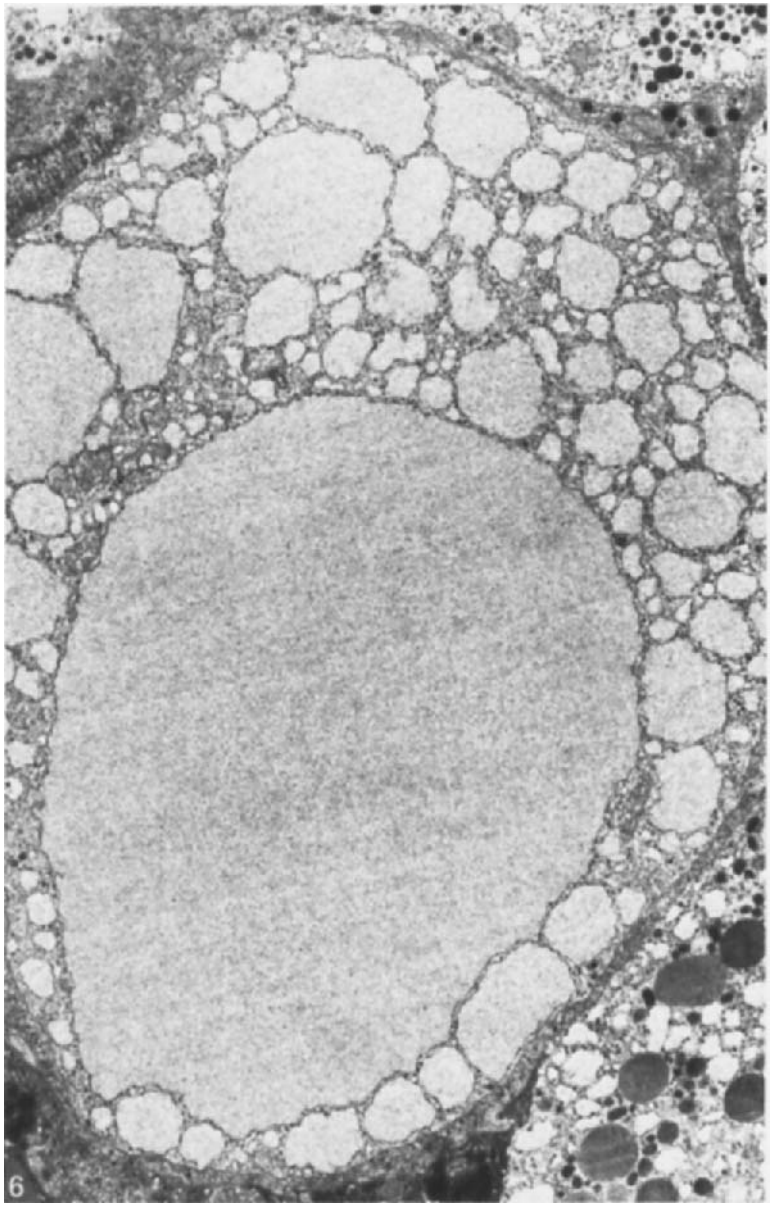


Fig. 6. Electron micrograph of a cisternal gonadotropic cell from a rainbow trout (*Salmo gairdneri*) pituitary. The cytoplasm contains very few granules and no globules. It is filled with dilated cisternae of the GER, and in this extreme case some of these have fused to form a big vacuole. Technical details are as in Fig. 2.

ary sex characteristics begin to develop in the males, a new cell type appears in the PPD and the RPD, the globular basophil, which after staining with AB and PAS contains blue granules and purplish red globules. Chestnut supposed that the latter type might function in the final phase of gonad maturation. Cook and van Overbeeke (1972), studying the pituitary of the sockeye salmon during the upstream migration, described two types of basophils. One type, which had a few small granules (diameter, 80–300 nm) appeared to be "foamy" because of the numerous dilated cisternae of the GER. The other type was characterized by dark granules (diameter, 200–375 nm) and globules (diameter, 400–2000 nm) which were relatively electron translucent. These vacuolar and globular basophils were also observed in the pituitary of sexually maturing and adult coho salmon by Leatherland and Sonstegard (1980), and by Ueda and Hirashima (1979) in adult masu salmon (*Oncorhynchus masou*). Unlike the data from the *Salmo* species, intermediate stages between these two types have not been found in these *Oncorhynchus* species. Therefore, Chestnut, Cook and van Overbeeke, and Ueda and Hirashima assume that the two cell forms represent two different types of gonadotrops. However, Leatherland and Sonstegard suggest that one of them produces thyrotropin instead of gonadotropin.

Ueda (1980) recognizes two types of closely related basophils in the PPD of the whitespotted char (*Salvelinus leucomaenis*). These globular and vesicular cells both contain small dark granules, more electron-translucent globules, and somewhat rounded or irregular cisternae of the GER. Globular cells appear in immature, 1-year-old fish, first in the central PPD, but later, during gonad maturation also in the ventral PPD and the RPD. The diameters of the cell granules increase from a range of 150–350 to a range of 200–400 nm; the globules increase from a range of 400–1000 to a range of 800–1500 nm. Likewise, the cisternae of the GER, which are at first small in size and number, become larger and more numerous, patricularly during spawning. The vesicular cells develop somewhat later, and remain restricted to the central and dorsal PPD. These cells have granules of 100–300 nm in diameter, globules with a diameter of 500–800 nm, and a great number of variously dilated cisternae of the GER. The activity of the vesicular cells is particularly obvious after spawning; that is, before the initiation of exogenous vitellogenesis in the oocytes. The problem with these two types of gonadotrops is that their distribution and particularly their ultrastructural characteristics overlap. Therefore, even a careful study of the text and the micrographs of Ueda's publications does not at first support Ueda's statement that: "The vesicular cells were clearly demarcated from the globular cells at every stage of gonadal development."

Jasinski (1973) described the ultrastructure of glandular cells, including the gonadotrops, in the pituitary of the pondloach (*Misgurnus fossilis*). In a

related species, the loach *Misgurnus anguillicaudatus,* Ueda and Takahashi (1977, 1980) pointed to globular and vesicular basophils in the PPD as the gonadotrops. However, the small and overlapping morphological differences between these two forms do not support the researchers' concept of two separate cell types. Further, experimental results do not support this idea. Ovulation induced by clomiphene was accompanied by a decrease in the number of granules and a strong dilatation of the GER cisternae in both cell types. In females, gonadectomy had very much the same effect, but only in the globular cells. In males castration had a similar, but less pronounced effect on the vesicular cells only. Treatment of females with estradiol and of males with testosterone affected both cell types in such a way that the differences between the two disappeared. However, a very important point emerging from the studies of Ueda and Takahashi is that the size of the granules and globules and the extent of the GER cisternae may change considerably with the physiological condition of the cell, and, therefore, must be interpreted with utmost care when distinguishing between cell types.

Whether Leatherland (1972) was correct in assuming that the basophils in the PPD of the goldfish (*Carassius auratus*) (with granules 125–420 nm in diameter, globules of 875–1170 nm in width, and few if any distended GER lacunae) belong to a cell type different from the basophils in the same area (with more and larger granules of 375–840 nm diameter, globules of 1600–2000 nm diameter, and as clearly distended cisternae of the GER) is difficult to evaluate. Both cell types are presumed to be gonadotrops, but only the one with the larger granules and globules has been observed to change during the ovulation period and following gonadectomy. Probably, the gonadotrop with the larger granules and globules is the same cell type as that designated as the only gonadotrop in the pituitary of the goldfish by Nagahama and Yamamoto (1969b), Nagahama (1973), Kaul and Vollrath (1974a), and Ueda and Takahashi (1977). However, these researchers report a much smaller diameter of the secretory granules (little more than 200 nm) of these cells in adult goldfish. Even so, it is this cell type that shows an increasing granulation and accumulation of globules during the maturation of the gonads. Further, there is a distinct decrease in the number of granules on natural or experimentally induced ovulation and a large increase in number and size of the GER cisternae. Reciprocally, the administration of estradiol and progesterone each led to coalescence of the globules to large, irregular masses (Kaul and Vollrath, 1974a). Huge masses of secretory material have also been observed by a group of Chinese scientists (1978) in the only type of gonadotropic cell in the grass carp (*Ctenopharyngodon idellus*). These masses, having a diameter of over 2 μm and electron-translucent contents in untreated adult grass carp, may attain sizes of approximately

5–10 μm following administration of LHRH. In other respects, the LHRH treatment led to the same results as described for the goldfish by Kaul and Vollrath (1974a) and Lam *et al.* (1976), i.e., a decrease in granulation and a strong dilatation of the GER. In all cases, LHRH did not influence the adenohypophysial cell types except the gonadotrops. This is in agreement with the results of Pan *et al.* (1981) who observed that in the mud carp (*Cirrhrinus molitorella*) anti-LHRH binds to the nuclei and cytoplasm of gonadotropic cells only.

Leatherland *et al.* (1974) described two types of basophils in the PPD of five African *Tilapia* species. The cells which are mainly concentrated in the ventral portion of the PPD and which contain both small and large secretory vesicles produce gonadotropin. This was confirmed for *Tilapia mossambica* (*Sarotherodon mossambicus*) by Sasayama and Takahashi (1975) and by Chiba *et al.* (1978a). Another type of basophil, with smaller granules, is located more dorsally and is presumed to be thyrotropic; Bern *et al.* (1974) consider these cells to be a second type of gonadotrop.

Two types of gonadotrops have also been postulated by Slijkhuis (1978) for the stickleback (*Gasterosteus aculeatus*). One of these types was identical with the gonadotrop in the ventral PPD, as described by Follénius (1968) and Benjamin (1974). Leatherland (1970) and Honma *et al.* (1976) demonstrated an increase in size and granulation of these gonadotrops during sexual maturation. However, among these gonadotrops, Slijkhuis observed cells with less electron-translucent cytoplasm. He supposed that these were also gonadotrops, although there were no signs of changes in secretory activity of these cells during gonad maturation. It may be necessary to test the validity of the functional name given to this cell type by appropriate experiments.

A single type of gonadotropic cell, located in the ventral PPD, has been observed by Kasuga and Takahashi (1970) in the medaka (*Oryzias latipes*) and by Zambrano (1971) in the Gobiid fish, *Gillichthys mirabilis*. Those of *Oryzias*, with granules of 150–200 nm diameter and occasional globules of about 800 nm, changed in correlation with the gonadal cycle; in *Gillichthys*, gonadotrops, which have only one type of secretory granules (diameter, 150–170 nm), showed degranulation and dilatation of the GER cisternae after castration. Likewise, the pituitary of the bagre (*Rhamdia hilarii*) appears to have only one type of gonadotrop. In a light microscopical study and in studies of thin sections and freeze fractures at the electron microscopical level, Val-Stella and Sesso (1980a,b) estimated variations in the number of ventral basophils in the PPD and described changes in the vesicles of the GER of these cells. These changes were closely related to alterations in the gonads during the spawning season. In the gonadotrops of mature fish, the cisternae of the GER were observed to fuse, forming one large vacuole, the contents of which were in direct contact with the inner nuclear membrane.

Signs of active formation of secretory granules (about 200 nm in diameter) and of their exocytosis were also observed in these gonadotropic cells.

A single type of gonadotropic cell has also been described for the pituitary of some catfishes. Baker *et al.* (1974), Joy and Sathyanesan (1979), and Peute *et al.* (1982a,b) demonstrated one type of globular gonadotrop in the PPD of the Indian catfish (*Heteropneustes fossilis*), *Clarias batrachus* and the African catfish (*Clarias lazera*). However, in the channel catfish (*Ictalurus punctatus*), Massoud *et al.* (1980) observed not only this cell type in the PPD, which seemed to be active during the seasonal reproductive cycle of mature fish, but also a type of basophil in the PI, which differentiated in immature fish and was functional throughout life without much seasonal variation in activity. Massoud and co-workers believed that these basophils in the PI must represent a second type of gonadotropic cell. In a very different species, the platyfish (*Xiphophorus maculatus*), some of the PAS-positive cells in the PI are also presumed to have some function in gonadotropin production. Schreibman *et al.* (1979, 1982), Schreibman and Margolis-Kazan (1979), Margolis-Kazan and Schreibman (1981), and Margolis-Kazan *et al.* (1981) observed that just as in the ventral basophils of the PPD, secretory granules in these cells cross-react with antisera to the β chain of carp-GTH and to LHRH. However, without experimental evidence some doubt must remain about the gonadotropic function of PI basophils. Indeed, unspecific reactions with anti-teleost-gonadotropins in PI cells have been noted by Burton *et al.* (1981) in the winter flounder (*Pseudopleuronectes americanus*) and by Peute *et al.* (1982a,b) in the African catfish (*Clarias lazera*).

Experimental evidence has been provided for gonadotropin production in ventral basophils which are located in the PPD of several Poeciliidae, including the green sailfin molly and the black molly (*Poecilia (Mollienisia) latipinna*) In a detailed ultrastructural account, Batten *et al.* (1975) described only one type of cell in the ventral PPD, which on the basis of earlier experimental work (Ball and Baker, 1969) could be identified as a gonadotrop. The cells of this type had secretory granules with an average diameter of 200 nm and a marked halo between contents and envelope. In some animals, the gonadotrops contained globules that were more electron translucent than the granules and up to 700 nm in diameter. The cytoplasm of these cells varied in electron density and in amount of more or less dilated GER cisternae. Peute and de Bruyn (1976) and Peute *et al.* (1976) treated black mollies with methyltestosterone and observed an increase in the number of lysosomes and partly filled or empty secretory vesicles together with a decrease in the number of completely filled granules. These changes, which were probably indications of an increased breakdown of intracellular gonadotropin, were restricted to one cell type, the basophils of the ventral

PPD. This points to the possibility that the ventral basophils of the PPD are the only gonadotrops in *Poecilia latipinna*. Further, in the pituitary of another representative of the Poeciliidae, *Gambusia spec.*, the ventral basophils of the PPD are the only cells that with certainty produce gonadotropin (Chambolle *et al.*, 1981). Here too, these cells are of one type and possess secretory granules of 100–300 nm diameter and a clear halo between the limiting membrane and the relatively dense contents. These cells also contain relatively electron-lucent globules, which are maximally 1500 nm in diameter and which have irregular cisternae of the GER that are especially abundant at the end of ovarian vitellogenesis.

In summary, it may be concluded that it is relatively easy to indentify one type of gonadotrop in the teleost adenohypophysis. This gonadotrop is usually concentrated in the ventral zone of the PPD, has PAS- and often AB-positive secretory granules with a diameter of 200–300 nm and much larger globules with less electron-dense contents. The cisternae of the GER are somewhat rounded or irregular in shape and increase in number and size during gonad maturation. However, it is by no means certain that this is the only type of gonadotropin-producing cell. In several teleost species, a second type has been observed, but, unfortunately, there is very little uniformity in the description of this second putative gonadotrop, and there is a need for more experimental evidence about its function. A firm basis for two different types of gonadotrops in teleosts must depend on positive results of histophysiological studies combined with immunocytochemical research, using antisera against two different teleost gonadotropins.

At present, only Idler and associates have been successful in isolating two different fractions of teleost pituitaries with different gonadotropic potencies (Chapter 5, this volume), and only they are in the possession of these two gonadotropins, a maturational, high (Con AII) glycoprotein gonadotropin and a vitellogenic, low (Con AI) glycoprotein gonadotropin. They have prepared antibodies against both gonadotropins and against a Con AI and Con AII thyrotropin of the winter flounder (*Pseudopleuronectes americanus*). Light microscopical studies of the pituitary of the winter flounder by Burton *et al.* (1981) showed that the pars distalis contains two types of basophils, one restricted to a region, located between the RPD and the PPD, the other concentrated in the ventral PPD and scattered among acidophils in the central and dorsal PPD. This closely resembles the situation described for the flounder (*Pleuronectus flesus*) by Benjamin (1975).

In PAS-OG stained sections of winter flounder pituitaries, collected during the spawning season, the two types of basophils show signs of secretory activity. Moreover, those located in the intermediate zone contain material which cross-reacts with anti-flounder-Con AII-GTH and anti-flounder-Con AII-TSH, but not with anti-flounder-Con AI-GTH and anti-flounder-Con

AI-TSH. The basophils of the PPD show immunofluorescence when treated with anti-flounder-Con AII-GTH and anti-flounder-Con AII-TSH and also when treated with anti-flounder-Con AI-GTH. The anti-flounder-Con AI-TSH could only be demonstrated in basophils within a narrow, rostroventral zone between the RPD and the PPD. During vitellogenesis the basophils in the intermediate zone nearly completely lose their PAS-positive contents, and are thus very difficult to distinguish in PAS-OG stained sections. At the same time the basophils of the PPD increase in number. However, the latter lose their affinity for anti-flounder-Con AII-GTH, and show cross-reactivity for anti-flounder-Con AI-GTH only. At the same time, the basophils of the intermediate region no longer respond to anti-flounder-Con AII-GTH, but acquire material that cross-reacts with anti-flounder-Con AI-GTH. This seems to indicate that three out of four hormones can be produced by both types of basophils, and that only the Con AI-TSH originates from a limited group of basophils in the rostroventral region of the PPD. However, Con AII-TSH can be traced in the basophils of the intermediate zone as well as in those of the PPD. During vitellogenesis Con AI-GTH is the only gonadotropin in the two types of basophils, but during the spawning season Con AI-GTH and Con AII-GTH are produced by both basophilic cell types. In other words, the pituitary of the winter flounder appears to have two morphologically distinct types of gonadotrops and two morphologically distinct types of thyrotrops. However, this does not mean that there is a separate cell type for each of these hormones. On the contrary, the two types of basophils at times may produce one, two, or even three of these high or low glycoprotein hormones simultaneously. This, of course, should be verified, using specific antibodies to the β subunits of the two gonadotropins and thyrotropins, and by the culture of separate populations of cells, as has become common for the identification of the gonadotrops in the mammalian pituitary (a.o. Denef *et al.*, 1980; Tougard *et al.*, 1980; Halmi, 1981, and literature cited by these authors). Anticipating the results of such future studies, one might look for morphological criteria for the production of two different gonadotropins in one cell type, and might attempt to find these among the heterogenous inclusions in the cytoplasm of the globular gonadotrops.

VI. THE FUNCTION OF SECRETORY GRANULES AND GLOBULES

It is generally agreed that, in common with other peptide secreting cells, the gonadotrops in the teleost pituitary synthesize their secretory products in the GER and the Golgi system and store them in secretory vesicles from which they can be evacuated into the intercellular space by exocytosis. In

this concept, the gonadotropic cells are believed to produce one glycoprotein with hormonal functions, and to store this glycoprotein gonadotropin in the relatively small, usually spherical granules with AB- and AF-positive basophilic contents and of varying electron densities. However, as has been explained in the proceding discussion, with very few exceptions the gonadotropic cells in the pituitary of teleosts are characterized by the fact that in addition to these secretory granules they also contain much larger, spherical, or irregularly shaped globules. These are less numerous than the granules, contain material of relatively low electron density, and often stain more readily with OG, erythrosin and other dyes for marking acidophilic cells than with PAS and AB. Such globules are seldom, if ever, found in other cell types, and therefore must have some special function in the metabolism of the gonadotropic cells. Several researchers have noted this problem, and have attempted to formulate solutions, including the possibility mentioned previously that the granules and globules form different hormones. However, no one has to date been able to provide a definite statement concerning the significance of the globules as separate from the granules.

In discussing the literature, published before 1969, including their own results with the sailfin molly (*Poecilia latipinna*) and those of Olivereau with several salmonids, Ball and Baker (1969) suggested that the globules might be so-called R-granules, containing lytic enzymes, just as they have been demonstrated to be in the gonadotrops of amphibians and reptiles (van Oordt, 1968, 1974, 1979; Doerr-Schott, 1970, 1976a,b; Holmes and Ball, 1974). This opinion, also expressed by Batten *et al.* (1975) and by Leatherland *et al.* (1974) in studies of the pituitary of five African species of *Tilapia*, is based on the fact that globules accumulate in gonadotropic cells when the secretory activity of these cells declines (e.g., after spawning and when oocyte growth is arrested). Strong evidence in favor of this opinion has been provided by Peute *et al.* (1976) who studied effects of methyltestosterone on the gonadotrops in the black molly [*Mollienisia (Poecilia) latipinna*], and found that a loss of immunoreactive gonadotropin, as observed by Goos *et al.* (1976), was on the one hand accompanied by a loss of electron-dense material from the small secretory granules, and on the other hand by an increase in the number of globules. Several of these globules contained membrane material, suggestive of an augmented breakdown of secretory granules by lysosomal activity. Likewise, Olivereau and Chambolle (1978, 1979) demonstrated that in the gonadotrops of estradiol-treated eels (*Anguilla anguilla*), large globules appear much later than small secretory granules, and because there was no sign of hormone extrusion from those gonadotropic cells, the globules were considered to be indicative of an intracellular breakdown of secretory material. At any rate, according to Olivereau and Nagahama

(1982), the globules in the eel do not react with anti-salmon-GTH, in contrast to the small granules.

Ueda and Takahashi (1978) treated Japanese eels (*A. japonica*) with salmon pituitary powder, and described a gradual increase in size and number of globules and small granules. However, in one specimen this treatment led to ovulation, and that was accompanied by a discharge of granules, but not of globules. A similar effect was noted by Ueda and Takahashi (1977) in goldfish (*Carassius auratus*) treated with clomiphene. Likewise, Nagahama and Yamamoto (1969b) and Nagahama (1973) demonstrated that natural spawning in the goldfish is preceded by a loss of small granules only, and that the globules do not change until about 60 days after spawning when the gonadotrops show signs of degeneration. However, Kaul and Vollrath (1974a) noticed that, immediately after spawning, the goldfish gonadotrops were devoid of both granules and globules; a treatment with LHRH mainly led to a decrease in number of small granules. Lam *et al.* (1976) repeated these experiments, and obtained similar results; they concluded that the small granules of the goldfish are involved in gonadotropin release and that the function of the globules remains unknown. At present, there is no definite evidence that in the goldfish the globules are lysosomes.

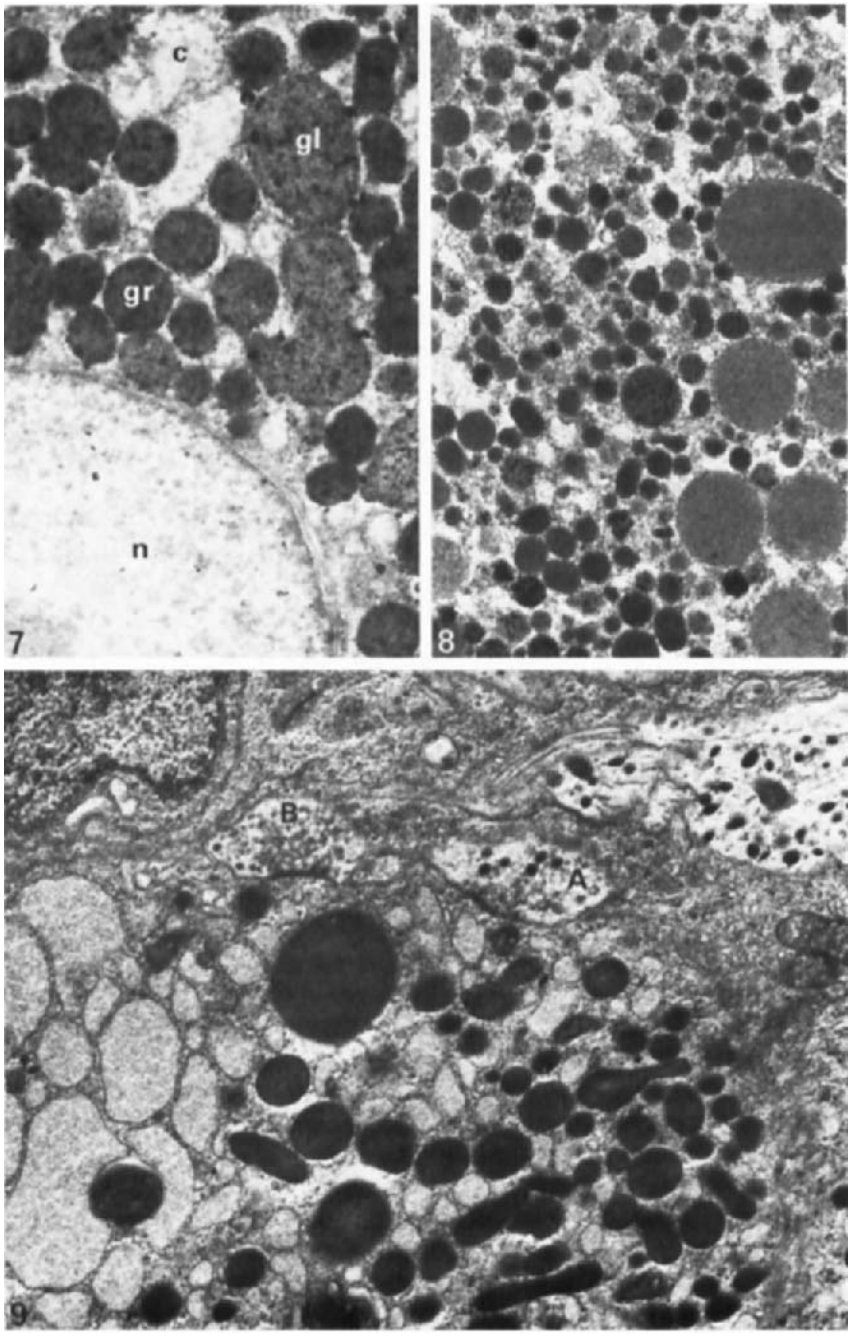
Kaul and Vollrath (1974a) observed that, in goldfish, the globules may coalesce after estradiol administration and form large irregular masses exhibiting a fine striation of possibly tubular nature. Such large heterogenous masses were also found by a group of Chinese scientists (1978) in the gonadotrops of the grass carp (*Ctenopharyngodon idellus*); these masses were distinct from the small granules and globules. The administration of LHRH led to a decrease in the number of granules and globules, and an increase in the size and number of the huge granules, reaching a width of 5–10 μm. The researchers assumed that the small granules secrete an LH-like hormone and the globules secrete an FSH-like hormone. Boddingius (1975) defended the opposite view, arguing that in the rainbow trout [*Salmo irideus (gairdneri)*] the small granules predominate during gametogenesis and the globules dominate during ovulation and spawning.

Boddingius (1975) further believes that the granules and globules may represent different forms of packaging of one gonadotropic hormone. If this is true, one should observe small granules coalescing to form globules, as suggested by Cook and van Overbeeke (1972) and others. Actually, such intermediate stages have been demonstrated. At the light microscopical level, Baker *et al.* (1974) observed condensation of granules into denser irregular masses in the gonadotrops of the Indian catfish (*Heteropneustes fossilis*). In electron micrographs of gonadotropic cells of the rainbow trout van Putten *et al.* (1981) found signs of fusion not only of granules with other

granules and globules with one another (Fig. 2), but also of granules with globules (Fig. 3). Concomitantly, during the reproductive cycle (Peute *et al.*, 1978), following ovariectomy (van Putten *et al.*, 1981) and as a result of induced maturation (Gielen *et al.*, 1982a,b), in gonadotropic cells of the rainbow trout changes in the amount of granules and globules go hand-in-hand with changes in the storage of glycoprotein gonadotropin. Further, using immunocytochemical methods, the glycoprotein gonadotropin can be demonstrated in the globules as well as in the granules; this has been shown in the rainbow trout by Leunissen *et al.* (1980, 1982), in the platyfish (*Xiphophorus maculatus*) by Margolis-Kazan *et al.* (1981) and in the African catfish (*Clarias lazera*) by Peute *et al.* (1982a,b; Fig. 7).

It would be incorrect to conclude from the foregoing that the globules are nothing but coalesced granules. This already follows from the fact that their contents are usually less electron dense than those of the granules. Indeed, it appears that the granules can enlarge and take up electron-translucent material not previously found in the granules. At any rate, in all three species mentioned, the concentration of immunoreactive glycoprotein gonadotropin is higher in the granules than in the globules. Therefore, obviously, the granules and globules do not represent a resting stage in the secretory process. On the contrary, it seems that they play a crucial role in hormone synthesis; if we assume, as is generally done, that the prohormone is constructed in the GER, it must be realized that this prohormone does not change into material that in ultrasections can react with an antiserum to teleost glycoprotein GTH or its β chain before it arrives in the secretory granules and globules. In the rainbow trout, Peute and de Bruyn (see van Oordt, 1979) demonstrated that it is these granules that at the same time contain the lytic enzyme acid phosphatase (Fig. 8). This may indicate the proteolytic cleavage of a prohormone preceding hormone extrusion, as has been described for β-cells in the endocrine pancreas by Novikoff and Novikoff (1977). Acid phosphatase is present in the globules in much smaller amounts than in the granules. This shows that, at least in the rainbow trout, the globules cannot be considered as lysosomes. Indeed, in teleost gonadotrops, lysosomes often are distinct organelles, different from the secretory globules (Kasuga and Takahashi, 1970).

If the globules are not involved in the intracellular breakdown of excess hormone, then one must question their function. One might speculate that a second gonadotropin is formed in the globules. The contents of the globules are less basophilic and contain immunoreactive glycoprotein gonadotropin in lower concentration than the granules. That raises the possibility of a low glycoprotein gonadotropin, comparable to the vitellogenic gonadotropin, isolated by Idler and his associates from pituitaries of several teleosts (Chap-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ter 5, this volume). Such a situation would be comparable to that observed by Tougard *et al.* (1980) in the gonadotropic cells of rat pituitaries where the contents of some of the larger granules stained with anti-rat-β-LH and with anti-rat-β-FSH. However, it is just as likely that the two gonadotropins and the glycoprotein thyrotropin, which, as noted by Burton *et al.* (1981) in the winter flounder (*Pseudopleuronectes americanus*), are formed in the same cell types, are simultaneously present in the granules as well as in the globules. It should be realized that the granules and the globules are not indispensable for gonadotropin secretion, because strongly activated gonadotropic cells continue to secrete gonadotropin after the granules and globules have disappeared (see van Putten *et al.*, 1981).

At present one can only speculate about the specific functions of the granules and the globules. Further research is necessary regarding the chemical nature of the hormone or hormones secreted by the gonatotropic cells and their synthetic pathway before questions concerning the exact function of organelles often considered responsible for the storage of secretory products can be answered. At the same time, it will be necessary to unravel the exact role of peripheral hormones and neurohormones in the regulation of the secretory activity of the gonadotropic cells. Present knowledge of this regulation is discussed in Chapter 3 of this volume. However, a very short survey of some peculiar morphological aspects regarding the central regulation of gonadotropin secretion is pertinent here.

Fig. 7. Electron micrograph of a portion of a globular gonadotropic cell from the pituitary of an African catfish (*Clarias lazera*), fixed in a 1:1 mixture of 2% formaldehyde and 4% glutaraldehyde, followed by a postfixation in 2% OsO_4 in 0.2 *M* cacodylic buffer, and embedded in Epon (Ladd). The thin section was etched on a drop of 15% H_2O_2, and subsequently stained with anti-carp-β-GTH, using Sternberger's double-antibody immunoenzyme technique. A positive reaction can be observed in the granules (gr) and the globules (gl), but not in the nucleus (n) and the cisternae (c) of the GER (magnification × 17,100). The technical assistance of M. G. A. de Mol Moncourt-de Bruyn and L. W. van Veenendaal is acknowledged.

Fig. 8. Electron micrograph of aportion of a globular gonadotropic cell from a rainbow trout (*Salmo gairdneri*) pituitary, fixed in 6.25% glutaraldehyde. An acid phosphatase reaction was conducted on 50-μm thick cryosections, which were subsequently dehydrated and embedded in Epon (Ladd). A positive reaction is mainly visible in most of the granules, and to a much lesser extend in the globules (magnification × 13,300). The technical assistance of M. G. A. de Mol Moncourt-de Bruyn and L. W. van Veenendaal is acknowledged.

Fig. 9. Electron micrograph of a portion of a globular gonadotropic cell from a pituitary of an African catfish (*Clarias lazera*). In the upper right-hand corner of the photograph nerve fibers approach the cell. A type A fiber (A) and a type B fiber (B) make synaptoid contact with the gonadotrop. Fixation and embedding are as in Fig. 7. Staining was done with uranylacetate and lead citrate (magnification × 16,200). The technical assistance of M. G. A. de Mol Moncourt-de Bruyn and L. W. van Veenendaal is acknowledged.

VII. THE INNERVATION OF THE GONADOTROPIC CELLS

In most groups of vertebrates, the pars distalis of the pituitary is connected with the neurohypophysis by blood vessels only; a capillary plexus in the median eminence leads to portal vessels that divide into a network of sinuses between the cell strands of the pars distalis. Teleosts have an "encapsulated median eminence" (Kerr, 1968) in the anterior neurohypophysis, and centrifugal capillaries penetrate from there into the pars distalis of the adenohypophysis. These capillaries run in perivascular channels, and carry neurosecretory material from axon endings in the anterior neurohypophysis to the glandular cells in the pars distalis (see reviews by Ball and Baker, 1969; Perks, 1969; Holmes and Ball, 1974). In eels (Knowles and Vollrath, 1966a,b), tench (*Tinca tinca,* Vollrath, 1967) and salmonids (Fridberg and Ekengren, 1977), there are no other means of contact between the neurosecretory system and the endocrine cells in the pars distalis; however, in all other teleost studied, neurosecretory axons penetrate the basal laminae separating the adenohypophysis from the neurohypophysis, proceed into the endocrine parenchyma of the pars distalis, contact the hormone producing cells, and may terminate with synapses on these cells or on the basement membrane of the intravascular channel system.

The neurosecretory fibers entering the pituitary from the hypothalamus differ in stainability of their secretory material and in the diameter of their granules. Knowles (1965) and Knowles and Vollrath (1966a,b) have described two fiber categories, the type A and type B fibers. Type A fibers are Gomori-positive, which means that their neurosecretory contents stain with Gomori's chrome-alumhaematoxylin, AB, AF, and aldehyde thionin; they store "elementary neurosecretory granules" of 100–200 nm diameter. The type B fibers are Gomori-negative and contain "large granulated vesicles" of 60–100 nm diameter, which have a clear halo between the electron-dense core and the bounding membrane. Sometimes subtypes can be recognized, especially among the type A fibers. For example, in the sailfin molly (*Poecilia latipinna*) Batten and Ball (1977) counted as many as five subtypes of type A fibers.

Axon terminals and preterminal portions of the neurosecretory fibers have been seen both in direct contact with the gonadotropic cells and in indirect contact, i.e., separated from these cells by a basal lamina. Thus, in the threespined stickleback (*Gasterosteus aculeatus*), Leatherland (1970) and Follénius (1972) observed a direct nonsynaptic innervation by type B fibers; a similar arrangement has been described by Bern *et al.* (1971, 1974) for *Tilapia mossambica* (*Sarotherodon mossambicus*), by Abraham (1974) for the mullet (*Mugil cephalus*), and by Ekengren *et al.* (1978a) for the

roach (*Rutilus rutilus*). Synaptoid contacts between type B fibers and gonadotrops have been demonstrated by Vollrath (1967) in the sea horse (*Hippocampus cuda*), by Kasuga and Takahashi (1970) in the medaka (*Oryzias latipes*), by Bern *et al.* (1971) and by Zambrano (1971) in *Gillichthys mirabilis*, by Jasínsky (1974) in the pond loach (*Misgurnus fossilis*), by Kaul and Vollrath (1974b) in the goldfish (*Carassius auratus*), by Peute *et al.* (1976), and by Batten and Ball (1977) in the molly (*Poecilia latipinna*), by Ekengren *et al.* (1978a) in the roach (*Rutilis rutilus*), and by Peute *et al.* (1982b) in the African catfish (*Clarias lazera;* Fig. 9). However, very often the gonadotrops are innervated by type A fibers also. This has been observed by Leatherland (1969) in *Cymatogaster aggregata*, by Kasuga and Takahashi (1970) in the medaka (*Oryzias latipes*), by Leatherland (1972) and by Kaul and Vollrath (1974b) in the goldfish (*Carassius auratus*), by Bern *et al.* (1974) in *Tilapia mossambica* (*Sarotherodon mossambicus*), by Abraham (1974) in the mullet (*Mugil cephalus*), by Peute *et al.* (1976) and by Batten and Ball (1977) in the molly (*Poecilia latipinna*), and by Peute *et al.* (1982b) in the African catfish (*Clarias lazera;* Fig. 9). However, these type A fibers seldom make synaptoid contacts with gonadotropic cells.

A double innervation appears to be a general feature of the gonadotropic cells in the teleost pituitary. However, such an innervation is not necessarily the same for all cells and at all times of the reproductive cycle. In the roach (*Rutilus rutilus*), Ekengren *et al.* (1978a) observed that type B fibers innervate gonadotrops adjacent to the neurohypophysis only. Neuroendocrine fibers did not penetrate deeper layers of the PPD, leaving the majority of the gonadotropic cells without neuroglandular connections (Båge *et al.*, 1974). Moreover, synaptoid contacts could be demonstrated with vesicular, but not with globular gonadotrops. Similarly, in *Tilapia mossambica* (*Sarotherodon mossambicus*), Bern *et al.* (1971) found an innervation by type B fibers of one of two types of gonadotropic cells; both were innervated by type A fibers. Abraham (1974) explained that in the mullet (*Mugil cephalus*) an innervation by type A fibers is particularly obvious during the spawning season, and suggested that when type A fibers are not observed between the gonadotrops, it may be attributable to the fact that the fish are not caught in the reproductive phase. Finally, Kaul and Vollrath (1974b) described a gradual decrease in the number of granules in type A and type B fibers innervating gonadotropic cells in the goldfish (*Carassius auratus*) pituitary during the spawning and postspawning seasons, and an increase in the amount of type B granules in fibers innervating gonadotropic cells after an estradiol treatment.

Knowles and Vollrath (1966a,b) have postulated that the double innervation of adenohypophysial cells reflects a separate regulation of synthesis and release of their secretory products. Morphological studies have not supported this hypothesis; the problem is an uncertainty of the origin of the

neurosecretory axons and of the contents of their granules. It has been suggested that the type A fibers are peptidergic axons coming from the preoptic nucleus, and that the type B fibers are aminergic and originate in the nucleus lateralis tuberis (NLT; see reviews by Ball and Baker, 1969; Holmes and Ball, 1974). However, such a view does not take into account the complexity of these nuclei, which are composed of several sections and contain different types of perikarya. It, moreover, ignores the possibility of other neurosecretory centers innervating adenohypophysial cells. Indeed, cytochemical studies using the Falck-Hillarp technique have demonstrated that in salmonids, aminergic neurons are not present in the NLT (Ekengren and Terlou, 1978; Terlou and Ekengren, 1979), but are mainly concentrated in the nucleus recessus lateralis (NRL) and the nucleus recessus posterioris (NRP; Terlou *et al.*, 1978). A similar situation has been observed in *Gillichthys mirabilis* (Swanson *et al.*, 1975), the eel (*Anguilla anguilla*; Fremberg *et al.*, 1977), and the sailfin molly (*Poecilia latipinna;* Batten *et al.*, 1979). Thus, aminergic fibers innervating gonadotropic and other cells in the adenohypophysis appear to originate in the NRL and NRP, and possibly also in scattered perikarya in the vicinity of these nuclei. However, as Ekengren (1975) has noted for the roach (*Rutilus rutilus*), only a small portion of the type B fibers is aminergic. Other type B fibers are axons of Gomori-negative peptidergic neurons, which in the roach (Ekengren, 1973) and the goldfish (*Carassius auratus;* Peter and Nagahama, 1976) have been thought to come from the NLT. According to Peute *et al.* (1976) in the black molly [*Mollienisia* (*Poecilia*) *latipinna*] the Gomori-negative peptidergic neurons in the NLT pars lateralis have secretory vesicles with a diameter of more than 100 nm. Axons of these type A fibers are presumed to innervate the gonadotropic cells in the pituitary. This conclusion is based on the fact that the diameter of the granules in axon endings near gonadotropic cells is about the same as the diameter of the secretory granules in the perikarya of the NLT pars lateralis, but is not based on observations of entire axons running from the NLT to the gonadotropic cells. The problem is that by definition these Gomori-negative peptidergic neurons of the NLT cannot be visualized with classical stains for neurosecretory material. However, recently Gielen *et al.* (1982c) and Gielen and Terlou (1982), applying Sternberger's double-antibody immunoenzymecytochemical technique with an antiserum against glucagon as first antibody, succeeded in selectively staining almost all of the NLT pars lateralis neurons and their axons running toward the pituitary in the rainbow trout (*Salmo gairdneri*). It appears that the axons of these NLT neurons do not enter the rostral neurohypophysis, but do enter the neurointermediate lobe to terminate in the protrusions of the neurohypophysis in the PI. Therefore, it is unlikely that these NLT neurons have anything to do with the direct innervation of gonadotropic cells. However, Gomori-nega-

tive peptidergic neurons, situated behind the NLT pars ventromedialis, which react with antigastrin, do penetrate the protrusions of the neurohypophysis in the PPD (Notenboom *et al.*, 1981), and on that ground cannot be excluded as a possible source of gonadotropin releasing hormone. Further, Schreibman *et al.* (1979) observed neurons staining with anti-LHRH in the NLT pars posterior of the platyfish (*Xiphophorus maculatus*), but no reaction with this antibody was observed in the caudal hypothalamus of the rainbow trout. However, in the latter species, neurons located in the area dorsalis pars medialis of the telencephalon and fiber tracts from this region toward the neurohypophysis can be visualized with anti-LHRH as first antibody in the double-antibody immunofluorescence technique (Goos and Murathanoglu, 1977). In the platyfish, neurons reacting with anti-LHRH are present in the ventral telencephalon (Schreibman *et al.*, 1979). It is obvious that these neurons must also be considered as neurosecretory elements possibly directly innervating gonadotropic cells. It is beyond the scope of this discussion to review the experimental evidence in favor and against the role of various neurosecretory centers in the regulation of gonadotropin secretion in teleosts (See Chapter 3, this volume). However, it should be emphasized that the direct innervation of gonadotropic and other cell types in the adenohypophysis is a unique feature of the pituitary in many teleosts, and that this provides an extra dimension to the study of the central regulation of gonadotropin secretion. Cytochemical studies at the ultrastructural level may prove useful in tracing the neurosecretory cells involved in the regulation of production, storage, and release of gonadotropin, and in determining some of the active principals secreted by these neurons.

VIII. CONCLUSION

In conclusion, it may be useful to outline the current knowledge of the gonadotropic cells in the pituitary of teleosts, and in doing so to note possibilities for future research on this topic.

Recently, Licht (1982) suggested that in most groups of vertebrates gonadotropic cells originate in the zona tuberalis of the adenohypophysis; good examples are the cartilagenous fishes, the amphibians and the reptiles. The primordium of the teleost adenohypophysis does not have a paired lateral lobe and does not develop a pars tuberalis and a zona tuberalis (Wingstrand, 1966). However, the ventral portion of the PPD may be homologous to a zona tuberalis, and is the place where, at least in some species, the first gonadotropic cells appear to develop. More evidence, based on pituitary ontogenesis in a number of teleost species, should verify this point. Very often the gonadotrops remain restricted to the ventral PPD, but they may

also spread out into other regions of the pars distalis mixing with other cell types, and may even reach the PI.

As in all other gnathostomes, the gonadotrops in the teleost pituitary are as a rule relatively large, somewhat rounded cells. They are basophilic, which means that they contain PAS-positive glycoproteins. This secretory material is often AB-positive also, and as in other fishes, amphibians and reptiles (van Oordt, 1979), it is stored not only in granules but also in globules. At the electron microscopical level the dense-cored granules are round or oval and have an average diameter of 100–500 nm. The globules have more electron-translucent contents, have a round or irregular shape and measure 0.5–3.0 μm diameter. Moreover, the gonadotrops are characterized by the presence of irregular cisternae of the GER. Both types of dense-cored vesicles contain gonadotropin, but it is unlikely that storage of secretory material is their only function. The process of hormone synthesis, beginning in the GER and the Golgi system, may continue in the granules and globules. In fact, little is known of the processes of hormone synthesis and release in the gonadotropic cells. Research in this field is badly needed, if only to provide an answer to the question why these cells contain two types of secretory vesicles.

It remains uncertain whether the teleost pituitary contains one or two types of gonadotropic cells. Some researchers have described two distinct morphological cell types, such as globular and cisternal gonadotrops. Others have found intermediate stages between these two. However, there is at present only one publication on the presence of two gonadotropic hormones in teleost pituitary cells, and that points to the possibility of two subtypes both involved in the secretion of the two hormones. If this observation is confirmed for several species, the situation would appear to resemble that found in tetrapods where two subtypes can produce two gonadotropic hormones both successively and simultaneously (van Oordt, 1979; van Oordt and Peute, 1983; Mikami, 1982).

The gonadotropic cells are often found in close apposition with neuroendocrine axons. The physiological significance of such a situation is unknown; all that can be said is that it appears to be unique among the vertebrates, and that the direct innervation of the gonadotropic cells appears to change with the secretory activity of these cells during the reproductive cycle. This aspect deserves further investigation. Some of the axons innervating gonadotropic cells consist of fibers with large granulated vesicles of less than 100 nm diameter. These often make synaptoid contacts with the gonadotrops. Other axons in direct contact with gonadotrops contain elementary granules larger than 100 nm diameter. There is a double direct innervation of gonadotropic cells. However, neurohormones may also reach the gonadotrops by the circulatory system, the intervascular channel system,

and the intercellular fluid. Moreover, gonadal hormones are known to influence gonadotropic cells (Chapters 7 and 9, this volume, and Chapter 7, Volume 9B, this series). Therefore, the regulation of gonadotropin synthesis, storage, and release is a very complex process, and studying effects of one factor only, as is so often done, may give a false impression of its role in these processes. Studies on the influence of combinations of hormones on dispersed pituitary cells, like those of Denef *et al.* (1980) in the rat, should be introduced in the study of the regulation of gonadotropin secretion in teleosts.

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5

TELEOST GONADOTROPINS: ISOLATION, BIOCHEMISTRY, AND FUNCTION

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I. INTRODUCTION

The paramount importance of the pituitary gland in the control of teleost reproduction has been extensively reviewed by Dodd (1960), Hoar (1969), Lam *et al.* (1978), and by Pickford and Atz (1957). Hypophysectomy leads to gonadal atrophy. Until 1975, data from chemical fractionation studies and bioassays supported the concept that the teleost pituitary elaborated a single gonadotropin which controlled all phases of the reproductive cycle including vitellogenesis, oocyte maturation, ovulation, spermatogenesis, androgen production, and spermiation (Burzawa-Gerard, 1974; Donaldson, 1973; Fontaine, 1975). However, histological studies of the teleost pituitary revealed one type of gonadotrop in some fish and more than one type in other species. Reinboth (1972) stressed that histological findings should not be overlooked.

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The lack of unanimity in the histological observations provided the impetus for continuing the search for more than one type of gonadotropin in teleost. Since 1975, reports on the isolation of gonadotropins from more teleostean species have appeared, and the results have shed some light on the controversial issue of the number of gonadotropins in this important class of vertebrates.

In the various investigations reported, pituitary glands were collected fresh from fish whenever possible, or as soon as possible after death, to prevent postmortem degradation of hormones. This was done in the case of Pacific salmon (Breton *et al.*, 1978; Donaldson *et al.*, 1972; Idler *et al.*, 1975b; Yoneda and Yamazaki, 1976), winter flounder (Ng and Idler, 1979), Tilapia (Farmer and Papkoff, 1977; Hyder *et al.*, 1979), and trout (Breton *et al.*, 1976). Acetone powder of carp pituitaries was obtained from a commercial source (Burzawa-Gerard, 1971). Acetone powder from pike eel pituitaries was similarly prepared (Huang *et al.*, 1981).

II. ISOLATION

A. Methods

The scope of this chapter is confined to teleosts (and chondrosteans); selachians and agnathans are discussed elsewhere (e.g., Fontaine and Burzawa-Gerard, 1978; Idler and Ng, 1980; Crim, 1982; and Ball, 1981). The action of mammalian hormones on fish has not been emphasized.

1. Solvent Fractionation

Solvent fractionation and salting out was used as a preliminary purification step in the isolation of gonadotropins (GtH) from pituitaries of carp (Burzawa-Gerard, 1971), chinook salmon (Donaldson *et al.*, 1972), sturgeon (Burzawa-Gerard *et al.*, 1975a), chum salmon (Yoneda and Yamazaki, 1976), Tilapia (Farmer and Papkoff, 1977; Hyder *et al.*, 1979), and pike eel (Huang *et al.*, 1981). The method of alcoholic percolation (Bates *et al.*, 1959) and the method of extraction with ethanolic ammonium acetate buffer followed by precipitation with ethanol (Stockell Hartree, 1966) were employed in these studies.

2. Affinity Chromatography

Idler and co-workers innovated the usage of affinity chromatography on Con A–Sepharose in their isolation of gonadotropins (Idler *et al.*, 1975b; Campbell and Idler, 1976, 1977; Ng and Idler, 1978a,b, 1979; Idler and Ng,

1979; Idler and Hwang, 1978). The Con A–Sepharose chromatography was later adopted by other research groups (Pierce *et al.*, 1976; Breton *et al.*, 1978). Fish pituitary extract yielded a broad peak unadsorbed on Con A–Sepharose (designated Con AI fraction) and an adsorbed fraction (designated Con AII fraction) which could be eluted as a sharp peak by inclusion of α-methyl-D-glucoside in the buffer (Idler *et al.*, 1975b; Pierce *et al.*, 1976). The protease inhibitor Trasylol and divalent cations including calcium, magnesium, and manganese were included in the elution buffer for better performance (Ng and Idler, 1978a). The immobilized lectin selectively adsorbs glycoproteins with α-D-glucopyranosyl end groups or internal 2-*o*-linked-D-mannopyranosyl residue (Goldstein *et al.*, 1965), leaving behind in the unadsorbed fraction proteins with either a low carbohydrate content or a lack of sugars requisite for binding to the lectin. Rechromatography of the Con AI fraction on Con A–Sepharose was always performed to minimize contamination with Con AII material. Idler and Hwang (1978) found that the inclusion of ethylene glycol (50% v/v) in the elution buffer optimized the recovery, from Con A–Sepharose, of maturational hormone which was contained in the Con AII fraction. In the opinion of Idler and Ng, affinity chromatography on Con A–Sepharose has advantages over the conventional ethanolic extraction and precipitation method (Stockell Hartree, 1966) because it yields a glycoprotein Con AII preparation minimally contaminated with Con AI material.

The usefulness of other immobilized lectins with different carbohydrate binding specificities in the isolation of teleost gonadotropins has been tested (Idler and Ng, 1979). Lentil lectin–Sepharose has a carbohydrate specificity similar to that of Con A–Sepharose (Allen *et al.*, 1976), but it has a lower affinity (Stein *et al.*, 1971). Wheat germ lectin–Sepharose and *Helix pomatia* lectin–Sepharose are specific for *N*-acetylglucosamine and *N*-acetylgalactosamine, respectively. It was found that the Con AII fraction was adsorbed on lentil lectin–Sepharose, but the Con AI fraction was unadsorbed on lentil lectin–Sepharose, wheat germ lectin–Sepharose and *Helix pomatia* lectin– Sepharose. Breton (1981) demonstrated that wheat germ lectin adsorbed the gonadotropin which was also adsorbed on Con A–Sepharose. Unfortunately, he concluded "unlike Idler (Idler and Ng, 1979) we found that salmon GtH can be retained on an immobilized lectin, such as wheat germ lectin." This statement misquotes the reference. It was the gonadotropin in the Con AI fraction (containing the carbohydrate poor or vitellogenic* GtH) that was not adsorbed on wheat germ lectin–Sepharose; the gonadotropin in the Con AII fraction (containing the carbohydrate-rich

*The term vitellogenic is used to describe that hormone which regulates the prematurational development of the ovary and does not refer to the process of vitellogenesis as it relates to liver synthesis of the glycolipophosphoprotein vitellogenin.

or maturation GtH) was adsorbed on Con A–Sepharose. Therefore, both groups found the same gonadotropin fraction to be adsorbed on the immobilized lectins.

3. Gel Filtration

Gel filtration was included as one of the steps of fractionation to separate proteins mainly on the basis of molecular size. Sephadex G-100 or G-75 was used by most researchers (Breton, 1968; Grönlund, 1969; Burzawa-Gerard, 1971; Clemens *et al.*, 1964; Donaldson *et al.*, 1972; Sundararaj *et al.*, 1972a,b; Burzawa-Gerard *et al.*, 1975a; Idler *et al.*, 1975b; Campbell and Idler, 1976; Yoneda and Yamazaki, 1976; Farmer and Papkoff, 1977) although some employed Ultrogel AcA 54 or AcA 44 (Breton *et al.*, 1976; Ng and Idler, 1978a,b, 1979). In the case of a species such as flounder, where the peak following the void volume has a relatively high molecular weight (MW 62,000), Ultrogel allows a better separation than Sephadex G-75 (Ng and Idler, 1978a). The buffer used was either the volatile NH_4HCO_3 buffer (Donaldson *et al.*, 1972; Farmer and Papkoff, 1977), or Tris-Cl at a near neutral pH (Idler *et al.*, 1975a; Campbell and Idler, 1976, 1977; Ng and Idler, 1978a,b, 1979; Idler and Ng, 1979), or phosphate buffer (Breton, 1968).

4. Ion-Exchange Chromatography

Diethylaminoethyl (DEAE)-cellulose (Burzawa-Gerard, 1971; Breton *et al.*, 1976; Donaldson *et al.*, 1972; Pierce *et al.*, 1976; Yoneda and Yamazaki, 1976; Farmer and Papkoff, 1977; Huang *et al.*, 1981) or a DEAE ion exchanger such as DEAE-Bio-Gel A (Idler *et al.*, 1975c; Breton *et al.*, 1978; Idler and Ng, 1979; Ng and Idler, 1979) or DEAE-Sephadex A-50 (Burzawa-Gerard *et al.*, 1975a) was used to purify teleost gonadotropins on the basis of charge properties.

The buffer used was either NH_4HCO_3 (Idler *et al.*, 1975a; Farmer and Papkoff, 1977), Tris buffer (Breton *et al.*, 1976), or sodium glycinate buffer (Donaldson *et al.*, 1972; Burzawa-Gerard *et al.*, 1975a). A concentration gradient was used to elute the adsorbed material.

Carboxymethyl ion-exchanger chromatography was utilized by Pierce *et al.* (1976), by Idler and Ng (1979), and by Hyder *et al.* (1979). The buffer used was ammonium acetate buffer at an acidic pH. Amberlite CG-50 chromatography was performed by Farmer and Papkoff (1977).

5. Preparative Polyacrylamide Gel Electrophoresis

Preparative polyacrylamide gel electrophoresis (PAGE) was included as one of the steps of the purification scheme by Burzawa-Gerard (1971) and by Huang *et al.* (1981).

B. Species

1. SALMONIDS

Early attempts to isolate gonadotropin from salmon pituitaries have been described by Donaldson *et al.* (1972). Otsuka (1956) extracted the pituitaries with 40% ethanol and then further fractionated the extract with rivanol and acetone. He claimed to have isolated fractions with biological activities similar to those of mammalian follicle stimulating hormone (FSH) and luteinizing hormone (LH). Robertson and Rinfret (1957) extracted the pituitaries with a solution of acetic acid and acetone, and precipitated gonadotropin with acetone. Using a similar method Schmidt *et al.* (1965) obtained a partially purified preparation of salmon gonadotropin. Grönlund (1969) extracted the pituitaries with phosphate-buffer saline and chromatographed the extract on Sephadex G-25. Gonadotropic activity was located in the retarded peak. A partially purified gonadotropin preparation from the chinook salmon *Oncorhynchus tshawytscha*, designated SG-G100, was obtained by Donaldson *et al.* (1972). Following an initial extraction of the pituitaries with an ethanolic ammonium acetate buffer and subsequent precipitation with ethanol, the crude gonadotropin precipitate was dissolved in buffer and chromatographed on Sephadex G-100. Activity was located in the ascending one-half of the second peak and the pooled fractions after lyophilization constituted SG-G100. The SG-G100 was further purified on DEAE-cellulose using sodium glycinate buffer (pH 9.2) to yield a retarded fraction containing gonadotropic activity designated SG-DEAE3. Pierce *et al.* (1976) further purified SG-G100 by another method. The SG-G100 was subjected to affinity chromatography on Con A–Sepharose to remove Con AI material which accounted for about one-third of the protein content of SG-G100. The Con AII fraction was next subjected to ion-exchange chromatography on CM cellulose and then on DEAE cellulose. Pierce and co-workers found two related fractions termed SG-DEAE I and SG-DEAE II which gave similar tryptic peptide maps and amino-acid compositions. Based on the finding of cross-reactivity between the *S*-carboxymethylated salmon gonadotropin fractions and an antiserum raised against reduced, *S*-carboxymethylated bovine α-LH subunit, they suggested that salmon maturational hormone (the gonadotropin isolated from the Con AII fraction) contained a sequence related to the α subunit of mammalian pituitary glycoprotein hormones. The homology became discernible only after unfolding of the polypeptide chain because there was no cross-reaction against an antiserum to bovine LH, an antiserum to bovine thyroid stimulating hormone (TSH), or antisera to the subunits of LH and TSH. Despite the high degree of purification, the gonadotropic fractions had very low biological activity presumably because of denaturation.

Yoneda and Yamazaki (1976) utilized a method similar to that of Donaldson *et al.* (1972) to purify gonadotropin from the pituitaries of the chum salmon *Oncorhynchus keta*. The pituitaries were extracted with 40% ethanol, and ethanol was then added to the extract to give a final concentration of 85%. The precipitate thus obtained was dried with acetone, dissolved in buffer, and chromatographed on Sephadex G-100 using 0.1 *M* NH_4HCO_3 at pH 8.3. Gonadotropic activity was located in the second peak, which was then chromatographed on DEAE-cellulose equilibrated and eluted with Tris-Cl buffer. The column was eluted either with a linear or with a stepwise concentration gradient of NaCl. The purified material was found in an adsorbed fraction.

Breton *et al.* (1976) prepared a gonadotropin preparation from the pituitaries of the rainbow trout *Salmo gairdneri* using gel filtration and DEAE-cellulose chromatography followed by rechromatography on Sephadex.

Idler and co-workers were the first group to present evidence of the existence of more than one type of gonadotropin in the chum salmon pituitary using teleost assays. After an initial fractionation of the pituitary extract on Con A–Sepharose into a Con AI fraction and a Con AII fraction, the Con AII fraction was subjected to gel filtration on Sephadex G-75. Gonadotropic activity resided in the ascending portion of the retarded peak designated G-75 fraction II (Idler *et al.*, 1975b). The G-75 fraction II was resolved by DEAE-Bio-Gel A chromatography into two gonadotropic fractions with identical molecular weight which exhibited distinct behavior in isoelectric focusing and which were sex specific in stimulating cyclic adenosine 5′ monophosphate (cAMP) production by trout gonadal tissue.

Subsequently, Campbell (1978) demonstrated that the Con AI fraction from chinook salmon pituitaries stimulated incorporation of vitellogenin into trout oocytes *in vitro*. Ng and Idler (1978b) and Idler and Ng (1979) isolated two forms of vitellogenic hormone from the Con AI fraction of chum salmon pituitaries, from two chromatographic fractions with MW 25,000 and 45,000, respectively. Salmon vitellogenic hormone was adsorbed on DEAE-Bio-Gel A in 5 m*M* NH_4HCO_3 at pH 9, and unadsorbed on CM–Bio-Gel A in 3 m*M* ammonium acetate at pH 6.

Breton *et al.* (1978), employing chromatographic procedures similar to those utilized by Idler *et al.* (1975b,c), found that the male chinook salmon pituitary Con AII fraction contained a maturational hormone which differed from its female counterpart in biological specific activity of stimulating oocyte maturation in female trout. There also may be a sex-specific gonadotropin in sturgeon (Goncharov *et al.*, 1980).

2. Plaice and Flounder

Campbell and Idler (1976, 1977) were the first to report the purification of two distinct gonadotropic fractions, with vitellogenic and maturational

ovulatory activities, respectively, from pituitaries of the American plaice *Hippoglossoides platessoides*. The pituitary extract was initially fractionated on Con A–Sepharose into a Con AI fraction and a Con AII fraction. The Con AI fraction was further purified on Sephadex G-75 and vitellogenic activity was located in tubes with the range of MW 25,000–35,000. Ng and Idler (1978a, 1979) conducted the purification further and detected vitellogenic activity in a large MW fraction (MW 62,000) and a small MW fraction (MW 28,000). The two fractions had comparable vitellogenic activities. They were both adsorbed on DEAE-Bio-Gel A in 5 m*M* ammonium bicarbonate at pH 9, and were eluted at comparable NH_4HCO_3 concentrations. Further purification could be achieved by chromatography on carboxymethyl cellulose. Vitellogenic activity was located in the fraction unadsorbed on the ion exchanger in 3 m*M* ammonium acetate at pH 5.5.

Plaice pituitary Con AII fraction with maturational ovulatory activity was purified successively by gel filtration and ion-exchange chromatography on DEAE-Bio-Gel A and CM-Bio-Gel A. Gonadotropic activity was detected in a large MW fraction (MW 62,000) and a small MW fraction (MW 28,000) which were subsequently adsorbed on DEAE-Bio-Gel A and unadsorbed on CM-Bio-Gel A.

Vitellogenic and maturational hormones were isolated from pituitaries of the winter flounder *Pseudopleuronectes americanus* with similar procedures (Ng and Idler, 1978b, 1979). The hormones existed in a big and a small molecular-weight form with chromatographic characteristics similar to those of the corresponding plaice gonadotropin.

3. Cyprinids

A partially purified gonadotropin preparation was prepared from acetone-dried pituitaries of the carp *Cyprinus carpio* by Clemens *et al.* (1964). An aqueous extract of the pituitary powder was chromatographed on Sephadex G-75 and the gonadotropic preparation had a molecular weight of 50,000.

Carp gonadotropin has also been prepared by Burzawa-Gerard (1971). The methodology employed involved alcoholic percolation, gel filtration on Sephadex G-50 to remove low-molecular-weight material, ion-exchange chromatography on DEAE-cellulose, gel filtration on Sephadex G-100, and preparative electrophoresis.

Haider and Blum (1977) presented evidence for the existence of two gonadotropins in the goldfish *Carassius auratus*. One electrophoretic band obtained by electrophoresing the pituitary extract stimulated only spermatogenesis while another band stimulated both spermatogenetic and interstitial tissue activities. Both bands stimulated ^{32}P uptake into the fish testis. Interestingly, Haider and Blum could find only one electrophoretic band possessing gonadotropic activity when they subjected the carp pitui-

tary extract to electrophoresis. Two types of gonadotropins, vitellogenic hormone and maturational hormone, were isolated from the carp pituitary by Idler and Ng (1979) with the same procedure they used to isolate vitellogenic hormone and maturational hormone from the pituitaries of salmon, plaice, and flounder (Idler and Ng, 1979).

A saline extract of punctius carp pituitary, on chromatography on a gel-filtration column, yielded several peaks, one of which possessed ovulatory activity (Sinha, 1971; Sundararaj and Samy, 1974).

4. Sturgeon

Sturgeon (*Acipenser stellatus*) gonadotropin was prepared by Burzawa-Gerard *et al.* (1975a) using a protocol similar to that used in the isolation of carp gonadotropin (Burzawa-Gerard, 1971) involving alcoholic percolation, gel filtration on Sephadex G-100, and ion-exchange chromatography on DEAE-cellulose. The only difference in methodology is that DEAE-cellulose chromatography was conducted at pH 9.4 instead of pH 7.7 in view of the finding that sturgeon gonadotropin was less acidic than carp gonadotropin and less stable at high ionic strength (Burzawa-Gerard *et al.*, 1976). Sturgeon gonadotropin was active in eliciting frog spermiation and also amphibian and sturgeon oocyte maturation. It is noteworthy that sturgeon gonadotropin was more active in amphibian spermiation and ovulation assays than in the goldfish assay.

5. Other Teleosts

a. Catfish. Sundararaj and Samy (1974) subjected a saline extract of catfish pituitaries to gel filtration on Sephadex G-100. Maturational ovulatory activity was detected in the second peak. A more highly purified preparation was obtained by Burzawa-Gerard *et al.* (1980) using the procedure described for carp gonadotropin (Burzawa-Gerard, 1971).

b. Whitefish. Breton (1968) extracted an acetone powder of *Coregonus lavaretus* pituitaries with saline at pH 7. The extract was dialyzed prior to chromatography on Sephadex G-100 in 0.1 *M* phosphate buffer at pH 6.3. Gonadotropic activity (measured in the ability to induce spermiation) was located in the ascending portion of the second peak.

c. Mudfish. A mudfish pituitary extract was fractionated by either preparative polyacrylamide gel electrophoresis or DEAE-cellulose chromatography followed by gel filtration on Sephadex G-50 (Hattingh and Du-Toit, 1973). Two fractions obtained from preparative electrophoresis possessed both gonadotropic and exophthalmic activities. A fraction retarded on

DEAE-cellulose, when analyzed by polyacrylamide gel electrophoresis, was shown to contain these two gonadotropic and exophthalmic fractions.

d. Tilapia. Farmer and Papkoff (1977) employed an alkaline extraction procedure for *Tilapia* pituitaries. The extract was chromatographed successively on Amberlite CG-50 and DEAE-cellulose. The gonadotropic material was adsorbed on Amberlite CG-50 and unadsorbed on DEAE-cellulose. After precipitation of inert material with 0.2 *M* metaphosphoric acid, the material was purified on Sephadex G-100 as the final step. No distinct peak was observed. Tubes with gonadotropic activity were pooled and lyophilized. It is interesting to note that *Tilapia* gonadotropin resembled LH in both its biological activity and chromatographic behavior. One fraction which resembled FSH in its chromatographic behavior had not been assayed for biological activities.

Hyder *et al.* (1979) applied the scheme of pituitary hormone fractionation developed by Stockell Hartree for mammalian (Stockell Hartree, 1966) and avian (Stockell Hartree and Cunningham, 1969) gonadotropins to purify *Tilapia* gonadotropin. An initial extraction of the pituitaries with 6% ammonium acetate at pH 5.1 was followed by precipitation with ethanol. The precipitate was redissolved and chromatographed on carboxymethyl cellulose at pH 5.5 in 4 m*M* ammonium acetate. Gonadotropic activity, assayed by the ability to stimulate spermatogenesis and interstitial cell size and nuclear size, was concentrated in the unadsorbed fraction (CM I) although the adsorbed fraction (CM II) also had some gonadotropic activity. As far as chromatographic characteristic on carboxymethyl cellulose is concerned, the gonadotropic fraction resembled mammalian FSH. However, steroidogenic activity reflected in stimulation of interstitial tissue also concentrated in this fraction. Hyder (1970) noticed a temporal separation between spermatogenetic and interstitial cell activities in the pond specimens of *Tilapia* that he collected, and proposed on the basis of this observation distinct gonadotropins regulating the two events of spermatogenesis and interstitial cell activity. The chromatographic evidence is, therefore, apparently not in line with his earlier hypothesis.

e. Pike Eel. Huang *et al.* (1981) extracted pike eel (*Maraenesox cinereus*) pituitaries with 40% ethanol–6% amonium acetate at pH 5.1 according to the method of Stockell Hartree (1966). A crude glycoprotein precipitate was obtained by adjusting the ethanol concentration to 80%. After desalting on Sephadex G-25, the proteins were subjected to preparative polyacrylamide gel electrophoresis. The bands were sliced and eluted. Bioassay revealed that the proteins differed in biological potency although they had similar sodium dodecyl sulfate (SDS) electrophoretic patterns and amino-acid compositions. Chromatography on DEAE-cellulose instead of preparative poly-

acrylamide gel electrophoresis yielded a peak unadsorbed in 5 m*M* ammonium bicarbonate with no gonadotropic activity, and adsorbed proteins could be eluted with stepwise increasing concentrations of 30, 50, 80, and 120 m*M* ammonium bicarbonate. These peaks, when analyzed by polyacrylamide gel electrophoresis, were observed to comprise combinations of the isohormones which could be obtained by preparative electrophoresis. The peak eluted by 80 m*M* NH_4HCO_3 had the highest biological activity. It was speculated that the isohormones arose as a result of differences in the carbohydrate content and/or microheterogeneity of amino acids as in the case of mammalian pituitary glycoprotein hormones (Huang *et al.*, 1981).

III. BIOLOGICAL ACTION

A. Bioassays

A variety of bioassays have been utilized to monitor gonadotropic activity during isolation. Testosterone output by rat Leydig cells *in vitro* was employed as an assay for *Tilapia* gonadotropin (Farmer and Papkoff, 1977). Pike eel gonadotropin was also active in this assay but much less so than the *Tilapia* hormone (Huang *et al.*, 1981). The 1-day-old cockerel testicular radiophosphate-uptake assay was used in the isolation of salmon gonadotropin by Donaldson *et al.* (1972), Idler *et al.* (1975b), and Pierce *et al.* (1976). The pike eel gonadotropin was also active in this avian assay (Huang *et al.*, 1981). However, Ishii and Yamamoto (1976) found that a salmon pituitary extract was inactive in stimulating hypertrophy of chick Sertoli cells. The discrepancy in their findings may have been attributable to the dosage or to the assay parameters employed.

Amphibian assays involving spermiation and oocyte maturation were used to monitor gonadotropic activity during isolation of carp and sturgeon gonadotropins (Burzawa-Gerard, 1971; Burzawa-Gerard *et al.*, 1975a,b).

There was also a variety of isolation procedures utilizing teleost assays. Yoneda and Yamazaki (1976) purified chum salmon gonadotropin with the goldfish spermiation assay (Yamazaki and Donaldson, 1968a). Stimulation of testicular growth (Schmidt *et al.*, 1965) and gonadal cAMP (Idler *et al.*, 1975b) in immature trout, stimulation of lipovitellin production (Grönlund, 1969), oocyte maturation *in vitro* (Breton *et al.*, 1978; Hirose, 1980) and *in vivo* (Ng and Idler, 1978b, 1979; Idler and Ng, 1979; Sundararaj and Nayyar, 1976), testicular interlobular and intralobular histology (Hyder *et al.*, 1979), testicular steroidogenesis (Huang *et al.*, 1981), and vitellogenesis (Sundararaj *et al.*, 1972a,b; Campbell and Idler, 1976; Campbell, 1978; Ng and

Idler, 1978a) have all been used. Mukherjee and Bhattacharya (1981) found gonadotropin induced a depletion of free cholesterol in the mature ovary of murrel (*C. punctatus*) and this formed the basis of an assay in the range of 1–10 μg.

Despite the finding of Donaldson (1973) that salmon-gonadotropin preparation SG-G100 was active in a variety of species, it is advisable to use, wherever possible, the same or a closely related species in biological studies, to circumvent the problem of biological specificity. When Fontaine *et al.* (1972) compared the potencies of their carp-gonadotropic preparation (Burzawa-Gerard, 1971) with salmon-gonadotropin SG-G100 (Donaldson *et al.*, 1972) in stimulating adenylate cyclase activity in the goldfish, he found that the carp gonadotropin was 36-fold more potent than SG-G100. Therefore, the discrepancy in potencies is attributable to the fact that the goldfish is taxonomically closer to carp than to salmon. The possibility must be taken into account that the carp-gonadotropic preparation and SG-G100 may be of different degrees of purity, but it is very likely that the same result would obtain after the differences in the extent of purification have been allowed for. Thus carp (*Cyprinus carpio*) maturational hormone was about 5 times more active than salmon maturational hormone in stimulating cAMP production by grass carp (*Ctenopharynogodon idellus*) ovaries *in vitro* (Idler and Ng, 1979). Carp gonadotropin was more active than catfish gonadotropin in stimulating the production of cAMP in eel ovary and both were much more active than was sturegon gonadotropin (Dufour *et al.*, 1979). It has been the interest of many comparative endocrinologists to test the effect of mammalian gonadotropins on fish reproduction. There is a discrepancy between the actions of mammalian and teleost gonadotropins on vitellogenesis in the catfish. Induction of vitellogenin into the circulation was achieved by mammalian gonadotropins, but no incorporation of the yolk precursor into the oocytes resulted (Nath and Sundararaj, 1981). This phenomenon again illustrates the principle of zoological specificity of gonadotropins. In the assay of maturational activity using the germinal vesicle breakdown of *Oryzias latipes* oocytes *in vitro* as the assay parameter, it was found that ovine LH was less potent than SG-G100 and the slopes of the dose-response curves were different (Hirose, 1980). Carp gonadotropin and mammalian LH differed in their actions in stimulating eel ovarian cAMP (Fontaine *et al.*, 1981). Carp gonadotropin produced a 30-fold stimulation, but mammalian LH achieved maximal two- and-one-half-fold stimulation, and the assay was much more sensitive to carp gonadotropin than to LH. Pike eel gonadotropin was much more active in stimulating testosterone production by carp testis *in vitro* than by rat Leydig cells (Huang *et al.*, 1981). Although it is generally accepted that mammalian LH and human chorionic gonadotropin (HCG) are active in teleost (Anand and Sundararaj, 1974; Sundararaj and Goswami, 1966), yet

spermatogenesis in the goldfish could not be stimulated by mammalian LH (Billard *et al.*, 1970). Luteinizing hormone and HCG were inactive in inducing *in vitro* oocyte maturation in the Indian carp *Labeo rohita* and *Cirrhina mrigala* (Sundararaj *et al.*, 1981). The HCG was also not able to enhance vitellogenesis in the goldfish (Yamazaki, 1965) or to increase estradiol output from plaice ovaries *in vitro* (Yaron and Barton, 1980). None of the mammalian gonadotropins tested by Idler *et al.* (1975b) elevated cAMP production by the trout gonad.

In contrast to the previously held consensus that mammalian FSH was inactive in the teleost and that any activity observed was attributable to contamination with LH, Bona-Gallo and Licht (1981) found that ovine FSH was active in stimulating testosterone secretion *in vitro* by testicular preparations of *Salmo gairdneri*, *Cichlasoma citrinellum*, and *Sarotherodon mossambica*, and in the case of *Salmo*, ovine FSH was even more active than LH. Mammalian FSH was also found to stimulate hepatic vitellogenin production in the catfish *Heteropneustes fossilis* (Nath and Sundararaj, 1981).

Salmon gonadotropin SG-G100 had no effect on the processes of luteinization and progesterone secretion in cultured monkey granulosa cells (Channing *et al.*, 1974). Carp gonadotropin was not active in the rat HCG augmentation test for mammalian FSH and the rat ovarian ascorbic acid depletion test for mammalian LH (Burzawa-Gerard, 1974). Therefore, mammalian bioassays could seldom be utilized to monitor gonadotropic activities during the isolation of teleost gonadotropins.

B. Biological Activities

1. Spermatogenesis

Both SG-G100 and carp gonadotropin have been shown to stimulate spermatogenesis in the teleost (Sundararaj *et al.*, 1971; Burzawa-Gerard, 1974; Leloup-Hatey *et al.*, 1981). An effective photothermal period to elevate plasma GtH and induce spermatogenesis in rainbow trout is a gradually decreasing photoperiod from 16 hr to 8 hr at about 16° C (Breton and Billard, 1977). Salmon maturational hormone reinitiated spermatogenesis in the hypophysectomized flounder (Ng *et al.*, 1980b) and induced spermatogenesis in the rainbow trout (Upadhyay, 1977). *Tilapia* gonadotropin stimulated spermatogenesis in hypophysectomized *Tilapia* (Hyder *et al.*, 1979). Maturational hormone stimulated 11-ketotestosterone and testosterone production in the male (Ng and Idler, 1980). The high concentration of 11-ketotestosterone in males and barely detectable levels in females suggests that it plays an important role in regulating testicular activities (Campbell *et al.*, 1980). A probable role of maturational hormone and 11-ketotestosterone in

spermatogenesis was suggested by high levels of the hormones coincident with precocious gonadal development in male Atlantic salmon (Dodd *et al.*, 1978). It has been reported that androgens stimulate spermatogenesis in the teleost (Billard, 1974; Lofts *et al.*, 1966; Nayyar *et al.*, 1976; Remacle, 1976). However, in other cases androgens are inactive. Intraperitoneally administered testosterone did not stimulate gonadal growth in juvenile male rainbow trout (Crim and Evans, 1979) and a similar observation was made by Upadhyay (1977). Probably a local concentration of androgens in the testis, produced in response to maturational hormone, is much more effective in stimulating spermatogenesis than exogenous androgens.

2. Spermiation

In the hypophysectomized goldfish, spermiation could be induced by SG-G100, and androgens mimicked the stimulatory action of SG-G100 on spermiation (Yamazaki and Donaldson, 1968b, 1969). The advent of spermiation in trout was accompanied by an increase in the circulating titer of androgens (Sanchez-Rodriguez *et al.*, 1978). Therefore the action of teleost gonadotropin in inducing spermiation may be linked to the hormonal effect on testicular steroidogenesis. This may be true in the case of salmon maturational hormone which induced spermiation as well as testicular steroidogenesis in the hypophysectomized flounder (Ng *et al.*, 1980b).

Carp gonadotropin was capable of eliciting spermiation in the amphibian (Burzawa-Gerard, 1974).

3. Testicular Steroidogenesis

Tilapia gonadotropin was found to be quite active in stimulating testosterone output from rat Leydig cells (Farmer and Papkoff, 1977). Pike eel gonadotropin was less so (Huang *et al.*, 1981). When compared with mammalian LH, both carp gonadotropin and SG-G100 had a much lower potency in stimulating androgen release from isolated quail testicular cells (Jenkins *et al.*, 1978). The SG-G100 was not steroidogenic in the male turtle *Chrysemys picta* (Lance *et al.*, 1977).

The SG-G100 caused an increase in the activity of 3β-hydroxysteroid dehydrogenase in the goldfish testis (Yamazaki and Donaldson, 1969). Carp gonadotropin stimulated testicular steroidogenesis in the immature eel *Anguilla anguilla* (Leloup-Hatey *et al.*, 1981). Pike eel gonadotropin enhanced *in vitro* testosterone production by carp testis (Huang *et al.*, 1981).

Administration of maturational hormones from plaice, flounder, salmon, and carp restored testicular steroidogenesis in the hypophysectomized flounder. The plasma levels of both 11-ketotestosterone and testosterone were elevated. Administration of vitellogenic hormones produced no

steroidogenic effect (Ng and Idler, 1980). Salmon maturational hormone greatly enhanced 11-ketotestosterone production in immature trout. The regulatory role of maturational hormone in testicular steroidogenesis was further demonstrated by the inhibitory effect of an antiserum to maturational hormone on the process (Ng and Idler, 1980).

Teleost maturational hormone did not alter the ratio of free androgen to its bound form (Ng and Idler, 1980). Likewise Leloup-Hatey *et al.* (1981) found that both the free form of testosterone and its glucuronoconjugate increased under stimulation of SG-G100.

4. Vitellogenesis and Ovarian Estrogen Secretion

Hormonal control of the vitellogenic process and ovarian estrogen secretion has been covered in detail in Chapter 8, this volume.

5. Oocyte Maturation and Ovulation

Various teleost gonadotropin preparations induced oocyte maturation and ovulation in teleost, e.g., SG-G100 (Sundararaj *et al.*, 1972a,c), carp gonadotropin (Burzawa-Gerard, 1974), and pike eel gonadotropin (Huang *et al.*, 1981). Maturational hormones from salmon (Breton *et al.*, 1978; Idler and Ng, 1979), carp (Idler and Ng, 1979), plaice (Ng and Idler, 1978a), and flounder (Ng and Idler, 1979) pituitaries but not the vitellogenic hormones (Ng and Idler, 1978a, 1979; Idler and Ng, 1979) accelerated the process of oocyte maturation and ovulation. Antisera to maturational hormones inhibited the process in salmon and flounder (Ng *et al.*, 1980a).

In view of the numerous reports of isohormones among mammalian and even teleost gonadotropins (Huang *et al.*, 1981), it is surprising that only one band showed ovulatory activity when pituitary proteins of two cyprinids, two salmonids, and two sturgeons, were separated by polyacrylamide gel electrophoresis and tested for their ability to stimulate ovulation of a loach (Burlakov and Labedeva, 1976).

Precocious induction of oocyte maturation and ovulation with SG-G100, followed by successful fertilization of the ova was achieved in the coho salmon *Oncorhynchus kisutch* (Jalabert *et al.*, 1978). There was a latent period between the initial sharp rise in plasma maturational hormone and the time of ovulation in the goldfish *Carassius auratus* (Stacey *et al.*, 1979).

Gonadotropin was active *in vitro* in trout (Jalabert *et al.*, 1974; Breton *et al.*, 1976), medaka *Oryzias latipes* (Hirose, 1971; Hirose and Donaldson, 1972), but not in the catfish *Heteropneustes fossilis* (Sundararaj and Goswami, 1977) and not in the yellow perch *Perca flavescens* (Goetz, 1976).

Gonadotropins exhibit species specificity which may be reflected in a

time delay in response or in failure to bring about a response. Therefore, ovulation of loach (*Misgurnus fossilis* L.) was induced 24 hr after the injection of GtH from two cyprinids, 48 hr after GtH from two salmonids was administered, and there was no ovulation when the pituitary proteins of pike and pike-perch were given (Burlakov and Labedeva, 1976). The *in vitro* maturation of carp oocytes was induced by pituitary homogenates from six teleosts in addition to carp. Within the confines of a 24-hr period, trout and pike were less effective than the other species tested, but no statistical treatment of the data was given (Epler *et al.*, 1979). Partially purified salmon and carp GtH induced migration and breakdown of the germinal vesicle (GVBD) in walleye oocytes, but were relatively ineffective on closely related yellow perch (Goetz and Bergman, 1978).

The mechanism of gonadotropic action on the maturing oocyte has been elucidated mainly by using SG-G100. Maturation of trout oocytes treated with maturational gonadotropin requires the presence of the follicle whereas appropriate steroids can mature the naked oocyte (Jalabert, 1976). This was confirmed for the medaka, *Oryzias latipes*, and the presence of granulosa cells of large pre- and postovulatory follicles were implicated in oocyte maturation. Removal of the follicle cells did not prevent the action of steroids on the oocyte (Iwamatsu, 1980), in contrast to another report on the same species (Hirose, 1972); the differences appear to be attributable to a difference in technique for removal of the follicle. Progestogens including 17α-hydroxyprogesterone and especially 17αOH, 20β-dihydroprogesterone have been implicated as the maturational steroids by *in vitro* and *in vivo* studies on a number of teleost species including trout, goldfish, carp, salmon, and flounder (Fostier *et al.*, 1973; Campbell, 1975; Jalabert *et al.*, 1978; Ng and Idler, 1980; Nagahama *et al.*, 1980).

In retrospect, perhaps it is not surprizing that 17αOH, 20β-dihydroprogesterone plays an important role in the spawning female because the first natural product from which it was isolated was the blood of spawning female Pacific salmon (Idler *et al.*, 1960). Although the quantity is larger in the female, this steroid has been reported in mature males (Schmidt and Idler, 1962), and no one seems to have looked for a possible function. It was found that 17αOH, 20β-dihydroprogesterone administered alone induced oocyte maturation, but induced only partial ovulation or no ovulation. There was a synergistic action between the pituitary and either 17α-hydroxyprogesterone or 17αOH,20β-dihydroprogesterone on ovulation (De Montalambert *et al.*, 1978; Jalabert *et al.*, 1978). These steroids were shown to be present in circulation in trout undergoing oocyte maturation and their presence seemed to be correlated with the maturational process (Campbell *et al.*, 1980). Incubation of Ayu (*Plecoglossus altivelis*) oocytes with SG-G100 led to an enhanced production of 17αOH,20β-dihydroprogesterone (Suzuki *et al.*,

1981). *In vitro* production of the maturational steroid by amago salmon (*Oncorhynchus rhodurus*) ovaries was demonstrated by Tamaoki *et al.* (1981).

However, in other species, such as the catfish *Heteropneustes fossilis* and the yellow perch, another mechanism of gonadotropic action operates. Induction of oocyte maturation could not be achieved by incubation of the oocytes with gonadotropin. Presence of interrenal tissue in the culture was necessary before gonadotropin could exert its action. Sundararaj and Goswami (1977) have postulated a mechanism by which gonadotropin stimulates the interrenal to produce a maturational steroid, which is probably cortisol in the Indian catfish. The finding of Truscott *et al.* (1978) is consistent with the foregoing hypothesis. Plasma cortisol in the catfish could be elevated by gonadotropin (SG-G100) treatment in both sexually regressed and gravid catfish. The interrenal origin of cortisol was demonstrated by the finding that ovariectomy in sexually regressed fish could not inhibit the action of mammalian LH on plasma cortisol. Another possible role of corticosteroids including cortisol is to render the oocytes more sensitive to gonadotropin in those species having progestational maturational steroids (Jalabert, 1976).

In the sturgeon *Acipenser stellatus*, triiodothyronine restored the ability of the follicular epithelial cells, lost after exposure to unfavorable conditions, to respond to gonadotropin by stimulating oocyte maturation (Dettlaff and Davydova, 1979).

C. Gonadal Receptors

The presence of binding sites in the flounder ovary for vitellogenic hormone and maturational hormone was demonstrated with the immunofluorescence technique (Ng *et al.*, 1980b). Vitellogenic oocytes, which have follicular envelopes, and the large immature oocytes due to become vitellogenic in the following year both bound vitellogenic hormone. Binding sites for maturational hormone were located in the perinuclear region and the follicular envelope of vitellogenic oocytes. Binding sites also existed in the interstitial tissue between oocytes. The locations of the binding sites correlated well with the physiological actions of the hormones.

Aida and Ishii (1981) demonstrated a specific binding of radioiodinated salmon gonadotropin to testicular receptor preparations of salmon, rainbow trout, and goby. However, specific binding to testicular preparations of flounder, *Tilapia*, goldfish, and carp was low. The specific binding was dependent on temperature, pH and tissue concentration, and was favored at a high temperature and a pH of 7.4. Mammalian FSH had no inhibitory effect on the binding, but huge doses of mammalian LH and HCG were needed to displace radioiodinated salmon gonadotropin from binding to the testicular

preparation. The presence of multiple binding sites with different affinities, or a negative cooperativity between binding sites, was indicated by a curvilinear Scatchard plot of the binding data.

Radioiodinated carp gonadotropin, after being purified on Con A–Sepharose, was used for studies of binding to ovarian receptors in the goldfish (Cook and Peter, 1980c). Autoradiography localized the hormone in the thecal region surrounding mature oocytes. The radioiodinated hormone and the cold hormone competed for the same binding sites.

IV. CHEMISTRY

A. Hormones

Vitellogenic hormones existing in a high- and a low-molecular-weight form were isolated from plaice, flounder, and salmon pituitaries. Large and small forms of maturational hormone were also isolated from plaice and flounder pituitaries. The small forms all were MW 25,000–28,000, but the molecular weights of the large form ranged from 45,000–62,000. All the teleost gonadotropins isolated to date have a molecular weight around 25,000–35,000, but it should be borne in mind that the carbohydrate moieties of these gonadotropic glycoprotein hormones may affect the value of the molecular weight as determined by gel filtration. The gonadotropins whose molecular weight were thus determined include those of trout (Breton *et al.*, 1976), salmon (Donaldson *et al.*, 1972; Pierce *et al.*, 1976), sturgeon (Burzawa-Gerard *et al.*, 1975a), carp (Burzawa-Gerard, 1971), *Tilapia* (Farmer and Papkoff, 1977), and whitefish (Breton, 1968). Salmon maturational hormone had a MW 42,000 as determined by gel filtration (Idler *et al.*, 1975b,c). Idler *et al.* (1975a) discovered in chum salmon pituitaries two forms of maturational hormone with distinct behavior in isoelectric focusing and sex specificity in stimulating cAMP by trout gonadal tissue. Maturational hormone isolated from male chinook salmon pituitaries was shown by Breton *et al.* (1978) to be different from the hormone from female glands in biological specific activity of stimulating oocyte maturation in the female trout. Recently Huang *et al.* (1981) obtained four types of gonadotropin from pike eel pituitaries with the same amino-acid composition and SDS electrophoretic pattern. The carp gonadotropic preparation of Burzawa-Gerard (1971), after adsorption to Con A–Sepharose, could be eluted as several fractions by applying a gradient of α-methyl-D-glucoside (Burzawa-Gerard, 1982). The isolated sturgeon gonadotropin (Burzawa-Gerard *et al.*, 1975a,b) existed as a mixture of several components with similar biolog-

ical activities and chemical characteristics including molecular weight, sialic acid content, and the NH_2-terminal amino acid, but with different isoelectric points (Burzawa-Gerard, 1982).

The inclusion of dithiothreitol in the buffer (Idler *et al.*, 1975b,c) minimized oxidation and may have contributed to preserve the biological activity of salmon maturational hormone (Pierce *et al.*, 1976). Ethylenediaminetetraacetic acid (EDTA) in the buffer functioned to minimize conversion to larger molecular species. However, sodium chloride and dithiothreitol did not reduce the extent of aggregation at the concentrations tested (Ng and Idler, 1978a).

The total hexose content of maturational hormone preparations from various species including plaice, flounder, salmon, and carp ranged from 6% to 15%, compared with the content of 1–2% for vitellogenic hormones from the same species. The amino sugar and sialic acid contents were also higher for maturational hormones than for vitellogenic hormones (Idler and Ng, 1979; Ng and Idler, 1979). The sugar content reported for carp gonadotropin (Burzawa-Gerard and Fontaine, 1972), salmon gonadotropin (Yoneda *et al.*, 1977), *Tilapia* gonadotropin (Farmer and Papkoff, 1977), and pike eel gonadotropin fell in the range of values reported for maturational hormones (Idler and Ng, 1979; Ng and Idler, 1979).

Vitellogenic hormone was unadsorbed on wheat germ lectin–Sepharose and *Helix pomatia* lectin–Sepharose in accordance with its low hexosamine content (Idler and Ng, 1979). Differences in hexose contents account for the distinct chromatographic behavior of vitellogenic hormone and maturational hormone on Con A–Sepharose and lentil lectin–Sepharose.

The elution profiles of pituitary Con AI fractions from various species from DEAE-Bio-Gel A were similar. Vitellogenic hormone was eluted in approximately the same position in each profile (Idler and Ng, 1979; Ng and Idler, 1979). The same gonadotropic preparations of Donaldson *et al.* (1972), Pierce *et al.* (1976), and Yoneda and Yamazaki (1976) were all adsorbed on DEAE-cellulose. Similarly trout gonadotropin (Breton *et al.*, 1976), carp gonadotropin (Burzawa-Gerard, 1971), sturgeon gonadotropin (Burzawa-Gerard *et al.*, 1975a), and pike eel gonadotropin (Huang *et al.*, 1981) were adsorbed on DEAE-cellulose. Maturational hormones from salmon, carp, plaice, and flounder pituitaries were all adsorbed on DEAE-Bio-Gel A (Idler and Ng, 1979; Ng and Idler, 1979). The preparation of *Tilapia* gonadotropin of Farmer and Papkoff (1977) were unadsorbed on DEAE-cellulose in 30 m*M* ammonium bicarbonate at pH 9.

The amino acid compositions of gonadotropic preparations from *Tilapia* (Farmer and Papkoff, 1977), carp (Burzawa-Gerard, 1971), salmon (Yoneda *et al.*, 1977), pike eel (Huang *et al.*, 1981), and trout (Breton *et al.*, 1976) have been reported. Amino acid compositions of chum salmon, carp, and

American plaice maturational and vitellogenic hormones were reported by Idler and Ng (1979) and Ng and Idler (1979), and that of chinook salmon maturational hormone by Pierce *et al.* (1976). Both vitellogenic hormone and maturational hormone were rich in aspartic acid, glutamic acid, serine, and leucine. Generally speaking, vitellogenic hormones had more glutamic acid than maturational hormones, but maturational hormones were richer in their threonine contents.

The vitellogenic pituitary fractions did not cross-react to any significant extent in radioimmunoassays for the maturational fractions and vice versa, and the vitellogenic fractions were unable to stimulate oocyte maturation, indicating minimal cross-reaction. Incubation of maturational hormone with a massive dose of carbohydrate-splitting enzymes did not appear to affect its immunological potency, indicating that the immunological difference between the two gonadotropins was attributable to differences in their protein moieties. An antiserum to flounder maturational hormone and an antiserum to flounder vitellogenic hormone was localized in regions of the flounder pituitary which did not show considerable overlap. Flounder collected after initiation of the vitellogenic season had many active vitellogenic gonadotrops and few maturational gonadotrops; however, flounder caught near spawning had active maturational gonadotrops (Burton *et al.*, 1981).

The yields of gonadotropins from various fish pituitaries, including those of carp, sturgeon, pike eel (see Huang *et al.*, 1981), salmon (Idler *et al.*, 1975b), plaice, and flounder (Ng and Idler, 1979), were high compared with those of mammalian LH and FSH, which have been quoted by Huang *et al.* (1981) to be 0.6 and 0.003%, respectively.

B. Subunits

Preparations of carp gonadotropin contained as much as 20% subunits (Fontaine and Burzawa-Gerard, 1978). Carp gonadotropin could be dissociated into its subunits by denaturing agents such as 8 *M* urea and propionic acid. The subunits could be separated by chromatography on DEAE-Sephadex. Each subunit retained only a very small percentage of the biological activity of the intact hormone. The subunit I unadsorbed on DEAE-Sephadex had MW 14,000, the subunit II adsorbed on the ion exchanger had MW 17,000. Recombination of the subunits led to a restoration of a significant portion of the original biological activity (Burzawa-Gerard *et al.*, 1976; Jollès *et al.*, 1977).

Partial sequencing of the first 26 amino acids from the NH_2-terminal of subunit II of carp gonadotropin revealed a homology between the subunit and the β subunits of various mammalian LHs and FSH. Two forms of

subunit I of carp gonadotropin existed, differing by the presence or absence of a nonpeptide at the NH_2-terminal. Partial sequencing of the first 33 NH_2-terminal amino acids of the longer form revealed homology with the α subunits of mammalian FSH and LH. Radioimmunoassays specific for subunit I and subunit II, respectively, have been developed. Intact carp gonadotropin was found to cross-react in both assays (Fontaine and Burzawa-Gerard, 1978; Burzawa-Gerard, 1982).

Hybrid molecules could be formed by combination of β subunit of carp gonadotropin with the α subunit of mammalian LH, mammalian TSH, or sturgeon gonadotropin, and by combination of the α subunit of carp gonadotropin with the β subunit of sturgeon gonadotropin, but there was no association of the α subunit of carp gonadotropin with the β subunit of mammalian gonadotropin (Fontaine and Burzawa-Gerard, 1978).

It is generally agreed that the β subunit of teleost gonadotropins contributes to zoological specificity and that the α subunit contains the antigenic determinant common to both teleost and mammalian gonadotropins (Dufour and Burzawa-Gerard, 1978; Burzawa-Gerard and Kerdelhue, 1978; Breton, 1981).

Fontaine *et al.* (1981) proposed that the α subunit of carp gonadotropin possessed the intrinsic characteristic of enhancing adenylate cyclase activity and that the β subunit determined the characteristic of the interaction between the intact hormone and the adenylate cyclase receptor system.

Fontaine and Burzawa-Gerard (1977) hypothesized that a common ancestral molecule gave rise by gene duplication to an α subunit and a β subunit. Combination of the subunits yielded a molecule which initially possessed only gonadotropic activity, but later acquired thyroid stimulating activity. Subsequent duplication of the β gene produced a β_1 subunit which later evolved into LH and a β_2 subunit which later evolved into thyroid stimulating hormone (TSH). Duplication of the β_2 subunit gave rise to FSH. Burzawa-Gerard *et al.* (1980) further proposed a homology between their carp gonadotropic preparation and mammalain LH based on the finding of cross-reactivity from bovine LH and its β subunit in a radioimmunoassay for the β subunit of carp gonadotropin, and binding of the bovine LH β subunit by antisera to carp gonadotropin and its β subunit. However, there was only a very low degree of cross-reaction which was not parallel to the standard curve.

Schreibman and Margolis-Kazan (1979) used the immunoperoxidase method to localize cells producing gonadotropin and thyrotropin in the pituitary of *Xiphophorus maculatus*. Both types of cells could be localized using an antiserum to carp gonadotropin (Burzawa-Gerard, 1971). An antiserum raised against the α subunit also localized in both cell types whereas an

antiserum to the β subunit localized only in the gonadotrops. The results imply that in *Xiphophorus*, the α subunits of gonadotropin and thyrotropin are structurally similar.

A study conducted on sturgeon gonadotropin showed that although it could be dissociated into its subunits by methods similar to those employed for carp gonadotropin, the characteristics of the equilibrium between the hormone and its subunits were found to be dissimilar to those for the carp hormone and subunits (Burzawa-Gerard *et al.*, 1976).

Subunits of the pike eel gonadotropin were prepared by dissociation of the intact molecule in propionic acid and then separation by hydrophobic interaction chromatography on phenyl-Sepharose CL-4B (Lo *et al.*, 1981). Subunit I had MW 10,500 and tyrosine as the N-terminal amino-acid residue; subunit II had MW 15,000 and serine as the N-terminal amino-acid residue. By itself, subunit I had no biological activity. Subunit II had a slight 2.8% activity of the intact molecule. Recombination of subunits I and II in 0.05 *M* phosphate buffer (pH 7.4) led to a restoration of 75% of the activity. It was proposed that subunit I corresponded to the α subunit of mammalian gonadotropins, and subunit II corresponded to the β subunit.

Salmon gonadotropin was quite stable in solution: biological activity was retained after 6 months (Donaldson *et al.*, 1978).

The subunit nature of salmon maturational hormone was demonstrated by Donaldson *et al.* (1972), Idler *et al.* (1975c), and by Pierce *et al.* (1976). The hormone was separated into its subunits by incubating the hormone with 8 *M* urea at room temperature for 24 hr and then chromatographing the reaction mixture on DEAE-Bio-Gel A using Tris-Cl buffer at pH 7.8 (Breton, 1981). The subunit unadsorbed under such conditions had a smaller molecular weight (12,500) than the adsorbed subunit (17,000). By comparison with the characteristics of the subunits of mammalian gonadotropin, the unretarded subunit was noted to correspond to the α subunit and the retarded subunit to the β subunit. The individual subunits had very low maturational activity (5.8% of that of the intact hormone). Reassociation of the subunits did not bring about a full restoration of biological activity because of incomplete recombination in too short a time period allowed.

Carp gonadotropin and salmon maturational hormone had reassociation constants which were, respectively, 160 and 5 to 10 times higher than that of mammalian LH (Marchelidon *et al.*, 1978, 1979; Salesse *et al.*, 1978). The β subunit of salmon maturational hormone and a recombination of α and β subunits, had approximately the same radioimmunological potency as the intact hormone, whereas the α subunit gave a nonparallel inhibition curve and its potency was considerably lower than that of the intact hormone (Breton, 1981).

V. RHYTHMS AND REGULATION

Immunological specificity of teleost maturational hormone was demonstrated by Tan and Dodd (1978), by Ng and Idler (1979), by Bye *et al.* (1980), and by Breton (1981). Radioimmunological determination of pituitary and plasma gonadotropin content can generally be made only in closely related species and at this time there is no radioimmunoassay for the vitellogenic gonadotropin.

The availability of radioimmunoassays for salmon and carp maturational hormones (Crim *et al.*, 1973, 1975; Breton *et al.*, 1972) has made possible investigations of the variation in plasma and pituitary gonadotropin levels in salmonid and cyprinid fish throughout the annual reproductive cycle. The salmonid was chosen as a model that lays large yolky eggs and whose spawning is not too environmentally dependent; the cyprinid lays small eggs and spawns when environmental conditions are favorable. In the trout, an increase in pituitary and plasma concentrations of maturational hormone marked the beginning of spermatogenesis when androgens were barely detectable. As spermatogenesis progressed, there was a parallel increase in pituitary and plasma maturational hormone levels, although changes in the pituitary were not as pronounced. Plasma maturational hormone level increased with precocious gonadal development in the male Atlantic salmon parr (Crim and Evans, 1978). During vitellogenesis in the female salmonid there was not much change in the plasma level of maturational hormone. The plasma hormone level peaked around spawning but there was a big difference between the level attained by trout and coho salmon and that attained by pink salmon which was probably attributable to the degree of domestication and reproductive behavior of the particular species (see Billard *et al*, 1978). The high level of maturational hormone that persisted in the circulation after ovulation might be important in maintaining the fecundity of the oocytes retained in the body cavity (Jalabert and Breton, 1980) or it may prime the immature ovary for the next cycle. In the cyprinids a generally similar profile of seasonal variation in plasma gonadotropin level occurred (see Billard *et al.*, 1978). The circadian cycles in serum maturational hormone levels in the goldfish and the effects of photoperiod, temperature, and sexual conditions were investigated by Hontela and Peter (1978). The periovulatory changes in circulatory maturational hormone level were examined in detail by Stacey *et al.* (1979). It was found that the gonadotropin level rose during the day to a maximum value which lasted for about 8 hr during the night, and then declined in the morning after ovulation to a level which was still higher than that in the nonovulating fish.

Plasma and pituitary concentrations of maturational hormone in salmonids were demonstrated by Crim and Evans (1978) to be well correlated.

Gillet and co-workers (1977) showed that plasma GtH was temperature dependent in goldfish. Cook and Peter (1980b) investigated the dynamics of goldfish maturational hormone in the circulation. The pituitary maturational hormone content was studied in fish at different stages of sexual maturation in the annual reproductive cycle. The variation of plasma titer of the gonadotropin was studied with respect to changes in both the reproductive status of the fish and the ambient temperature. Whereas the pituitary content of maturational hormone increased with the progress of sexual maturation, the plasma level was contingent on both the sexual status and the ambient temperature, with gonadal maturation and high temperature favoring a high plasma level.

The rate of utilization of maturational hormone by its target tissue, reflected in ovarian uptake and the metabolic clearance rate, was enhanced, and therefore the initial disappearance half-time was lower in sexually maturing and matured fish than in regressed fish, indicating that maturational hormone had greater physiological significance to the sexually maturing and matured fish than to the sexually regressed fish.

The plasma level is thus a result of the effects of temperature, pituitary secretion rate, gonadal uptake rate, and metabolic clearance rate. It is in a dynamic equilibrium with the aforementioned forces rather than staying at a static level. The sialic acid content of the gonadotropin is a possible determinant of its metabolic clearance rate.

Regressed female goldfish did not exhibit daily fluctuation in the circulating maturational hormone level or showed a daily fluctuation which was smaller in magnitude than those exhibited by maturing and matured fish (Hontela and Peter, 1978). Peter and Crim (1979) hold the opinion that daily fluctuations in circulating gonadotropin level play a role in stimulating and maintaining gonadal development; merely high plasma concentrations of gonadotropin do not always elicit gonadal stimulation.

The temporal variation in gonadal responsiveness to maturational gonadotropin is indicative of a daily fluctuation in the number of gonadal receptors (Peter, 1981). Intraperitoneal administration of testosterone into juvenile rainbow trout led to an increase in the pituitary storage of maturational hormone (Crim and Evans, 1979), but it is not known if this phemonenon is related to the normal maturation process. Nonaromatizable androgens were not effective in stimulating pituitary maturational hormone accumulation, but 1,4,6-androstatriene-3,17-dione, an aromatase inhibitor, decreased the response to testosterone. All three estrogens, estradiol, estrone, and estriol, caused an accumulation of the pituitary gonadotropin by a positive-feedback mechanism (Crim *et al.*, 1981).

The combined effects of pinealectomy, and various photoperiod and temperature regimes on pituitary and plasma gonadotropin levels in the gold-

fish, were studied by de Vlaming and Vodicnik (1977), Vodicnik *et al.* (1978), and by Hontela and Peter (1980). Pinealectomy of goldfish exposed to a long photoperiod and warm temperature brought about a reduction in plasma maturational gonadotropin level and gonadal regression. Peter (1981) found that such treatment abolished the normal daily peak in serum maturational gonadotropin level. A daily peak in serum maturational gonadotropin level, which was normally absent in goldfish exposed to a short photoperiod and warm temperature, appeared after pinealectomy. The aforementioned findings suggest that the pineal regulates pituitary gonadotropin secretion probably by relaying photic information to the hypothalamus which then adjusts its secretion of gonadotropin releasing hormone.

Hypothalamic control of pituitary gonadotropin secretion has been studied by various investigators. Crim and Cluett (1974) found that mammalian LHRH elevated plasma maturational hormone in brown trout. Crim *et al.* (1976) discovered, in the goldfish, that intraventricular injection of hypothalamic extract stimulated maturational hormone secretion from the pituitary. Carp gonadotropin was more active than catfish gonadotropin in stimulating the production of cAMP in eel ovary. Both were much more active than was sturgeon gonadotropin (Dufour *et al.*, 1979). Dibutyryl cAMP, procine LHRH, and carp hypothalamic extract stimulated maturational hormone release from the carp pituitary (Yu *et al.*, 1981). The release of pituitary maturational hormone in the goldfish in response to a superactive LHRH analog D-Ala^{6}des-gly^{10} LHRH ethylamide (Peter, 1980) varied with the reproductive status of the fish; maturing and mature fish were more responsive than sexually regressed fish (Lin *et al.*, 1981). Similarly Crim (1981) found that castration produced an effect on pituitary maturational hormone level in maturing fish but not in postspawned fish, indicating that the hypothalamic–pituitary–gonadal axis operated at its optimal capacity when the fish were undergoing maturation. The existence of a gonadotropin releasing hormone as well as a gonadotropin release-inhibiting hormone in goldfish was indicated by measurement of plasma gonadotropin level after lesioning of appropriate areas of the brain. Dopamine mimicked the actions of this gonadotropin release inhibiting hormone (Peter *et al.*, 1981; Peter, 1982). A gonadotropin releasing hormone was demonstrated by radioimmunoassay (RIA) and high-pressure liquid chromatography (HPLC) in *Tilapia* by King and Millar (1980) and isolated from flounder hypothalami by Idler and Crim (1981) using as assay parameter the *in vitro* stimulation of gonadotropin release from trout pituitary and an RIA.

Breton and Billard (1980) have presented preliminary evidence for an inhibinlike factor in seminal plasma of rainbow trout. Administration of charcoal-treated seminal fluid partially diminished the increase in gonadotropin which followed castration between 2 and 4 hr.

The uptake and clearance of carp and salmon GtH, administered intraperitoneally, has been followed by RIA of plasma in goldfish and rainbow trout (Cook and Peter, 1980a; Crim and Evans, 1976). In both instances clearance was accelerated at higher temperatures.

VI. CONCLUDING REMARKS

Reports on purification of a single gonadotropin from various teleost species appear to be in contrast to the discovery of vitellogenic hormone and maturational hormone by Idler, Campbell, and Ng. The difference stems from the use of or lack of affinity chromatography (Con A–Sepharose) as one of the purification procedures and the fact that most workers did not monitor vitellogenic activity during the course of purification. The gonadotropins that were isolated, as judged from the spectrum of biological activities, correspond to maturational hormones. Some of the gonadotropin preparations (e.g., SG-G100) are known to consist of maturational hormone contaminated to some extent with vitellogenic hormone (Pierce *et al.*, 1976). A carp gonadotropin preparation of Burzawa-Gerard (1971) also contained 5% Con AI material (Burzawa-Gerard, 1982).

Maturational gonadotropin levels in plasma do not appear to correlate well with the reproductive processes. For example, levels of this hormone did not increase when pituitary levels increased and when plasma 11-ketotestosterone levels rose in precocious male salmon parr (Stuart-Kregor *et al.*, 1981). There was no substantial increase to correlate with other than the terminal phase of maturation in females. At this time it is not possible to define the relative importance of other gonadotropins and receptors.

Substantial evidence has been collected for the existence of two gonadotropins, vitellogenic hormone and maturational hormone, in teleost (Idler, 1982). The two hormones manifest distinctive chromatographic, compositional, immunological, and biological characteristics. Antiserum to each of these two hormones produces inhibitory effects on those aspects of fish reproduction which are consistent with the biological activities of the hormone. Immunofluorescent investigation into the pituitary gonadotropic cell types in the flounder, using antisera raised against flounder vitellogenic and maturational hormones, revealed differential spatial and temporal distributions of the vitellogenic and the maturational gonadotrops which correlated well with the physiological roles of the hormones in the reproductive cycle.

Efforts to equate mammalian with teleost gonadotropins have not been too successful. Some teleost gonadotropins have been found to resemble both mammalian FSH and LH in their amino acid compositions (Farmer and Papkoff, 1977; Yoneda *et al.*, 1977) and mammalian FSH (Hyder *et al.*, 1979)

and LH (Farmer and Papkoff, 1977) in chromatographic behavior. Certain teleost gonadotropins possessed luteinizing hormonelike biological activities (Farmer and Papkoff, 1977; Huang *et al.*, 1981), but in others separation of follicle stimulating hormonelike and luteinizing hormonelike biological activities were not clearcut (Hyder *et al.*, 1979). Although maturational hormone may be likened to mammalian LH in its ability to stimulate oocyte maturation and steroidogenesis, vitellogenic hormone is not comparable to mammalian FSH because mammalian gonadotropins are ineffective in stimulating vitellogenin incorporation into the teleost gonad (Nath and Sundararaj, 1981). Furthermore, vitellogenesis is absent from the process of ovarian growth and maturation in the mammal. Therefore, it is more appropriate to refer to teleost gonadotropins as vitellogenic* hormone and maturational hormone, names that reflect the physiological roles of the hormones in the teleost, rather than attempting to establish a resemblance between teleost and mammalian gonadotropins.

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6

THE FUNCTIONAL MORPHOLOGY OF TELEOST GONADS

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I. INTRODUCTION

Reproduction in teleost fishes is diverse. The teleosts are predominantly dioecious, but hermaphroditism, including juvenile hermaphroditism, and gynogenesis occur in some species (e.g., Hoar, 1969; Dodd, 1977; Lepori, 1980). Although this complexity of reproduction in teleosts is reflected in their wide range of gonadal structures, their fundamental structures (i.e., the morphology of germ cells and various somatic cell elements constituting the gonadal tissue) are similar. The basic and complementary tasks of the gonads of teleosts, like those of higher vertebrates, are to produce fertilizable gametes (i.e., eggs and sperm) necessary for successful reproduction and

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the pituitary-dependent synthesis and secretion of a variety of steroid hormones which regulate the development of germ cells. During the past 10 years, there has been a marked expansion in the knowledge of the morphology of the teleost gonads associated with these two fundamental functions. The processes involved in the formation of gametes have been extensively investigated by the use of improved techniques of histochemistry and electron microscopy. Some recent reviews on this subject are available (Dodd, 1977; Grier, 1981; Wallace and Selman, 1981; Billard *et al.*, 1982; de Vlaming, 1982). The sites of steroid production in the teleost gonads have also been studied using similar techniques (Guraya, 1976a,b; Nagahama *et al.*, 1982). More recently, the advent of *in vitro* techniques combined with improved technology in the area of hormone measurement has permitted the investigation of the contribution of various somatic components of the gonads in the production of steroid hormones. The discussion in this chapter is mainly concerned with some of the new information concerning the functional morphology of the teleost gonads. Some emphasis is placed on ultrastructural studies and on the role of the follicle layers in relation to follicular steroidogenesis. Discussion of much of the histological background and early publications has been omitted, because most of them have been reviewed in a previous volume of this treatise (e.g., Hoar, 1969).

II. MORPHOLOGY OF THE REPRODUCTIVE SYSTEM

A. Primordial Germ Cells and Sex Differentiation

The primordial germ cells in teleosts, as in other vertebrates, originate extragonadally and migrate to the gonadal region. The origin and the migration of the primordial germ cells in teleosts have been studied largely by standard histological methods. The pertinent literature is not reviewed here (see Hardisty, 1978). Most of the studies on the identification of germ cells during the early phases of gonadal development have been based on cell size and staining affinity using the light microscope; these criteria may not be completely reliable. Recent advances in electron microscopy have made it possible to determine some ultrastructural markers specific for the identification of the teleost primordial germ cells (Satoh, 1974; Bruslé and Bruslé, 1978a,b; Bruslé, 1980). Hogan (1978), who studied the ultrastructure of germ cells of the medaka, *Oryzias latipes*, determined two cytoplasmic markers for primordial germ cells and germ cells in the subsequent maturation stages. One of these markers is a fenestrated sheet of distinctive endo-

plasmic reticulum which usually follows the curvature of the nucleus. The second marker is the clump of granular material closely associated with mitochondria (mitochondria-associated granular material).

Gonads of cyclostomes and teleosts, unlike those of other vertebrates, lack medullary tissue. Therefore, the gonads of teleosts correspond only to the cortex of other vertebrates, and it has been suggested that the absence of a dual origin of the gonad accounts for the more wide-spread occurrence of intersexuality among the teleosts (e.g., Hoar, 1969). The onset of gonadal sex differentiation has been studied in several teleosts, and its timing varies according to species and sex. In general, using meiosis of germ cells as a criterion, gonadal differentiation becomes apparent earlier in females. In the medaka, it is signalled by differences in germ cell number. Because an earlier and more rapid proliferation of primordial germ cells occurs in female embryos (Satoh and Egami, 1972), differentiation of the gonad takes place at the time of hatching. In addition to germ cell morphology, associated somatic cell development such as the formation of the ovarian cavity or the testicular lumen (efferent duct) can also be used to recognize early sex differentiation in some species. For example, in *Tilapia mossambica (Sarotherodon mossambicus)* in which ovarian differentiation occurs about 20 days after hatching (rearing temperature 20° C), its onset is marked by both the meiotic activity of germ cells and the formation of the ovarian cavity. Early testicular differentiation first becomes apparent at the time of formation of the efferent duct; meiosis of germ cells does not occur until 50–60 days of age (Nakamura and Takahashi, 1973). Various aspects of sex differentiation in teleosts have been extensively reviewed (Yamamoto, 1969; Harrington, 1974; Reinboth, 1975; Haffen, 1977; Merchant-Larios, 1978; Lepori, 1980).

B. Male

1. Testis

In most teleosts, the testes are elongated paired organs attached to the dorsal body wall; in some species (e.g., poeciliids) they are combined into a single sac. A main sperm duct (vas deferens) arises from the posterior mesodorsal surface of each elongated testis and leads to the urogenital papilla located between the rectum and the urinary ducts. Testicular structure in teleosts is variable from species to species, although two basic types, lobular and tubular, can be identified according to the differentiation of the germinal tissue (Billard *et al.*, 1982).

The testis of the lobular type, which is typical of most teleosts, is composed of numerous lobules which are separated from each other by a thin

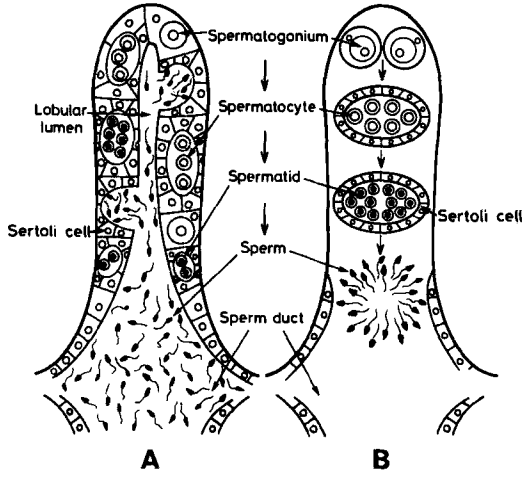

Fig. 1. Diagramatic representation of testis structure in teleosts: (A) Lobular type. (B) Tubular type.

layer of fibrous connective tissue (Fig. 1A); the arrangement of the lobules varies considerably (Roosen-Runge, 1977). Within the lobules, primary spermatogonia undergo numerous mitotic divisions to produce cysts containing several spermatogonial cells. During maturation, all of the germ cells within one cyst are at approximately the same stage of development. As spermatogenesis, and then spermiogenesis proceed, the cysts expand and eventually rupture, liberating sperm into the lobular lumen which is continuous with the sperm duct.

The other type of testicular structure, the tubular type, is restricted to the atheriniform group, such as the guppy, *Poecilia reticulata* (Billard *et al.*, 1982). The tubules are regularly oriented between the external tunica propria (blind end) and a central cavity into which the spermatozoa are released (Fig. 1B). Primary spermatogonia are located only at the blind end of the tubule. As spermatogenesis and spermiogenesis proceed, the germinal cyst moves centrally within the testis toward the vas efferens (efferent duct); there is no structure corresponding to the lobular lumen in this type (Roosen-Runge, 1977; Pilsworth and Setchell, 1981; Billard *et al.*, 1982). More recently, Grier *et al.* (1980) examined the testes of four orders of teleosts (Salmoniformes, Perciformes, Cypriniformes, and Atheriniformes) using conventional light microscopy and scanning and transmission electron microscopy, and classified testicular structure into two basic tubular types, unrestricted spermatogonial testis and restricted spermatogonial testis, according to the distribution of spermatogonia. The former type is common to

most teleosts, and the latter type, in which spermatogonia are totally restricted to the distal terminus of the tubule immediately beneath the tunica albuginea, is restricted to the Atheriniformes (Grier, 1981; cf. Billard *et al.*, 1982).

The teleost testis, as in mammals, is composed of interstitial and lobular (tubular) compartments (Fig. 2). The interstitium between lobules consists of interstitial cells, fibroblasts, and blood and lymph vessels. The lobular (tubular) component of the teleost testis contains two cell types: germ cells and distinct somatic cells lining the periphery of the lobule. The terminology of these interlobular somatic cells has long been confused (see Section IV,A). The term *lobule boundary cells* was first introduced by Marshall and Lofts (1956) in their work on the testis of the pike, *Esox lucius*, char, *Salvelinus willughbii*, and *Lebeo* (sp?). These cells arise not in the interstices, but within the walls of the lobule, and they stain positively for lipids and cholesterol. Marshall and Lofts considered that the lobule boundary cells were homologous with mammalian Leydig cells. Similarly, O'Halloran and Idler (1970) reported homology of the lobule boundary cells with mammalian Leydig cells in the testis of Atlantic salmon, *Salmo salar*. The term *lobule boundary cell* may be acceptable from an anatomical point of view, but the proposed functional homology of these cells with steroidogenic Leydig cells is now doubtful. The presence of cholesterol-positive lipids is not a sufficient criterion for the identification of steroid-producing cells. Furthermore, the lobule boundary cells, according to Marshall and Lofts (1956), generally occur in fish testes that do not possess typical interstitial cells. However,

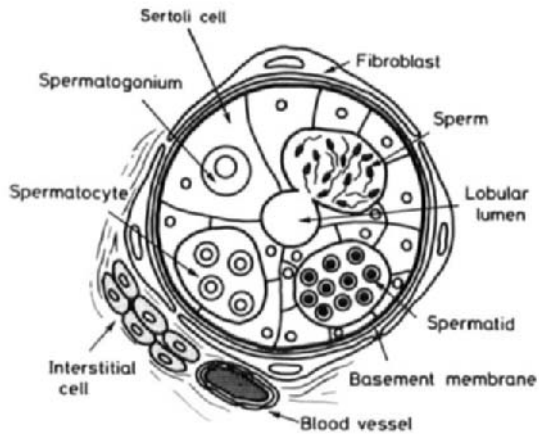

Fig. 2. Diagramatic representation of a portion of a testis of the tubular type demonstrating a close relationship between germ cells at various stages of development and Sertoli cells. Interstitial cells are located in the interstitium.

recent electron microscopical observations clearly indicate that there are some teleost species whose testes appear to have both interstitial cells and lobule boundary cells (Guraya, 1976b; Nagahama *et al.*, 1982).

In some teleost species, the lobule boundary cells are considered more accurately to be homologous with the Sertoli cells. In these species, lobule boundary cells are separated from the interlobular space by a thin but distinct basal lamina; these cells often occur in close proximity to spermatids and developing sperm (see Section III,A,1), and possess some ultrastructural features suggesting phagocytosis of residual bodies and degenerating germ cells and an involvement in the transport of metabolites (e.g., Billard, 1970b; Billard *et al.*, 1972; Gresik *et al.*, 1973b; Grier, 1975). Grier and Linton (1977), using histochemical and electron microscopical techniques, reinvestigated the testis of the northern pike, and found that the sudanophilic material was localized in the Sertoli-homologous cells which lay within the basement membrane. However, they could not identify interstitial Leydig cells within the interstitium, thus supporting the observation by Marshall and Lofts (1956); the cell organization of the pike testis may be atypical of teleosts as a whole (Grier, 1981). In conclusion, the term lobule boundary cell (with its implied steroidogenic role) for the intralobular somatic cells is misleading, because it is not justified on functional grounds and should therefore be regarded as having outlived its usefulness. Although the alternative terminology presently in use, Sertoli cell, and, more recently, cyst cell (Roosen-Runge, 1977; Billard *et al.*, 1982) are not totally satisfactory, the wide use of the term Sertoli cell in invertebrates and vertebrates is perhaps reasonable justification for its continued usage in teleosts, at least until the function of the intralobular somatic cells is established. The terminology in use for teleost testicular components has been discussed in detail by Grier (1981).

2. Sperm Duct

During the course of testicular morphogenesis, teleost sperm ducts (vas deferens) are formed by somatic cells derived from the coelomic wall. These structures, unlike those of most vertebrates, are in no way a part of the nephric duct or Wolffian duct. An ultrastructural study has demonstrated that the epithelial cells of the main duct of the black molly, *Mollienisia latipinna*, possess numerous microvilli with alkaline phosphatase activity at their apical surface and abundant mitochondria (van den Hurk *et al.*, 1974a). These observations suggest that these cells are involved in the transport of substances of low molecular weight, and therefore regulate the ionic composition and osmotic pressure of the seminal fluid. The sperm duct system of species with tubule type testes consists of two parts, efferent duct (vasa

efferentia) and main sperm duct (vasa defferentia). It has been suggested that the epithelial cells lining the efferent duct are derived from Sertoli cells during spermiation (e.g., Pandey, 1969; van den Hurk *et al.*, 1974b; Gardiner, 1978a; Grier *et al.*, 1978; Grier, 1981). A secretory nature of these cells has been suggested by electron microscopical studies (e.g., van den Hurk *et al.*, 1974a). Although the exact nature and function of this secretory substance is unknown, it may be involved in the immobilization of the sperm cells or the formation of sperm packets (spermatozeugmata).

3. Seminal Vesicle

Specific glandular structures, often referred to as seminal vesicles, which are composed of bilateral outgrowth of the common sperm ducts, are found in some teleosts. The seminal vesicles of *Gillichthys* have the appearance of greatly enlarged lobules, which contain a bright yellow fluid rather than the milky substance of the testes (Weisel, 1949). These seminal vesicles probably do not store sperm and are not part of the Wolffian-duct system; therefore, they are not homologous with the structures of the same name in higher vertebrates. They are probably responsible for the fluid which is of importance in sperm transfer or other breeding activities. However, a specialized structure comprising inconspicuous protrusions along the medial ventral wall of the sperm duct of goldfish has been reported. It is considered to be homologous with the seminal vesicle, serving essentially as a sperm reservoir but not as a glandular organ (Takahashi and Takano, 1972).

C. Female

1. Ovary

The female reproductive system of teleosts, unlike that of mammals, is highly variable, reflecting the wide range of reproductive patterns, including viviparity. In most teleosts, the ovary is a hollow paired organ; however, in some species, paired structures become fused into one solid, single organ during their early development. The ovary consists of oogonia, oocytes, and their surrounding follicle cells, supporting tissue or stroma, and vascular and nervous tissue (Fig. 3). Most teleosts are cyclical breeders and the ovary varies greatly in appearance at different times in the cycle. Three ovarian types have been classified according to the pattern of oocyte development (e.g., Wallace and Selman, 1981; de Vlaming, 1982). The synchronous ("synchronisme total") ovary contains oocytes all at the same stage of development; this type is found in teleosts which spawn only once and then die, such as anadromous *Oncorhynchus* species or catadromous eels. The group syn-

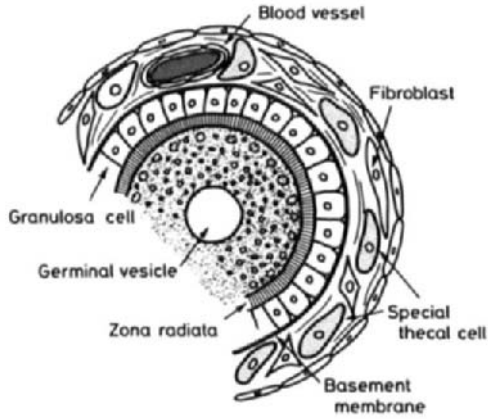

Fig. 3. Diagramatic representation of the follicle layer surrounding an early vitellogenic oocyte of salmonid fishes. The granulosa layer is separated from the thecal layer by a distinct basement membrane. The thecal layer is composed of fibroblasts, blood vessels, *etc.*, and large special thecal cells.

chronous ("synchronisme par groups") ovary consists of at least two populations of oocytes at different developmental stages; teleosts with this type of the ovary, such as the flounder, *Liopsetta abscura* and the rainbow trout, *Salmo gairdneri*, generally spawn once a year and have a relatively short breeding season. The asynchronous ("metachrone") ovary contains oocytes at all stages of development; this type occurs in those species (e.g., the medaka and the goldfish, *Carassius auratus*) which spawn many times during a prolonged breeding season.

Each oocyte during its early development becomes surrounded by a layer of follicle cells. With the growth of the oocytes, follicle cells multiply and form a continuous follicular layer (granulosa cell layer). Simultaneously, the surrounding stromal connective tissue elements also become organized to form the distinct outer layer of the follicular envelope (the thecal layer). Therefore, vitellogenic oocytes are surrounded by two major cell layers, an outer thecal layer and an inner granulosa layer which are separated from each other by a distinct basement membrane. The thecal layer contains fibroblasts, collagen fibers, and capillaries, and, in some species, special thecal cells (steroid-producing cells). A detailed description of these layers in relation to steroidogenesis is given in Section IV.

2. Ovarian Cavity

The teleost ovary generally has an ovarian cavity continuous with the oviduct; mature oocytes are discharged into the ovarian cavity and move to the oviduct. The development of the ovarian cavity has been investigated in

several teleosts and varies from species to species (e.g., Lepori, 1980). The ovarian cavity of the goldfish is formed by the fusion of the distal edges of the ovary with the dorsolateral coelomic wall (Takahashi and Takano, 1971). In a tilapia, *Tilapia mossambica*, the cavity is formed by the fusion of two stromal ridges growing from both the proximal and distal borders of the ovary (Nakamura and Takahashi, 1973).

In oviparous teleosts, the ovarian cavity has been regarded as merely a "stockroom" in which ovulated eggs are kept temporarily until they are spawned. Studies on the medaka have suggested that the cells lining the ovarian cavity have a secretory function, because they become progressively hypertrophied and actively secrete fluid (glycoprotein?) during ovarian maturation (Yamamoto, 1963). Ultrastructurally, these cells are characterized by many microvilli, well-developed tonofilaments, abundant agranular endoplasmic reticulum, and Golgi apparatus; cellular characteristics of macroapocrine secretion have also been observed (Takano, 1968). These observations suggest a significant participation of the lining cells of the ovarian cavity in maintaining ovulated eggs and in transporting them through the oviduct for spawning. The development and functional maintenance of epithelial cells lining the ovarian cavity has been shown to be dependent on certain sex hormones (Takahashi and Takano, 1971).

3. Viviparous Species

Various aspects of viviparity in teleosts have been the subject of several reviews (Hoar, 1957, 1969; Amoroso, 1960; Hogarth, 1976; Amoroso *et al.*, 1979; Wourms, 1981). Almost all viviparous teleosts have a single median ovary. The structure of the guppy (*Poecilia reticulata*) ovary is shown diagramatically in Fig. 4. The morphological aspects of oocyte development including the associated development of the follicle cells of viviparous species are essentially the same as those of related oviparous species (e.g., Takano, 1964; Jollie and Jollie, 1964a,b). The mature eggs are fertilized internally and the young are retained within the body of the female for a significant period to complete some or all of their embryonic development. In viviparous species, in which the yolk content of the egg is greatly reduced, the developing young depend largely on a continuous supply of maternal nutrients. Therefore, in certain viviparous species the ovary has not only a gametogenic and endocrine role, but also a nutritive role. Various structural adaptations occur for maternal–fetal nutrient transfer. Literature on this subject may be found in recent reviews (Hoar, 1969; Amoroso *et al.*, 1979; Wourms, 1981).

In teleosts, gestation is always intraovarian and consists of two types: follicular gestation and ovarian gestation. In the former (typical of the family Poeciliidae), fertilized eggs are not ovulated and development of the young

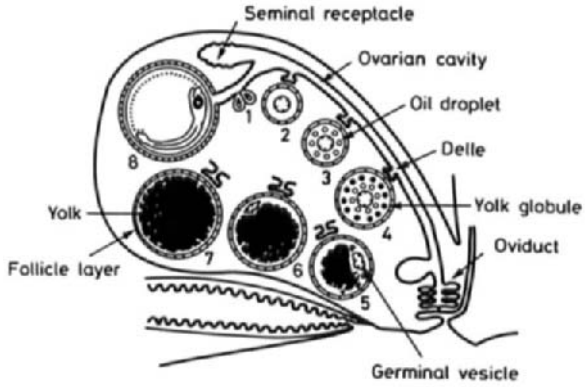

Fig. 4. Diagramatic representation of the ovary of the guppy (*Poecilia reticulata*) showing follicles at various stages of development (1–8), and the location of specialized structures, delle and seminal receptacle, for internal fertilization. (Courtesy of Dr. Takano.)

occurs within the ovarian follicles, in the latter, development proceeds within the ovarian cavity. A prolonged follicular gestation is found only in the poeciliids. Most poeciliids are able to store viable sperm for a relatively long period. Some obvious structural modifications occur in the ovarian cavity of viviparous species which are almost certainly associated with sperm storage and internal fertilization (e.g., Jalabert and Billard, 1969; Gardiner, 1978b). These include a localized expansion of the epithelium of the ovarian cavity overlying each developing oocyte to form a "delle," and the existence of a similarly formed, specialized, single "seminal receptacle" in the anterodorsal ovarian cavity (Jalabert and Billard, 1969; K. Takano, unpublished observations) (Fig. 5). Sperm are often found embedded head first in the apical cytoplasm of the epithelium lining the ovarian cavity. Recent electron microscopical studies of the specialized structure in the ovary of the guppy have revealed a much higher density of embedded sperm in each delle (K. Takano, unpublished observations) and in the seminal receptacle (Jalabert and Billard, 1969; K. Takano, unpublished observations). The delle terminates directly on the follicle, presumably to allow access of sperm to the oocyte during fertilization. Indeed, during fertilization the terminal end of the delle and the area of the follicle upon which it contacts breaks down to permit direct access of the sperm to the oocyte surface. This is undoubtedly related to the absence of a micropyle in this species. In another viviparous species, *Sebastes taczanowskii*, the formation of delle does not occur, but a micropyle is present (K. Takano and H. Ohta, unpublished observations).

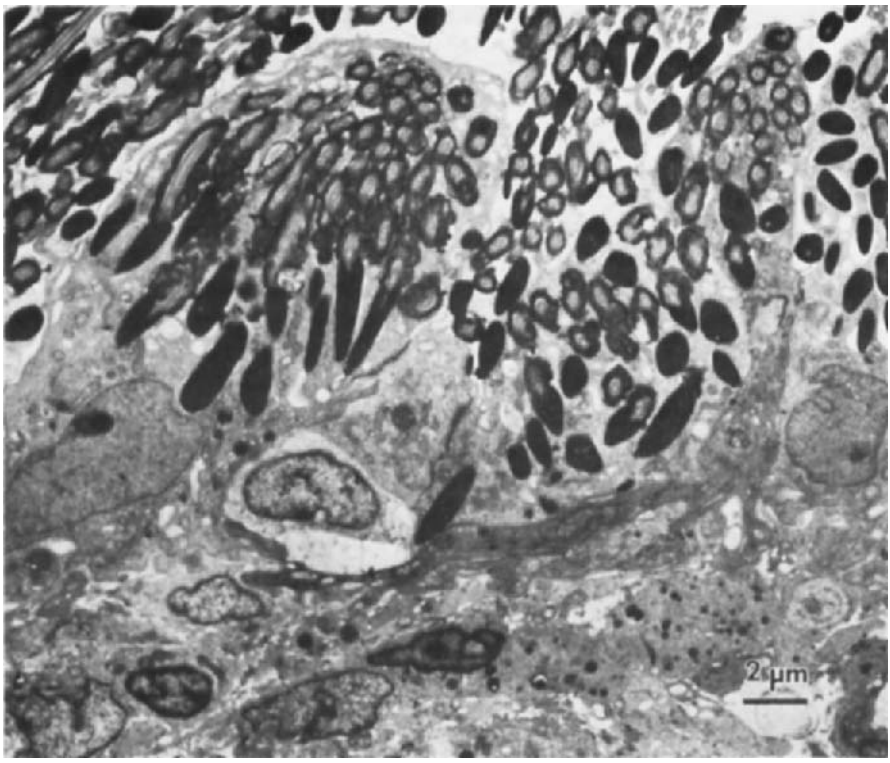

Fig. 5. Electron micrograph of a portion of the seminal receptacle of the guppy (*Poecilia reticulata*) ovary. Spermatozoa are deeply embedded in the apical cytoplasm of the epithelial cells. (Courtesy of Dr. Takano.)

III. GAMETOGENESIS

A. Spermatogenesis

1. Spermatogenesis and Spermiogenesis

In both testicular types, lobular or tubular, the development of germ cells takes place within cysts formed by Sertoli cells. Stages of spermatogenesis and spermiogenesis are distinguishable on the basis of their characteristic nuclear and cytoplasmic morphologies. As briefly described previously (see Section II), cyst formation begins with the mitotic division of

spermatogonia. Spermatogonia then transform into primary spermatocytes. The first meiotic division produces two daughter cells, secondary spermatocytes. The secondary spermatocytes then transform into spermatids through the second meiotic division. These spermatids, although possessing a haploid set of chromosomes, are still not capable of functioning as male gametes. They must undergo differentiation into spermatozoa, a process termed *spermiogenesis*. Figure 6 shows light micrographs of various stages of spermatogenesis in the testis of *Sarotherodon mossambicus* (Y. Nagahama and M. Nakamura, unpublished observations).

The duration of spermatogenesis has been determined in a few teleosts, and the results vary from species to species. The interval of the medaka

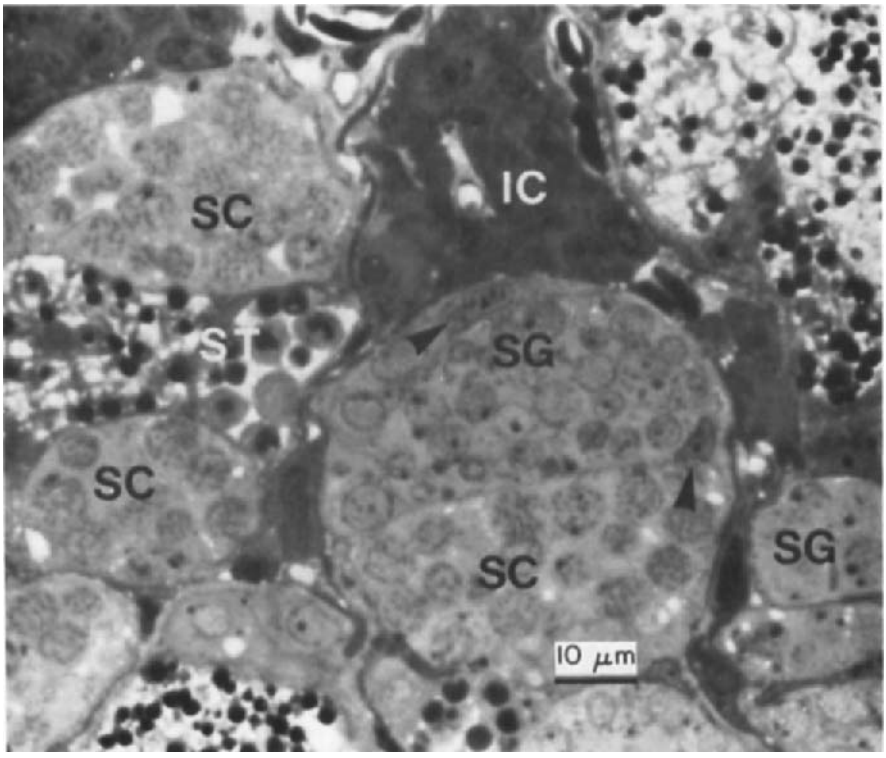

Fig. 6. A portion of the tilapia (*Sarotherodon mossambicus*) testis (1 μm Epon-embedded section, methylene blue, and azure II), showing germ cells at various stages of development, Sertoli cells (arrows), and interstitial cells (IC). (SG, spermatogonium; SC, spermatocyte; ST, spermatid).

spermatogenesis was determined by Egami and Hyodo-Taguchi (1967), who used tritiated thymidine and autoradiographic techniques; the minimum duration from early DNA synthesis in the leptotene spermatocyte to the early spermatid is 5 days at 25° C (12 days at 15° C) and the period from early spermatid to spermatozoon is 7 days at 25°C (8 days at 15°C). In the guppy, the time periods for the development from early leptotene to spermatozoon is 14.5 days at 25°C (Billard, 1968). In *Poecilia shenops* the duration from leptotene to mature spermatozoon is at least 21 days (DeFelice and Rasch, 1969).

Spermatogonia are located within the basal region of a spermatogenic cyst, and are always separated from the basement membrane by thin, intervening strands of Sertoli cell cytoplasm, a different configuration from that in mammals where type A spermatogonia have a broad attachment to the basal lamina of the seminiferous epithelium. At least two types of spermatogonia, i.e., primary spermatogonia and secondary spermatogonia (probably corresponding to type A spermatogonia and type B spermatogonia, respectively), have been identified in the teleost testis. The primary spermatogonia are generally the largest germ cells in the testis, but their diameter varies in size with the species and even within the same species. Three types of spermatogonia have been distinguished in the testis of the medaka; spermatogonia A stem (3–4 μm diameter), spermatogonia differentiated (5–10 μm diameter), and spermatogonia B (approximately 5 μm diameter) (Michibata, 1975). Electron microscopy clearly demonstrates intercellular cytoplasmic bridges connecting neighboring spermatogonia (Billard and Fléchon, 1969; Grier, 1975, 1976).

Despite a number of light microscopical investigations, there remains some controversy as to how spermatogonial renewal takes place in the teleost testis. The majority of investigations have concluded that the spermatogonia arise from germ cells ("stem cells") which have been dormant in the peripheral portions of the testis (see Roosen-Runge, 1977; Grier, 1981). An interstitial origin of spermatogonia has also been demonstrated in the testis of the rainbow trout (van den Hurk *et al.*, 1978a). Ruby and McMillan (1975) have shown, using [^{3}H]thymidine, that in the five-spined stickleback, *Culaea inconstans*, spermatogonia are derived from clumps of germ cells in the interstitial tissue.

The cytoplasm of primary spermatocytes is generally more electron dense than that of spermatogonia and neighboring cells are joined by intercellular bridges (e.g., Clérot, 1971). Their nuclei are characterized by abundant, irregularly condensed chromatin. During meiotic pachytene, synaptonemal complexes appear in the primary spermatocyte nucleus. Secondary spermatocytes are observed infrequently in a section of the testis because

their life span is relatively short, i.e., they rapidly divide to form spermatids. Secondary spermatocytes are smaller than the primary spermatocytes, and larger than their daughter cells, the spermatids.

Transformation of spermatids into mature spermatozoa (spermiogenesis) consists of a reorganization of nucleus and cytoplasm together with the development of flagellum; no cell division occurs in this process. Spermiogenesis has been observed in a number of teleosts by the use of electron microscopy. A wide variety of patterns have been observed. Because this aspect is perhaps of specialized cytological interest, a review of the literature is not given here; information may be found in a number of recent papers (e.g., Dadone and Narbaitz, 1967; Mizue, 1968, 1969; Stanley, 1969; Billard, 1970c; Asai, 1971; Grier, 1973, 1975, 1976; van Deurs and Lastein, 1973; Zirkin, 1975; Todd, 1976; Gardiner, 1978a; Grier *et al.*, 1978). Near or at the termination of spermiogenesis, spermatids or sperm nuclei of some teleosts with tubular-type testes (e.g., the Poeciliidae) become embedded within Sertoli cell cytoplasmic recesses (e.g., Billard, 1970b; van den Hurk *et al.*, 1974b; Grier, 1975). A similar association of germ cells with Sertoli cells is found in the Goodeidae, but in this case the developing spermatid flagellum becomes associated with the Sertoli cell layer (e.g., Grier *et al.*, 1978; Grier, 1981). No such associations occur between germ cells and Sertoli cells in species with lobular-type testes.

2. Spermatozoon

The morphology of spermatozoa has been studied in several species of teleosts (e.g., Billard, 1970a; Mattei, 1970). The teleost spermatozoa can be morphologically subdivided into head, neck piece, midpiece, and tail. They lack an acrosome which occurs in all other vertebrate groups; this may be related to the presence of an egg micropyle in teleost eggs. The heads are generally spherical or oval in shape; sickle or crescent-shaped spermatozoa occur in eels (Ginsburg and Billard, 1972; Colak and Yamamoto, 1974; Todd, 1976). The midpiece follows a common ultrastructural pattern, consisting of a central flagellum and a surrounding mitochondrial sheath. In most teleost spermatozoa, mitochondria are few, not modified, and situated in a low collar immediately behind the rounded nucleus. Aflagellated spermatozoa and biflagellated spermatozoa occur in some teleosts (e.g., Mattei, 1970). The tail flagellum of the spermatozoa of most species exhibits a typical 9 + 2 axonemal pattern, but the eel spermatozoon is remarkable in having a 9 + 0 pattern having no central axoneme (Colak and Yamamoto, 1974; Todd, 1976).

Sperm morphology appears to reflect the mode of fertilization. A primi-

tive type of spermatozoon is retained in those species in which fertilization occurs externally; the shape of the sperm nucleus of these species is rounded (Grier, 1981). Various modifications in sperm morphology occur in those species with internal fertilization (e.g., Billard, 1970a; Grier, 1981) in which the sperm nucleus is more elongated and the midpiece is well developed. A good example of this diversity in sperm morphology according to the mode of reproduction has been noted in two closely related species. In the medaka, with external fertilization, sperm morphology is primitive with a rounded nucleus and short midpiece. In the guppy, with internal fertilization, an elongation of both nucleus and midpiece is observed (Grier, 1981).

3. Blood–Testis Barrier

Recent electron microscopical investigations involving the use of an extracellular tracer, horseradish peroxidase, have demonstrated the existence of a blood–testis barrier in two species of teleosts, the guppy (Marcaillou and Szöllösi, 1980) and the cyprinodont *Aphanius dispar* (Abraham *et al.*, 1980). In cysts containing spermatogonia and spermatocytes, this tracer can penetrate freely beyond the Sertoli cell layer. However, in cysts containing ripe spermatozoa, the tracer penetrates only to the intercellular spaces between the Sertoli cells; tight junctional complexes near the lumina of the cysts appear to be a barrier to permeability between the vascular spaces of the stroma and the lumina of ripe cysts (Abraham *et al.*, 1980). Therefore, the blood–testis barrier is probably involved in the process of spermatogenesis, by controlling the environment within the closed compartment of the testicular lobule.

4. Spermiation

Spermiation in teleosts involves a hormone-dependent thinning, or hydration of the semen (Billard *et al.*, 1982). The precise physiological significance of hydration is not clear. Billard *et al.* (1982) suggested that this hydration of the semen, by increasing interlobular pressure, allows the sperm to migrate to the vas deferens where they are stored. However, the morphology of spermiation in teleosts has not yet been studied in detail. The ultrastructure of spermiation in amphibians has been well defined; this process corresponds to the separation of late spermatids from the Sertoli cells (Burgos and Vitale-Calpe, 1967). This definition may be valid in fish with a tubule-type testis. During spermiation in poeciliids, sperm embedded in Sertoli cells are voided as a discrete bundle, a spermatozeugmatum (e.g., Grier, 1975; Billard *et al.*, 1982).

B. Oogenesis

1. Oogenesis

Changes in various cellular organelles of the oocyte during oogenesis have been described in a number of teleost species (e.g., Wallace and Selman, 1981; de Vlaming, 1982). The first stage of the development of female gametes is similar to that found in spermatogenesis. Oogonia undergo proliferation by mitotic divisions and become primary oocytes when the chromosomes become arrested at the diplotene stage of the first meiotic prophase. Oocytes, unlike male gametes, then enter a period of growth which varies from species to species. Enlargement of oocytes is caused mainly by the accumulation of yolk.

Several criteria have been employed for staging the process of oogenesis; they are size, amount, and distribution of various cell inclusions, especially yolk granules, and morphology of the chromosomes. Yamamoto *et al.* (1965) divided the development of the oocyte of the rainbow trout into eight stages; each stage is defined cytologically by size, appearance of nucleus and nucleolus, and the type and localization of cytoplasmic inclusions. They are chromatin–nucleolus stage, perinucleolus stages (subdivided into early and late stages), oil drop stage, primary yolk stage, secondary yolk stage, tertiary yolk stage, and maturation stage (Figs. 7 and 8). The chromatin–nucleolus stage is characterized by a conspicuous nucleolus associated with chromatin threads. Concomitant with oocyte growth, the nucleus increases in size, and multiple nucleoli become located around the periphery of the nucleus (early perinucleolus stage). The late perinucleolus stage can be distinguished from the previous stage by the enlargement of the oocyte. During this period (diplotene stage of meiosis), lampbrush chromosomes are formed which disappear immediately prior to the breakdown of germinal vesicles during oocyte maturation. In the perinucleolus stage, most teleost oocytes, like those of other animals (Nørrevang, 1968; Guraya, 1979), accumulate a small juxtanuclear mass which is basophilic in histological sections (Fig. 7). These aggregates have been termed "yolk nucleus" or "Balbiani bodies." Recent electron microscopical studies have revealed that the yolk nucleus is not a homogeneous structure, and that it is composed of various cellular organelles such as mitochondria, Golgi bodies, smooth endoplasmic reticulum, multivesicular bodies, and lipid granules (e.g., Beams and Kessel, 1973; Guraya, 1979; Wallace and Selman, 1981). Further, annulate lamellae have also been described as a component of the yolk nucleus in several cyprinid species (Clérot, 1976). Although its role is as yet not clear, it has long been considered that the yolk nucleus functions as a center for the formation of organelles within the oocyte (Guraya, 1979).

2. VITELLOGENESIS

Teleost oocytes enter a prolonged growth phase which is dependent on pituitary gonadotropin. The enlargement of oocytes, attributable mainly to the accumulation of yolk, is very considerable; for example, the young oocyte of the rainbow trout is about 20 μm in diameter and the fully developed egg is about 4 mm. As in oocytes of most animals, yolk is an important constituent of teleost oocytes. There are three distinct types of yolk material in teleosts: oil droplets, yolk vesicles, and yolk globules. In general, the oil droplets first appear in the perinuclear area and then migrate to the periphery in later stages. The sequence of the appearance of this yolk material varies with species. In the rainbow trout the droplets appear soon after the commencement of the formation of yolk vesicles (Yamamoto *et al.*, 1965) (Fig. 8). The lipid droplets of the smelt, *Hypomesus japonicus*, appear after the formation of both yolk vesicles and globules (Yamamoto, 1956).

The PAS-positive (mucopolysaccharide or glycoprotein) yolk vesicles are generally the first structures to appear in the oocyte cytoplasm during the secondary growth of oocytes, and first appear in the outer and midcortical zones of the oocyte. Electron microscopy reveals the possible involvement of the endoplasmic reticulum and Golgi apparatus in the formation of yolk vesicles (e.g., Anderson, 1968; Shackley and King, 1977). In an autoradiographic study, Korfsmeier (1966) demonstrated in the zebrafish, *Brachydanio rerio*, that yolk vesicles rapidly incorporate both [^{3}H]histidine and [^{3}H]glucose. These results suggest that yolk vesicles are synthesized within the oocyte (autosynthetic). As the oocyte grows, the yolk vesicles increase in both size and number, and at maturity they move to the periphery of the oocyte, where they become known as the cortical alveoli (Wallace and Selman, 1981). It has been well established that cortical alveoli function in the cortical reaction at fertilization, the components of the cortical alveoli being released into the perivitelline space when the egg is inseminated.

Several ultrastructural studies have indicated that yolk globules are formed by the fusion of small, coated vesicles which first appear peripherally in the oocyte (e.g., Droller and Roth, 1966; Anderson, 1968; Ulrich, 1969; Wegmann and Götting, 1971; Gupta and Yamamoto, 1972; Shackley and King, 1977). As vitellogenesis proceeds, most of the cytoplasm of mature eggs becomes occupied by many dense yolk globules which are surrounded by a limiting membrane. During the later stage of vitellogenesis, the yolk globules of some teleosts fuse with each other to form a single mass of yolk; the transparency of eggs in certain species may be attributed to this fusion of yolk globules. Yamamoto and Oota (1967) have demonstrated, by electron microscopy, that the yolk globules of the zebrafish consist of a crystalline main body surrounded by a superficial layer and outer membrane. Similar

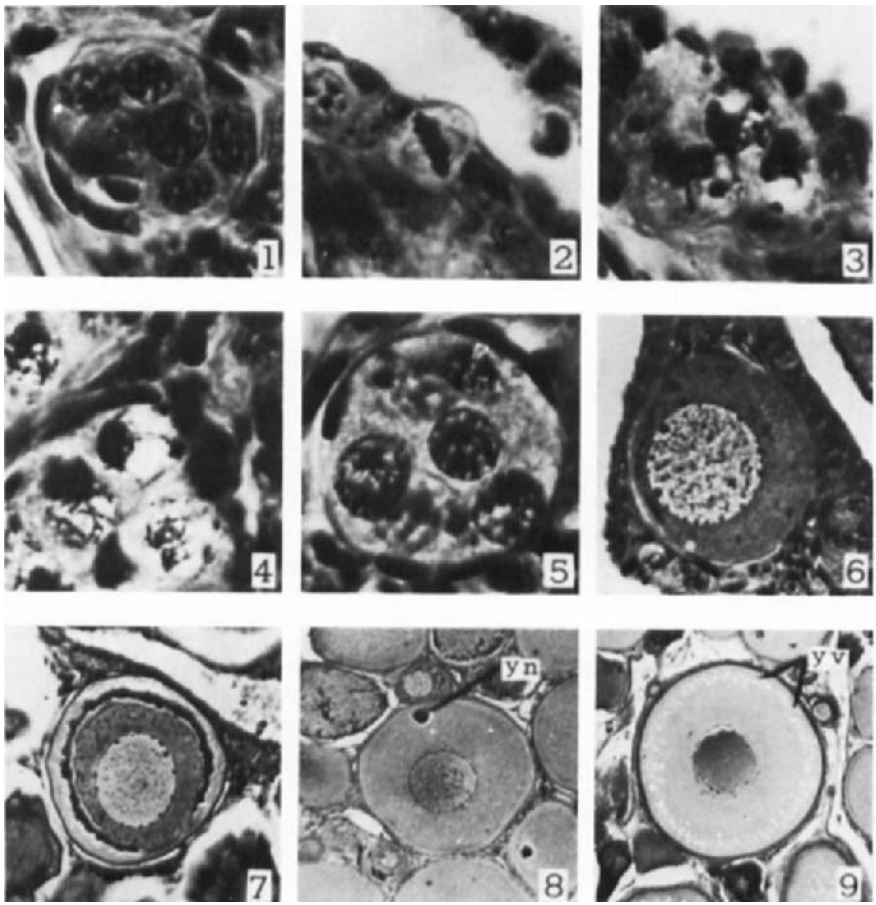

Fig 7

Figs. 7 and 8. Developmental stages of oogenesis in rainbow trout (*Salmo gairdneri*). 1–2, oogonium stage; 3–5, chromatin-nucleolus stage; 6–7, early perinucleolus stage; 8, late perinucleolus stage (yn, yolk nucleus); 9, yolk vesicle stage (yv, yolk vesicle); 10–11, oil drop stage (fd, fatty drop); 12, primary yolk globule stage (yg, yolk globule); 13, secondary yolk globule stage; 14, tertiary yolk globule stage; 15, migratory nucleus stage (n, germinal vesicle); 16, ripe

crystalline yolk has been described in the oocytes of a few species of teleosts (Ulrich, 1969; Gupta and Yamamoto, 1972; Flegler, 1977). The electron diffraction pattern of yolk globules of *Pelvicachromis pulcher* closely resembles that of *Xenopus laevis* (Lange, 1980).

In teleosts, as in other nonmammalian vertebrates, it has been demonstrated that a female-specific protein (vitellogenin), which is synthesized by the liver in response to 17β-estradiol, is released into the blood and then transported to the ovary (e.g., Wallace, 1978; see Chapter 8, this volume).

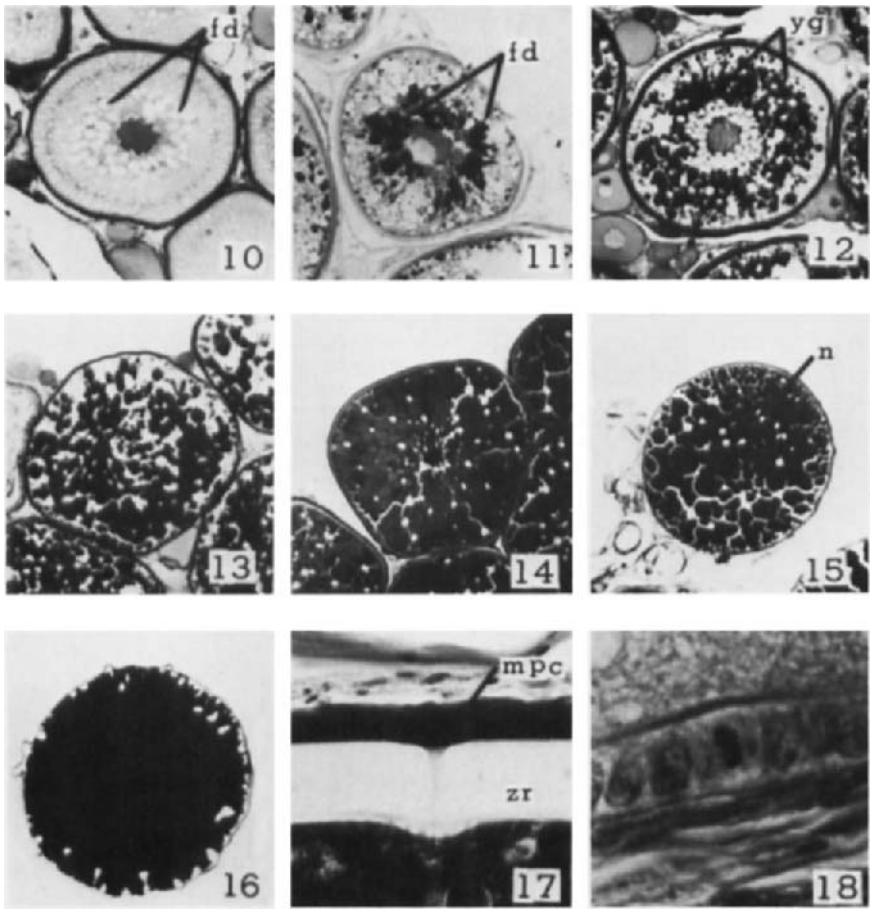

Fig 8

stage; 17, a portion of an oocyte at the migratory nucleus stage showing a micropylar cell (mpc) and zona radiata (zr); 18, a portion of follicle layers surrounding an oocyte at the yolk vesicle stage showing a granulosa cell in the process of mitosis. (Reproduced by permission from Yamamoto *et al.*, 1965.)

Ultrastructural evidence shows that protein yolk precursors are incorporated into the oocyte by micropinocytosis (Droller and Roth, 1966; Anderson, 1968; Ulrich, 1969; Wegmann and Gotting, 1971; Gupta and Yamamoto, 1972; Shackley and King, 1977). Autoradiographic studies by Korfsmeier (1966) with the zebrafish also demonstrated the transfer of protein from the blood to form yolk. Therefore, most yolk proteins appear to be synthesized outside the oocyte (heterosynthetic). In addition to this heterosynthetic process, an intraovarian origin of yolk proteins (autosynthetic) has also been suggested by an electron microscopical study (Yamamoto and Onozato,

1965). Yolk formation in teleosts, as in other animals (Nørrevang, 1968; Anderson, 1974), appears to consist of both autosynthetic and heterosynthetic types, or a combination of both, although in the oocytes of *Xenopus laevis* 99% of the yolk protein is derived of hepatic origin yolk protein (vitellogenin) (Wallace *et al.*, 1972).

It has been reported that salmon pituitary extract stimulates micropinocytotic activity at the oocyte surface (Campbell, 1978; Upadhyay *et al.*, 1978). Ultracytochemically, Na^+-, K^+-activated ATPase is localized on the oocyte and follicular microvilli and the egg membrane surrounding the primary oocytes of *Heterandria formosa* (Riehl, 1980). These results confirm that the microvilli are the sites of substance exchange between the follicle cells and the oocyte. The yolk proteins of the rainbow trout resemble those of amphibians and birds in that they consist of lipovitelline and phosvitin (Hara and Hirai, 1978). However, details of the mechanism and the site of

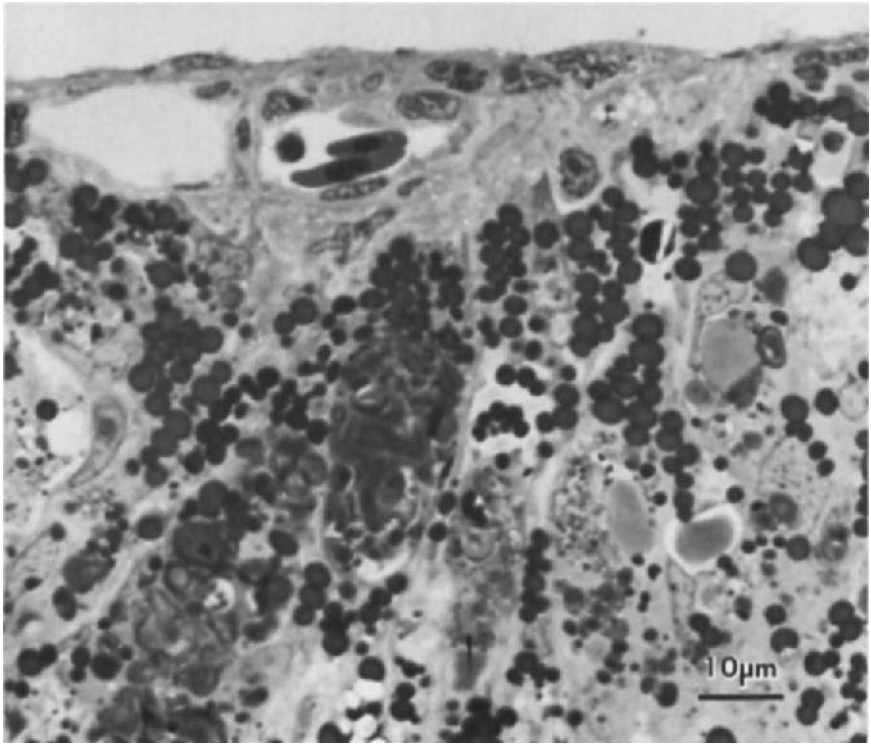

Fig. 9. A portion of an atretic follicle of the goldfish (*Carassius auratus*) (1 μm Epon-embedded section, methylene blue, and azure II) showing hypertrophied granulosa cells filled with various inclusions.

the conversion of vitellogenin into these yolk proteins is unknown. In particular, evidence for the roles of the follicular envelope and the oocyte in the cleavage of vitellogenin to lipovitelline and phosvitin is lacking.

3. Atretic Oocytes

Atretic oocytes (preovulatory corpora atretica) are a very common feature of the teleost ovary (Ball, 1960; Lofts and Bern, 1972; Browning, 1973; Guraya, 1976a; Saidapur, 1978) and may be caused by environmental stress (Ball, 1960). Several studies have dealt with the histological changes in follicular atresia during the annual reproductive cycle and under various experimental conditions (e.g., hypophysectomy or administration of sex steroids). Follicular atresia, which involves the hypertrophy of the granulosa cells (Fig. 9) and possibly the thecal cells may occur in follicles at any stage of oocyte development. Most investigators have divided teleost follicular atresia into four consecutive stages according to the original light microscopical description by Bretschneider and Duyvene de Wit (1947) in *Rhodeus amerus*. Khoo (1975) provided a detailed description of histological changes in follicular atresia in the goldfish after hypophysectomy, and classified five consecutive stages, α, β, γ, δ, and ϵ stages. After the complete reabsorption of all remnants of oocytes by hypertrophied granulosa cells (δ stage), the granulosa cells collapse into the atrium to form an irregular cellular mass, and yellow lutein pigments are observed among the cells (ϵ stage). Following this stage, some atretic cells appear to differentiate into oogonia (Khoo, 1975).

4. Oocyte Maturation

After the oocyte completes its growth, it is ready for the resumption of reduction divisions. In teleosts, like in other vertebrates, the fully grown oocyte possesses a large nucleus (germinal vesicle) in meiotic prophase. The germinal vesicle of this stage is generally located centrally or halfway between the center and the oocyte periphery. In general, the germinal vesicle cannot be seen by external observation because of the opaque cytoplasm. The use of certain fixatives increases yolk transparency and the germinal vesicle appears golden brown under transmitted light after this treatment (Goetz and Bergman, 1978). In the terminal phase of vitellogenesis, goldfish oocytes lose their spherical shape and become slightly flattened. The animal pole, on one of the flattened surfaces, is located around a small depression in the follicle and zona radiata, the micropyle. Under the dissecting microscope, this area can be easily distinguished, appearing as a number of small furrows in the follicle radiating out from the micropyle. In some species such as the yellow perch, *Perca flavescens*, and the Indian catfish, *Heteropneustes*

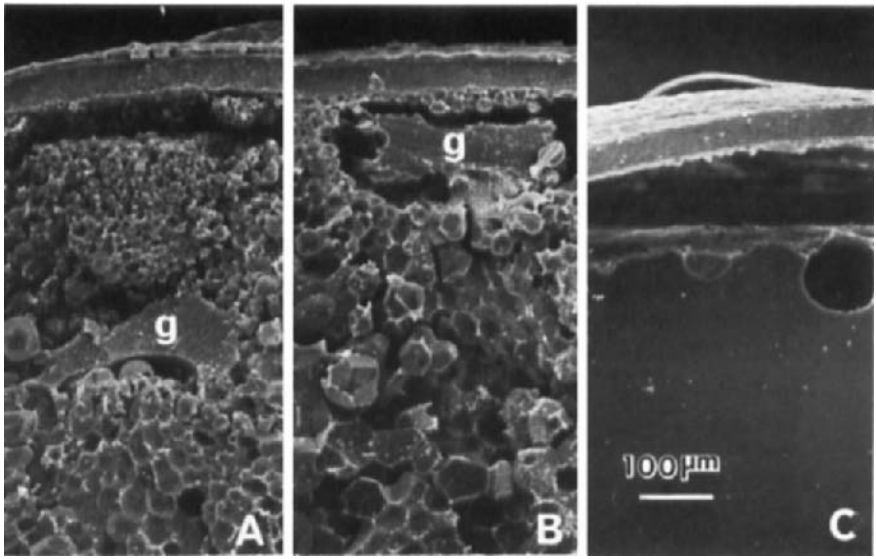

Fig. 10. Scanning electron micrograph of the fractured surface of rainbow trout (*Salmo gairdneri*) oocytes during salmon gonadotropin (SG-G100)-induced germinal vesicle breakdown *in vitro*. (A) A fully grown immature oocyte with the germinal vesicle (g) located halfway between the center and the oocyte periphery. (B) An oocyte 24 hr after the onset of incubation with salmon gonadotropin. The germinal vesicle (g) is located at the oocyte periphery. (C) An oocyte 72 hr after the onset of incubation with salmon gonadotropin. Note the disappearance of the germinal vesicle and the homogeneous ooplasm.

fossilis, the animal pole is quite conspicuous with an elevated cap (see Chapter 3, Volume 9B, this series; Goswami and Sundararaj, 1971).

Recent improvements in the *in vitro* incubation technique have allowed an observation of the various morphological events associated with oocyte maturation. Figure 10 shows the morphological changes occurring in the nucleus and the cytoplasm of oocytes during gonadotropin-induced *in vitro* oocyte maturation in salmonids. The germinal vesicle of immature oocytes is inconspicuous in salmonids. The first visible event associated with final oocyte maturation is the migration of the germinal vesicle to the animal pole where the micropyle is situated; at this stage the germinal vesicle becomes visible under the dissecting microscope. The membrane of the germinal vesicle then breaks down (germinal vesicle breakdown) and its contents become intermingled with the surrounding cytoplasm. Besides these changes in the nucleus, there are also several cytoplasmic processes occurring during oocyte maturation. These include the coalescence of lipid droplets and yolk globules, a further rapid size increase of the oocyte caused by hydration, and an overall increase in oocyte translucency.

Iwamatsu *et al.* (1976) and Iwamatsu and Ohta (1977) have observed ultrastructural changes in the cytoplasm of the medaka and the loach, *Misgurnus anguillicandatus*, oocytes during corticosterone-induced maturation *in vitro*. Fully grown immature oocytes of the loach, prior to exposure of corticosterone, are characterized by the existence of annulate lamellae, a mass of long mitochondria, an electron-dense layer beneath the vitelline surface, and numerous cortical alveoli and yolk platelets. The initial changes occurring in the cytoplasm (3 hr after exposure to this hormone) is the disappearance of the electron-dense layer and annulate lamellae, and changes in the shape of mitochondria from long to spherical ones. The ooplasm at the later stages of maturation is occupied by enlarged yolk platelets and tubular endoplasmic reticulum develop throughout the cytoplasm; cortical alveoli are formed aligned beneath the vitelline surface. Some of the earlier events occurring in the ooplasm may be associated with the appearance of a cytoplasmic factor (maturation-promoting factor) which has recently been detected in mature oocytes of the goldfish (T. Kishimoto and Y. Nagahama, unpublished observations).

5. Ovulation

After the completion of the first meiotic division, oocytes (now eggs) are expelled into the ovarian cavity or the peritoneal cavity, a process known as ovulation. Ovulated eggs continue meiosis up to the second meiotic metaphase, the point at which fertilization first becomes possible, the entire process or at least part, being hormone dependent (see Chapter 3, Volume 9B, this series). However, final oocyte maturation and ovulation are not always associated because oocytes of most teleosts do not undergo ovulation following steroid stimulation *in vitro*. Ovulation in teleosts involves several preparatory steps. First, the follicle layer must detach from the oocyte. Ultrastructural studies have revealed that both follicle cell microvilli and oocyte microvilli withdraw from the egg membrane near the time of ovulation and a wide space is formed between the follicle cell and the egg membrane (Flügel, 1967; Yamamoto and Yamazaki, 1967; Hirose, 1972; Pendergrass and Schroeder, 1976; Iwamatsu and Ohta, 1977). Although the mechanism directing the interruption of follicle–oocyte communication prior to ovulation is unknown, proteolytic enzymes have been suggested to be involved in disrupting follicle–oocyte connections (Oshiro and Hibiya, 1975, 1982). Prostaglandin $F_{2\alpha}$ is capable of inducing ovulation *in vitro* of goldfish oocytes that had been previously matured *in vivo* by injection of human chorionic gonadotropin (HCG) (Kagawa and Nagahama, 1981). Observations with the dissecting microscope and the scanning electron microscope have revealed that during prostaglandin-induced ovulation, oocytes are expelled into the incubation medium through a rupture in the follicle at the point of

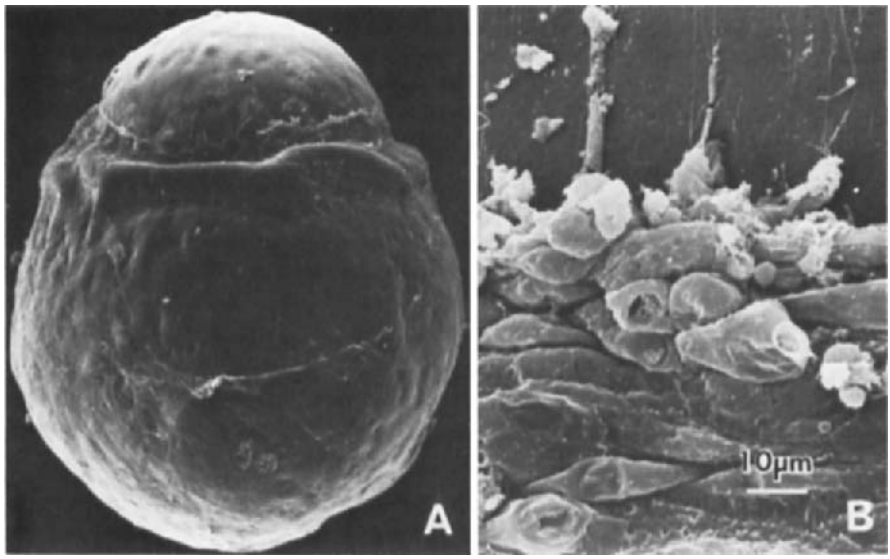

Fig. 11. (A) Scanning electron micrograph of a mature goldfish (*Carassius auratus*) oocyte during ovulation *in vitro* induced by prostaglandin $F_{2\alpha}$. (B) Detail of A showing shrinkage of the follicle layers away from the emerging oocyte.

attachment to the overlying epithelium. At the same time contraction of the ovarian follicle layer was observed (Kagawa and Nagahama, 1981) (Fig. 11). *In situ,* rupture at this point would allow the oocyte to be expelled from its enclosing follicle into the ovarian cavity. Ultrastructural studies of oocytes from some teleosts have demonstrated that some contractile elements such as microfilaments are present in the thecal cells near the time of ovulation (Szöllösi and Jalabert, 1974; Nagahama *et al.*, 1976; Pendergrass and Schroeder, 1976). Smooth musclelike cells have been reported in the thecal cells of the rainbow trout (Szöllösi and Jalabert, 1974). In the medaka, 50–70 Å microfilaments, which were sparse in the thecal cells at the start of incubations with hydrocortisone, became organized into bands after 5 hr and into bundles after 10 hr (Pendergrass and Schroeder, 1976). The addition of cytochalasin B, an inhibitor of microfilamentous contractile systems, into the incubation medium totally prevented hydrocortisone-induced ovulation *in vitro* of the medaka (Schroeder and Pendergrass, 1976) and partially inhibited prostaglandin-induced ovulation *in vitro* of the rainbow trout (Jalabert and Szöllösi, 1975). These results suggest that the microfilamentous system is necessary for ovulation in teleosts.

IV. STEROIDOGENIC TISSUES

Earlier biochemical approaches to steroidogenesis in fish gonads demonstrated in a few restricted species that teleost gonadal tissue is capable of synthesizing several different steroids (see Chapter 7, this volume). The recent advance in specific radioimmunoassay methods and the development of reliable *in vitro* incubation techniques have permitted a quantitative assessment of the production by gonadal tissue of various steroids in response to teleost and mammalian gonadotropin preparations (e.g., Yaron and Barton, 1980; Bona-Gallo and Licht, 1981; Kagawa *et al.*, 1982; Nagahama *et al.*, 1982). Various morphological methods, such as the histochemical demonstration of various hydroxysteroid dehydrogenases regulating the biosynthesis of steroid hormones, and electron microscopy have been applied to determine the site of steroid synthesis in the gonads of many species of teleosts (Hoar, 1969; Lofts and Bern, 1972; de Vlaming, 1974; Guraya, 1976a,b; Hoar and Nagahama, 1978; Nagahama *et al.*, 1982). Using these techniques. researchers have implicated at least five different cellular sites in testicular steroid production depending on the species studied: the interstitial cells, lobule boundary cells, Sertoli cells, germ cells, and the epithelial cells of testicular efferent ducts. Similarly, in the ovary, the granulosa cells, certain thecal cells, corpora lutea, corpora atretica, and interstitial gland tissue have been described as steroid producing (see ref. above).

A. Testis

1. Interstitial Cells or Leydig Cells

Interstitial cells are usually distributed singly or in small groups in the interstices between the lobules of the teleost testis. In *Poecilia latipinna*, interstitial cells are distributed around the efferent duct and at the periphery of the testis, but not between testis lobules (van den Hurk, 1973, 1974). The abundance of the interstitial cells varies with teleost species. Histochemically, 3β-hydroxy-Δ^5-steroid dehydrogenase (3β-HSD), an enzyme involved in steroid hormone synthesis, has been demonstrated in the interstitial cells of the testis of a number of teleosts (e.g., *Blennius* spp., Chieffi and Botte, 1964; *Gobius paganellus*, Stanley *et al.*, 1965; *Tilapia mossambica*, Yaron, 1966; Hyder, 1970; *Cymatogaster aggregata*, Wiebe, 1969; goldfish, Yamazaki and Donaldson, 1969; guppy, Takahashi and Iwasaki, 1973a; medaka, Takahashi and Iwasaki, 1973b; rainbow trout, van den Hurk *et al.*, 1978a,b). Electron microscopical observations have demonstrated that testicular interstitial cells of teleosts have ultrastructural features typical of

steroid-producing cells (e.g., guppy, Follénius and Porte, 1960; *Gasterosteus aculaetus*, Follénius, 1968; rainbow trout, Oota and Yamamoto, 1966; van den Hurk *et al.*, 1978a,b; *Cichlasoma nigrofasciatum*, Nicholls and Graham, 1972; medaka, Gresik *et al.*, 1973a; *Gobius jozo*, Colombo and Burighel, 1974; black molly, van den Hurk *et al.*, 1974b; coho, *Oncorhynchus kisutch* and pink, *O. gorbuscha*, salmon, Nagahama *et al.*, 1978; *Anguilla japonica*, Sugimoto and Takahashi, 1979); they are large polygonal cells characterized by extensive agranular endoplasmic reticulum and mitochondria with tubular cristae (Fig. 12). In medaka, interstitial cells appear before the spermatogonia differentiate (Yoshikawa and Oguri, 1979), and ultrastructurally they can be identified in the testis of *Tilapia nilotica* during or immediately after sex differentiation (M. Nakamura and Y. Nagahama, unpublished observations). Interstitial cells have also been iden-

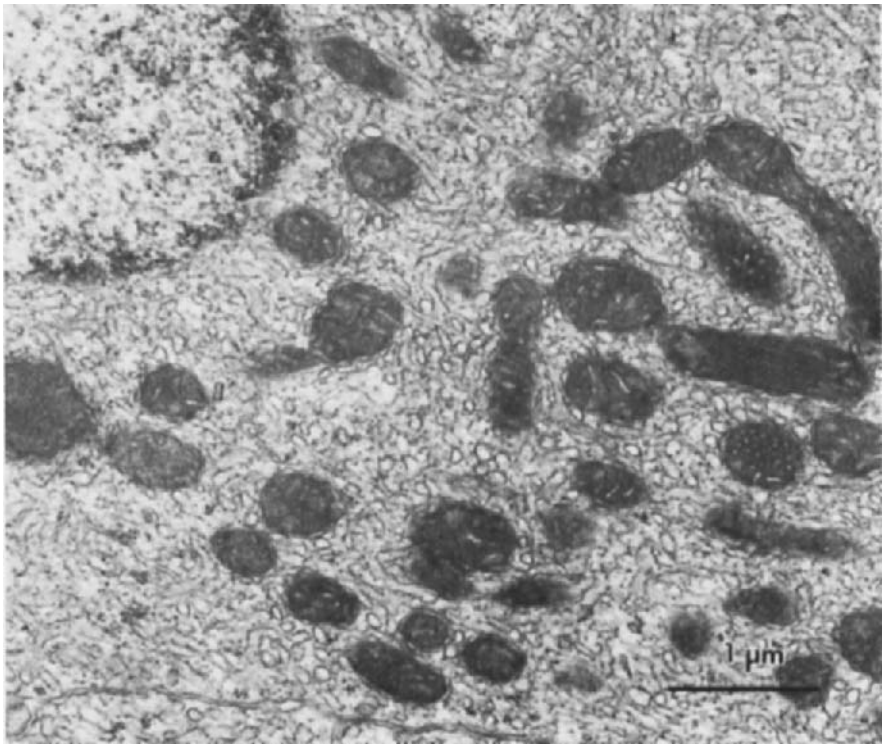

Fig. 12. Electron micrograph of a portion of an interstitial cell of *Tilapia nilotica* showing extensive agranular endoplasmic reticulum and numerous dense mitochondria with tubular cristae. (Courtesy of Dr. Nakamura.)

tified in immature rainbow trout (Oota and Yamamoto, 1966) and coho and pink salmon testes (Nagahama *et al.*, 1978). Nicholls and Graham (1972), who studied the ultrastructure of the testis of *Cichlasoma nigrofasciatum* found evidence for the origin of interstitial Leydig cells from fibroblastlike connective tissue elements.

The interstitial cells of immature silver eel, *Anguilla japonica*, were markedly stimulated by HCG, producing a pronounced increase in the size and number of mitochondria and an increased organization of agranular endoplasmic reticulum (Sugimoto and Takahashi, 1979). These morphological changes in the interstitial cells may be associated with enhanced steroid production observed in testicular tissues of the European eel, *Anguilla anguilla*, after administration of HCG (Eckstein *et al.*, 1982). Seasonal changes in the morphology of interstitial cells have also been reported in some teleosts (Guraya, 1976b; Grier, 1981). These observations clearly indicate that the interstitial cells are homologous with mammalian Leydig cells, and are the major site of androgen synthesis.

2. Sertoli Cells or Lobule Boundary Cells

Histochemical studies have revealed the presence of 3β-HSD activity in the Sertoli cells of *Cymatogaster aggregata* (Wiebe, 1969), *Fundulus heteroclitus* (Bara, 1969),and rainbow trout (van den Hurk *et al.*, 1978a,b) and in the lobule boundary cells of *Tilapia mossambica* (Yaron, 1966). However, most electron microscopical observations do not support these histochemical results. As described earlier (see Section II), the Sertoli cells of most of the teleosts studied to date have some ultrastructural features suggesting phagocytosis and an involvement in transporting metabolites (Fig. 13). Nevertheless, Sertoli cells or lobule boundary cells of certain species have been reported to contain some ultrastructural features commonly accepted as characteristic of steroid-producing cells, i.e., sparse tubular cristae of mitochondria and agranular endoplasmic reticulum, although much less developed than those of the interstitial Leydig cells, and many lipid droplets (e.g., Nicholls and Graham, 1972; van den Hurk *et al.*, 1974b, 1978a,b; Nagahama *et al.*, 1978).

In the amago salmon testis, Sertoli cells become hypertrophied during the later part of spermiation. This appears to be related to increased steroidogenic activity, because incubation of testicular fragments at this stage with gonadotropin results in a striking increase in the production of 17α,20β-dihydroxy-4-pregnen-3-one. Significantly, plasma levels of this steroid reach a peak during the spermiation stage (Ueda *et al.*, 1983). The relationship of Sertoli cell activity to 17α,20β-dihydroxy-4-pregnen-3-one production is currently being further explored.

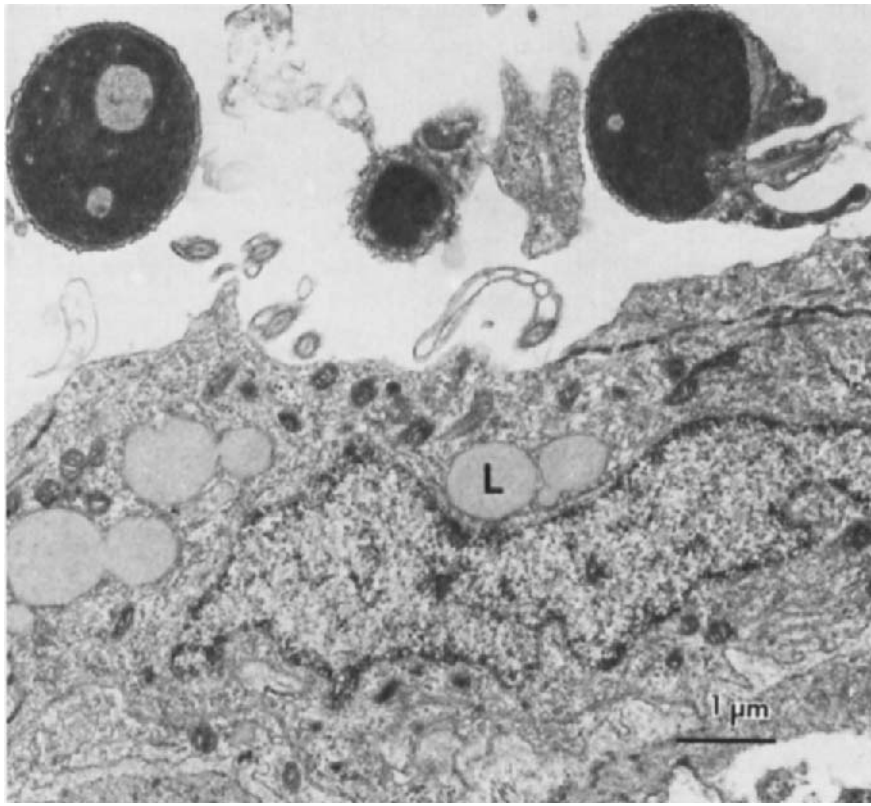

Fig. 13. Electron micrograph of a portion of a Sertoli cell in the goldfish (*Carassius auratus*) testis (L, lipid droplet).

B. Ovary

1. Preovulatory Follicles

a. Granulosa Cells. Histochemical studies have indicated that 3β-HSD activity is localized in the granulosa cells of guppy (Lambert, 1970), *Mugil capito* and *Tilapia aurea* (Blanc-Livni *et al.*, 1969; Blanc-Livni, 1971), *Acanthobrama terrae-sanctae* (Yaron, 1971), medaka (Iwasaki, 1973; Kagawa and Takano, 1979), *Monopterus albus* (Tang *et al.*, 1974), rainbow trout (van den Hurk and Peute, 1979), and loach (Ohta and Teranishi, 1982). Recently, a strong 3β-HSD activity has also been demonstrated in the micropylar cell of the ovarian follicle of the loach during the prematuration and spawning

season (Ohta and Teranishi, 1982). In addition, 17β-HSD activity has been detected in the granulosa cells of *M. capito* (Blanc-Livni *et al.*, 1969; Blanc-Livni, 1971), guppy (Lambert, 1970), and goldfish (Khoo, 1975).

However, these histochemical observations on granulosa cells are not supported by ultrastructural studies, because these cells contain features suggestive of protein synthesis, but not organelles associated with steroid-producing cells (Nicholls and Maple, 1972; Nagahama *et al.*, 1976, 1978; Hoar and Nagahama, 1978; Kagawa and Takano, 1979; van den Hurk and Peute, 1979; Kagawa *et al.*, 1981; Ohta and Teranishi, 1982). Two recent exceptions to the numerous observations on the nonsteroidogenic appearance of granulosa cells have been reported. Wallace and Selman (1980) have shown that during final oocyte maturation of *Fundulus heteroclitus*, the granulosa cells undergo specific cytological alterations. These changes include: (1) a proliferation of enormous Golgi complexes with accumulated secretory material, and (2) an increase in the number of cisternae of granular endoplasmic reticulum and free ribosomes. Wallace and Selman suggest the possible contribution of the granulosa cells to the production of maturation-inducing steroid. In the medaka, the appearance of "special granulosa cells" 14.5 hrs after the beginning of the light phase in oocytes destined for maturation has been reported (Iwamatsu and Ohta, 1981b). Mitochondria with tubular cristae were described, leading Iwamatsu and Ohta to suggest a transient steroidogenic role for these cells. It should be noted that typical steroidogenic, special thecal cells are not found in the thecal layers of both *Fundulus* (Wallace and Selman, 1980) and medaka (Iwasaki, 1973).

b. Special Thecal Cells. Bara (1965), who studied the distribution of 3β-HSD in the ovary of the mackerel, *Scomber scomber*, at different stages of the reproductive cycle, was the first to demonstrate the activity of this enzyme in certain cells of the thecal layer; activity was strongest at the beginning of vitellogenesis and became reduced in intensity as the oocytes matured. Subsequently, histochemical examinations have revealed a similar restricted occurrence of 3β-HSD activity in the thecal layer of the ovaries of zebrafish (Yamamoto and Onozato, 1968; van Ree, 1976), goldfish (Nagahama *et al.*, 1976) and *Mystus cavasius* (Saidapur and Nadkarni, 1976). The presence of 17β-HSD has also been reported in the thecal cells of the ovaries of *Trachurus mediterraneus* (Bara, 1974) and *Mystus cavasius* (Saidapur and Nadkarni, 1976). Ultrastructurally, the special thecal cells reported in a number of teleosts possess features which characterize steroidogenic tissue in general, that is, mitochondria with tubular cristae and a tubular agranular endoplasmic reticulum (zebrafish, Yamamoto and Onozato, 1968; *C. nigrofasciatum* and *Haplochromis multicolor*, Nicholls

and Maple, 1972; goldfish, Nagahama *et al.*, 1976; *Oncorhynchus kisutch* and *O. gorbusha*, Nagahama *et al.*, 1978; white-spotted char, *Salvelinus leucomaenis*, Kagawa *et al.*, 1981).

2. Postovulatory Follicles

Young postovulatory follicles of some teleosts are characterized by a highly vascularized thecal layer and hypertrophied granulosa cells. Moreover, both special thecal cells and granulosa cells often show evidence of 3β-HSD activity (Bara, 1965; Iwasaki, 1973; Lambert and van Oordt, 1974; Khoo, 1975; Nagahama *et al.*, 1976; Kagawa and Takano, 1979; van den Hurk and Peute, 1979; Kagawa *et al.*, 1981; Lang, 1981). Therefore, the close histological and histochemical resemblance of these postovulatory follicles to the mammalian corpus luteum might suggest steroid biosynthesis in these

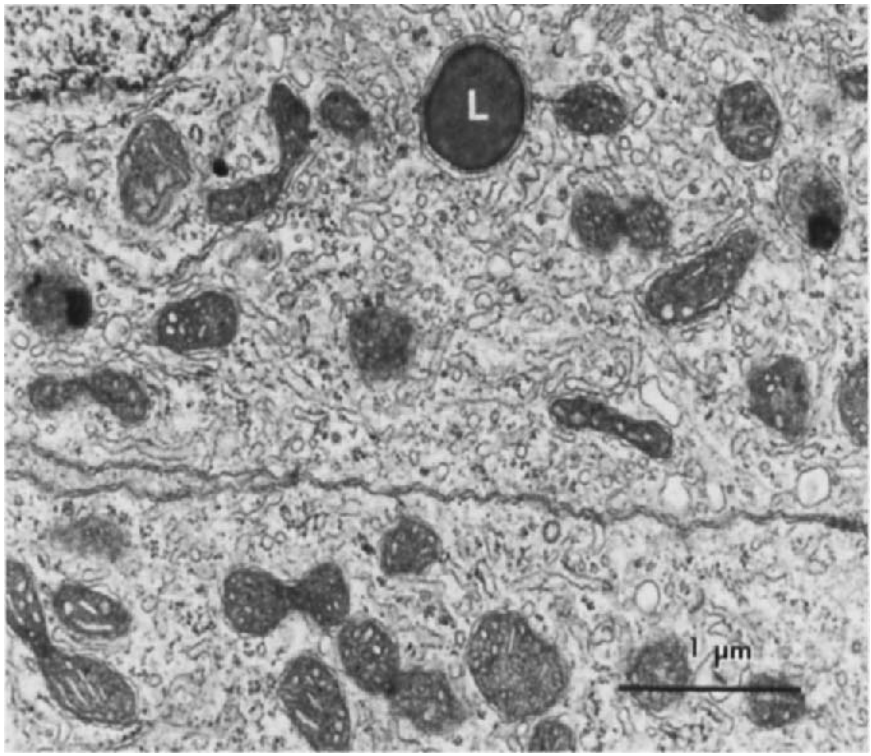

Fig. 14. Electron micrograph of a portion of the special thecal cells of the postovulatory follicle of coho salmon (*Oncorhynchus kisutch*) showing extensive agranular endoplasmic reticulum and large mitochondria with tubular cristae (L, lipid droplet).

tissues. Special thecal cells appear to maintain their steroidogenic activity for some time after ovulation (Fig. 14); the duration of maintenance of the activity varies from species to species (Nicholls and Maple, 1972; Nagahama *et al.*, 1976, 1978; van den Hurk and Peute, 1979; Kagawa *et al.*, 1981). In addition, the possible immediate transformation of granulosa cells to luteal cells has been suggested by ultrastructural observations on the young postovulatory follicles of *Cichlasoma nigrofasciatum* (Nicholls and Maple, 1972), goldfish (Nagahama *et al.*, 1976), rainbow trout (van den Hurk and Peute, 1979), white-spotted char (Kagawa *et al.*, 1981), and *Perca fluviatilis* (Lang, 1981). Khoo (1975), using [^{3}H]thymidine, suggested a differentiation of older luteal cells into oogonia in the goldfish.

Kagawa *et al.* (1981) reported an active steroidogenic appearance of the special thecal cells of postovulatory follicles shortly after ovulation in the white-spotted char, in parallel with high levels of plasma progesterone, thereby suggesting that the special thecal cells are the major sites of progesterone synthesis during the postovulatory period. Direct evidence of steroidogenesis in young postovulatory follicles was obtained by recent *in vitro* studies (Nagahama and Kagawa, 1982). Partially purified chinook salmon gonadotropin (SG-G100) stimulated progesterone production *in vitro* in the isolated young postovulatory follicles of the amago salmon, *Oncorhynchus rhodurus,* but not in the older ones. Recent *in vitro* data further indicate that the young postovulatory follicles of the amago salmon are also capable of producing 17α-hydroxyprogesterone, 17α,20β-dihydroxy-4-pregnen-3-one, and, to lesser extent, testosterone in response to gonadotropin (S. Adachi *et al.*, unpublished observations). Production of 17β-estradiol was not stimulated in either young or older postovulatory follicles. The demonstrated synthetic ability of young postovulatory follicles to produce progestogens and testosterone in response to gonadotropin and the presence of high concentrations of these steroids in the plasma of postovulatory females (H. Kagawa and G. Young, unpublished observations) leads to the question of the physiological significance of these steroids in the postovulatory period. Possibilities, such as ovarian maintenance, and/or an involvement in the spawning process exist, and certainly deserve further investigation.

3. Atretic Follicles (Corpora Atretica or Preovulatory Corpora Lutea)

The atretic follicle has also been described as the site of ovarian steroid biosynthesis in teleosts because of its glandular appearance (e.g., Ball, 1960; Hoar, 1969; Browning, 1973). However, the histochemical test for 3β-HSD has failed to demonstrate any enzyme activity in the atretic follicles of the

ovaries of mackerel (Bara, 1965) and *Poecilia reticulata* (Lambert, 1970). These histochemical data are supported by recent *in vitro* studies on the steroidogenic capacity of atretic follicles of the goldfish. Incubation of atretic follicles (α to β stages according to Khoo, 1975) with gonadotropin failed to elicit a detectable (more than 30 pg/ml) production of progesterone, testosterone, and 17β-estradiol. In the light of the numerous attempts and failures to demonstrate steroidogenesis in atretic follicles that have occupied investigators over the years, it seems likely that further *in vitro* studies will finally allow us to conclude whether atretic follicles are associated only with degeneration and resorption of yolk or additionally with some other process.

4. A Two Cell-Type Model for the Production of 17β-Estradiol and Maturation-Inducing Steroid (17α,20β-Dihydroxy-4-pregnen-3-one)

a. 17β-Estradiol. In teleosts, 17β-estradiol, which has been previously isolated from the ovaries of some species (e.g., Gottfried *et al.*, 1962; Lupo and Chieffi, 1963; Horvàth *et al.*, 1978; see also Chapter 7, this volume), is known to induce the synthesis and secretion of a female specific protein, vitellogenin, by the liver (e.g., Wallace and Selman, 1981). However, the site of 17β-estradiol synthesis has not been established. Recent studies suggest that estrogen biosynthesis occurs not only in the gonads but also in the brain, although the physiological importance of the latter observation is not yet understood (Callard *et al.*, 1981). The recent use of *in vitro* techniques has provided detailed information on the site of 17β-estradiol production. Intact follicles obtained from vitellogenic amago salmon produced 17β-estradiol when they were incubated with fish gonadotropins (Kagawa *et al.*, 1982). Unlike many teleosts, the thecal layer can be removed intact from the follicles of this species as a routine dissection procedure (Kagawa *et al.*, 1982) (Fig. 15). Taking advantage of this characteristic of the salmonid ovarian follicle, the role of the thecal and granulosa layers in 17β-estradiol synthesis has been investigated in detail by an *in vitro* incubation method. In these experiments, four different follicular preparations, intact early vitellogenic follicles (oocytes with complete follicle layers), thecal layers contaminated with less than 10% granulosa cells, pure granulosa layers and zona radiata, and thecal layer–granulosa layer cocultures, were incubated in the presence or absence of a partially purified salmon gonadotropin (SG-G100) or various precursor steroids. The SG-G100 enhanced 17β-estradiol production both by intact follicles and coculture preparations, but not by the isolated thecal or granulosa layers. Furthermore, isolated granulosa layers produced large amounts of 17β-estradiol when incubated in media in which thecal layers had been previously incubated with gonadotropin (G. Young *et al.*, un-

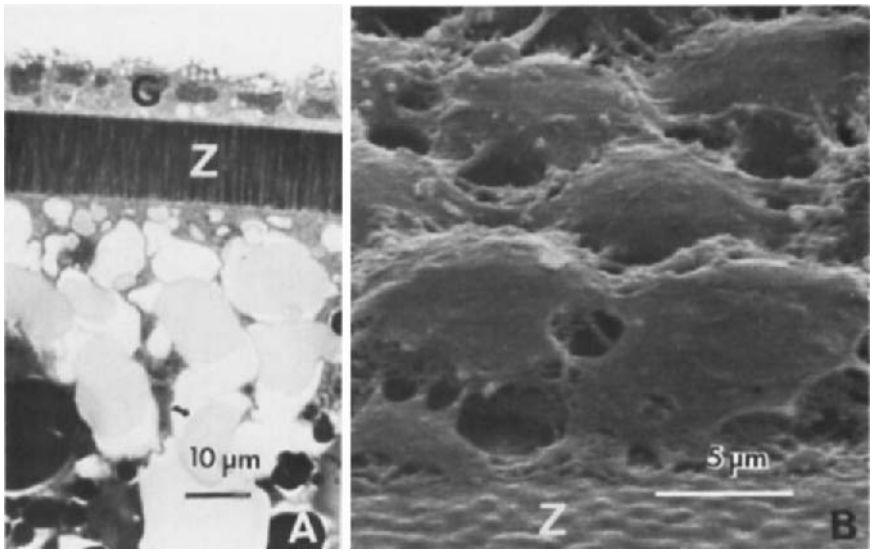

Fig. 15. (A) Light micrograph of a follicular preparation (Epon-embedded 1 μm section, methylene blue and azure II) from which the thecal layer has been removed (G, granulosa layer; Z, zona radiata). (B) Scanning electron micrograph of a granulosa layer preparation, consisting purely of granulosa cells (Z, zona radiata).

published observations). These results clearly indicate that both layers are necessary for gonadotropin-stimulated 17β-estradiol production (Nagahama *et al.*, 1982). Analysis of media from the same experiment (Kagawa *et al.*, 1982) indicated that SG-G100 greatly stimulated testosterone production by thecal layers, but only slightly stimulated production by the other follicular preparations. Using androstenedione or testosterone as substrates, much more 17β-estradiol was produced by granulosa layers compared to the production with 17α-hydroxyprogesterone, suggesting that the enzyme involved in the conversion of 17α-hydroxyprogesterone to androstenedione (17α-hydroxyprogesterone-C_{17}-C_{20} lyase) in the granulosa cells is weak (H. Kagawa, unpublished observations). These results further suggest that 17β-hydroxysteroid dehydrogenase is located not only in thecal layers but also in granulosa layers.

The conversion of testosterone to 17β-estradiol in the granulosa layer was not enhanced by addition of gonadotropin to the incubation medium, suggesting that in early vitellogenic follicles at least, all that is necessary for 17β-estradiol synthesis by the granulosa cell layers is the availability of suitable substrate. The mechanism of the induction or activation of the granulosa cell

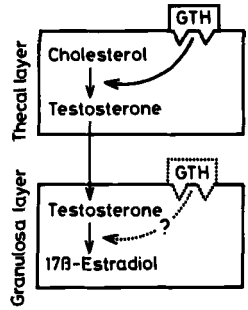

Fig. 16. Two cell-type model for the synthesis of follicular 17β-estradiol in the amago salmon (*Oncorhynchus rhodurus*) (see the text for details).

aromatase system is unknown. From these results, we have recently proposed a two cell-type model for the production of follicular estrogen, which implicates the vascularized thecal layer as the site of the biosynthesis of androgens from cholesterol in response to gonadotropin; androgens (androstenedione and/or testosterone) are transported to the granulosa layer and aromatized to 17β-estradiol (Kagawa *et al.*, 1982) (Fig. 16). A similar two cell-type model seems applicable to the production of follicular estrogen in the rainbow trout (Fig. 17) (Y. Nagahama, unpublished observations). This is the first direct evidence in lower vertebrates for an interraction between thecal cells and granulosa cells in the synthesis of follicular estrogens. In mammals, a similar model for the production of follicular estrogens involving thecal and granulosa cells, first proposed by Falck (1959), is now widely accepted (e.g., Dorrington, 1977). Only one other report concerning a two cell-type model has been presented for a nonmammalian vertebrate. Huang and Nalbandov (1979a,b) suggest that the granulosa cells of the chicken produce progesterone (or testosterone) which is converted to estrogens by the thecal cells. This two cell-type model for the production of follicular estrogen in the chicken is in sharp contrast to the fishes and mammals and thus is of evolutionary interest.

Because in the amago salmon only the special thecal cells possess the ultrastructural characteristics of steroid-secreting cells resembling testicular interstitial cells, it is reasonable to assume that these cells are the major cellular sites of estrogen precursor synthesis including testosterone. Nevertheless, aromatase and 17β-hydroxysteroid dehydrogenase activities have been demonstrated in the granulosa cells of both amago salmon and rainbow trout. Although the granulosa cells of the amago salmon, like most other teleosts (see Section IV,B), lack organelles associated with steroidogenesis, they contain features suggestive of protein synthesis. Further studies are

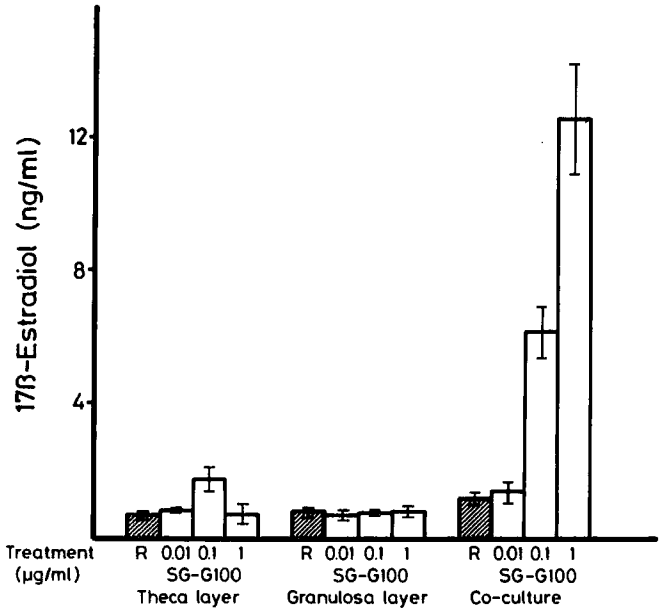

Fig. 17. Effects of chinook salmon gonadotropin (SG-G100) on 17β-estradiol secretion by rainbow trout (*Salmo gairdneri*) follicles at the early vitellogenic stage. Three different follicular preparations (10 follicles/ml) were incubated in Ringer alone (R, shaded) or Ringer with various concentrations of chinook salmon gonadotropin (0.01–1 μg/ml) for 18 hr. The vertical bars represent the mean ± SEM of the three replicates (see the text for details).

required before the ultrastructural characteristics of the granulosa cells can be linked to these enzymatic activities.

b. Maturation-Inducing Steroid. The stimulatory effects of gonadotropins on oocyte maturation and ovulation have been documented for a number of teleost species (see Chapter 3, Volume 9B, this series). In most teleosts, it is believed that gonadotropin stimulates the ovarian follicle layer to produce maturation-inducing steroid(s) which in turn acts on the surface of oocytes to induce maturation. This has been proved by *in vitro* studies using either defolliculated oocytes (Hirose, 1976; Jalabert, 1976; Iwamatsu, 1980), or folliculated oocytes treated with metabolic inhibitors (cyanoketone, an inhibitor of 3β-HSD, Young *et al.*, 1982; metopirone, an inhibitor of 11β-hydroxylase, Hirose, 1973). Studies in my laboratory and others have conclusively demonstrated that the maturation-inducing steroid of certain salmonids is 17α,20β-dihydroxy-4-pregnen-3-one (Tamaoki *et al.*, 1982; Nagahama *et al.*, 1983a; see Chapter 3, Volume 9B, this series). This steroid is a highly effective inducer of maturation *in vitro* (e.g., Nagahama *et al.*,

1983b; see Chapter 3, Volume 9B, this series). It is synthesized by the follicle (Suzuki *et al.*, 1981b) in response to gonadotropin (Fostier *et al.*, 1981; Suzuki *et al.*, 1981a; Nagahama *et al.*, 1983a; Young *et al.*, 1983), and elevated concentrations of this steroid are found in the plasma of females undergoing final oocyte maturation (Fostier *et al.*, 1981; Scott *et al.*, 1982; Young *et al.*, 1983). The identification of the maturation-inducing steroid of amago and rainbow trout as 17α,20β-dihydroxy-4-pregnen-3-one permitted a study of the role of the follicle layers in the production of this steroid, using *in vitro* incubation techniques, similar to those used for the studies on 17β-estradiol production. Three follicular preparations (thecal layer, granulosa layer, and coculture of thecal and granulosa layers) obtained from fully grown oocytes of both species were incubated with or without salmon gonadotropin. A specific radioimmunoassay was employed to measure the levels of 17α,20β-dihydroxy-4-pregnen-3-one released into the medium. *In*

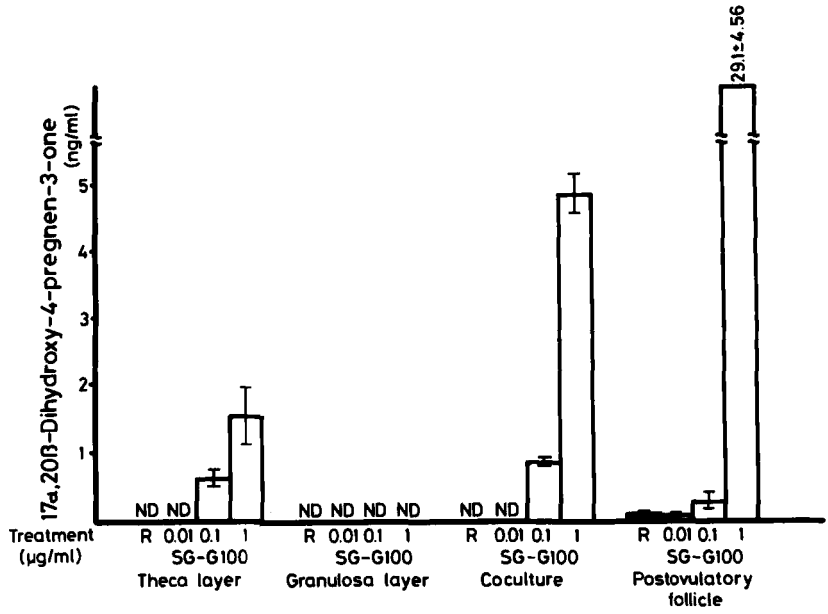

Fig. 18. Effects of chinook salmon gonadotropin (SG-G100) on 17α,20β-dihydroxy-4-pregnen-3-one production by rainbow trout (*Salmo gairdneri*) follicles. Production was assessed in three different follicular preparations from fully grown follicles and in postovulatory follicles. Ten preparations were incubated in Ringer alone (R, shaded) or Ringer with various concentrations of chinook salmon gonadotropin (0.01–1 μg/ml) for 18 hr. The vertical bars represent the mean ± SEM of the three replicates (ND, nondetectable) (less than 30 pg/ml) (see the text for details).

vitro production of 17α,20β-dihydroxy-4-pregnen-3-one by the coculture preparations was remarkably enhanced by gonadotropin, but gonadotropin only slightly enhanced production by thecal layers. No stimulation was observed in the granulosa preparations (Fig. 18). These results indicate that the interaction of both layers is necessary for the production of 17α,20β-dihydroxy-4-pregnen-3-one in response to gonadotropin. It is most probable that the relatively much lower concentration of 17α,20β-dihydroxy-4-pregnen-3-one produced by thecal layers was caused by the contamination of the thecal preparations with granulosa cells.

The concentrations of 17α-hydroxyprogesterone of media from the same experiment were determined, and the results showed that large amounts of 17α-hydroxyprogesterone were present in the thecal preparations, only small amounts in the coculture groups, but no detectable levels in incubates with granulosa layers. Furthermore, it has been shown that gonadotropin remarkably enhances the production of 17α,20β-dihydroxy-4-pregnen-3-one by granulosa cells incubated with 17α-hydroxyprogesterone, thereby indicating that gonadotropin acts directly on the granulosa layers to stimulate the activity of 20β-hydroxysteroid dehydrogenase (Young *et al.*, 1983). Considering these data taken together, a two cell-type model for the production of the maturation-inducing steroid by the teleost ovarian follicle has been proposed for the first time in any vertebrate (Nagahama *et al.*, 1983a; G. Young *et al.*, unpublished observations). Under the influence of gonadotropin, the thecal layer synthesizes precursors, possibly 17α-hydroxyprogesterone, which are transferred to the granulosa layer and converted to the maturation-inducing steroid, 17α,20β-dihydroxy-4-pregnen-3-one.

V. MORPHOLOGY OF EGG MEMBRANE—CHORION AND MICROPYLE

A. Egg Membrane

One of the most striking features observed during teleost oogenesis is the formation of a thick, highly differentiated zone (egg membrane, vitelline membrane, zona radiata, zona pellucida) lying between granulosa layers and oocytes. Depending on the species as well as the stage of oocyte growth, the egg membrane varies in its thickness; it is 7–8 μm in the fully grown oocytes of the goldfish and about 30 μm in the rainbow trout. These morphological differences of the membrane may reflect adaptations to diverse ecological conditions. In light micrographs, this membrane is characterized by its striated pattern; therefore, it has been designated as the zona radiata. Ultra-

structurally, the striated appearance of the membrane corresponds to the penetration of microvilli and processes from both the oocyte and the follicle cells (Fig. 19A). Histochemical examinations have revealed that the membrane consists mainly of carbohydrates and proteins (e.g., Guraya, 1978). The exact mechanism by which the egg membrane is formed in teleost oocytes is unknown. It has been a matter of confusion whether the membrane originates from the oocyte or the follicle cells, or both. In the oocytes of the seahorse, *Hippocampus erectus*, and the pipefish, *Syngnathus fuscus*, this membrane is believed to be formed by the oocyte; therefore, it is classified as a primary envelope (Anderson, 1967). These observations have been confirmed in some other species (Wourms, 1976; Flegler, 1977; Tesoriero, 1977; Dumont and Brummett, 1980). However, the follicle cells may contribute to the formation of certain parts of multilayered egg membranes. In two species of South American annual fishes, *Cynolebias ladigesi* and *C. melanotaenia*, the outer layer of the egg membrane is elaborated by the follicle cells, and is thus classified as a secondary envelope; the tubular components, which are synthesized and secreted by the follicle cells, are responsible for the formation of this layer (Wourms and Sheldon, 1976). In *Cichlasoma nigrofasciata*, the adhesive apparatus surrounding the zona pellucida, comprising filaments and mucous jelly coat, is synthesized in the follicle cells during vitellogenesis; this structure appears to be secreted directly from the granular endoplasmic reticulum (Busson-Mabillot, 1977). Various functions have been suggested for these structures; in *Cichlasoma* and *Fundulus* they serve as the attachment of the eggs to the substratum (Busson-Mabillot, 1977), and in *Cynolebias* they may serve as a chorionic respiratory system (Wourms and Sheldon, 1976).

B. Micropyle

The egg membrane of many teleosts is structurally modified to form a small opening, the micropyle, through which the sperm gains access to the enclosed egg (e.g., Laale, 1980). The literature describing micropyles has been reviewed by Riehl and Götting (1974). Both scanning and transmission electron microscopical studies have been conducted on micropyles of several teleosts (Szöllösi and Billard, 1974; Kuchnow and Scott, 1977; Riehl, 1977; Brummett and Dumont, 1979; Hosokawa, 1979; Stehr and Hawkes, 1979; Dumont and Brummett, 1980; Kudo, 1980; Hosokawa *et al.*, 1981; Iwamatsu and Ohta, 1981a) (Fig. 19A). The micropyle delineates the animal pole of the oocyte and varies in size from species to species. The outer diameter of the micropyle of *Fundulus heteroclitus* eggs is about 2.5 μm and 1–1.5 μm at its internal opening (Dumont and Brummett, 1980). The outer opening of funnel-shaped micropyle of the medaka is about 23 μm in diameter and its inner

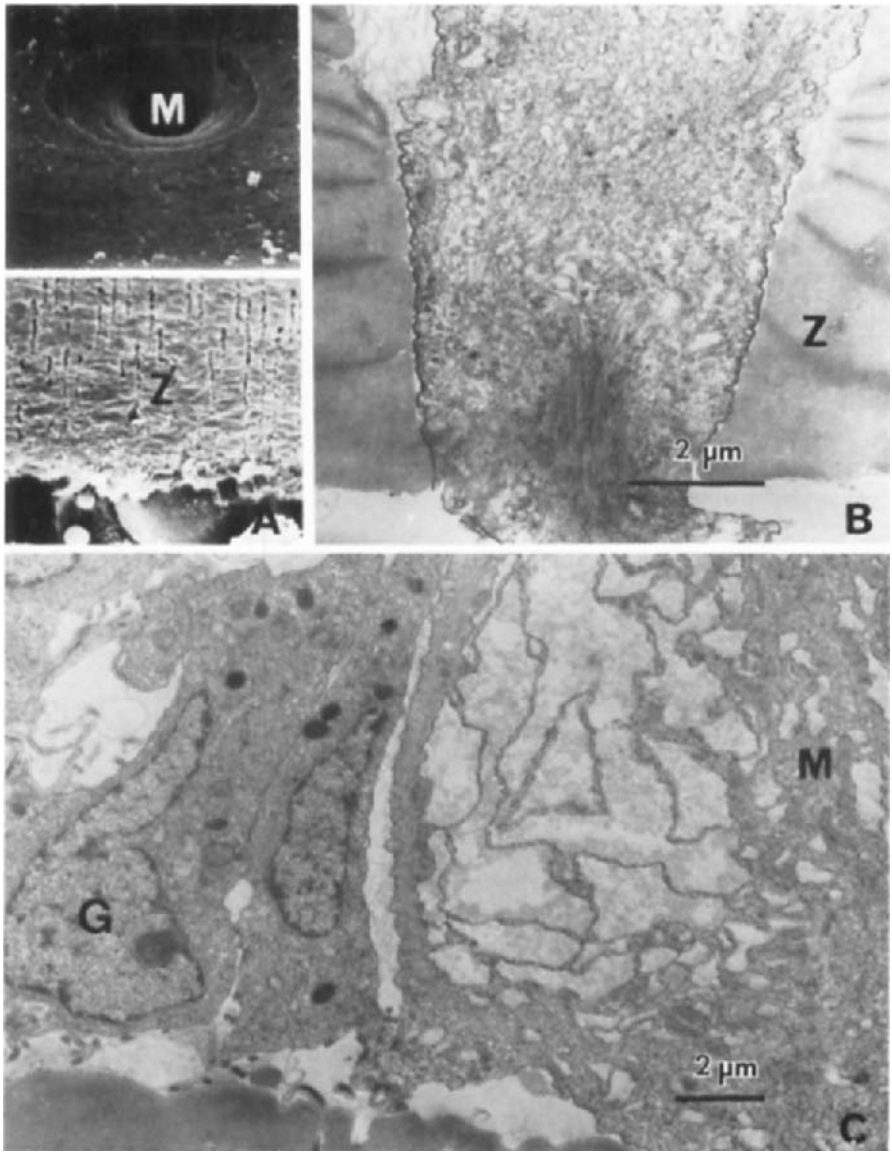

Fig. 19. The micropyle and micropylar cells of rainbow trout (*Salmo gairdneri*) and goldfish (*Carassius auratus*). (A) Scanning electron micrograph showing the appearance of the micropyle (M) and the zona radiata (Z). (B) Transmission electron micrograph of a goldfish micropylar cell process which extends from the granulosa layer through the zona radiata (Z) to the oocyte surface. (C) Transmission electron micrograph of a portion of the granulosa layer of goldfish in the vicinity of the micropyle. The micropylar cell (M) is morphologically distinct from the granulosa cells (G).

opening is about 2.5 μm (Iwamatsu and Ohta, 1981a). Very little is known of the morphological details of the formation of the micropyle. In *Noemacheilus barbatulus*, the micropyle first appears at a relatively early stage (late non-vitellogenic stage) during the formation of the egg membrane and is believed to be formed by a highly specialized follicle cell ("Sapfenzelle") (Riehl, 1977).

C. Micropylar Cells

The micropyle is occupied by a highly specialized micropylar cell until matured oocytes are discharged from their enclosing follicles at the time of ovulation. Numerous light microscopical studies have been conducted on these cells (e.g., Riehl and Götting, 1974; Laale, 1980) (Fig. 8). Transmission electron microscopical observations on the micropylar cells have been performed in *Noemacheilus barbatulus* and *Gobio gobio* (Riehl, 1977) and the loach (Ohta and Teranishi, 1982). The micropylar cells of the goldfish can be easily distinguished from granulosa cells by their characteristic morphology (Fig. 19B and C). The cells are large, triangular in shape, and are embedded in the micropyle canal. The micropylar cells possess various cell organelles, such as granular endoplasmic reticulum, mitochondria, small vesicles, Golgi apparatus, filaments, and microtubules, which have a characteristic distribution within the cytoplasm. The apical cytoplasm is occupied by a considerable amount of microtubules oriented, for the most part, parallel to the long axis of the cell. Centrioles are located in the apical cytoplasm immediately beneath the surface of the cell. A small process of the apical cytoplasm of the micropylar cells extends into the surface of the ooplasm. Well-developed cisternae of granular endoplasmic reticulum, often dilated with an amorphous material, are distributed in the basal cytoplasm. These specialized structures of the micropylar cells suggest that these cells function not only as the plug of the micropyle but also as secretory cells. In this connection, it is interesting to note that the aggregation of sperm occurs selectively in the area around the micropyle during the initial stages of fertilization (Suzuki, 1958).

VI. CONCLUDING REMARKS

Although our knowledge of the functional morphology of teleost gonads has substantially increased since the last related volume of this series (Hoar, 1969), a number of questions remain unresolved and this concluding section serves to highlight some of the current fundamental issues. Studies using the

most modern morphological techniques have helped to clarify several important morphological aspects of germ cell development in the teleost gonads. These studies will serve as the basis for further understanding of the kinetics of the gametogenic processes. Information presented in this chapter indicates that there are several processes of germ cell development which are closely associated with changes in cellular activities of somatic cell elements. Of particular interest is the kinetics of steroid-producing cell development during sexual differentiation, a subject which remains largely unexplored. In several species, sexual differentiation is accompanied by the almost simultaneous differentiation of steroid-producing cells. Detailed studies are necessary to determine whether steroid-producing cells differentiate prior to the onset of gonadal differentiation, and, if so, whether their appearance is directly related. Recent studies on biochemical aspects of vitellogenesis in teleosts have shown that the hepatic and ovarian yolk proteins are similar to those of amphibian species. A problem that remains is the site of cleavage of hepatic vitellogenin into yolk proteins prior to incorporation into the oocyte; morphological and biochemical studies of the ovarian follicle should help to resolve this question, particularly if isolated follicular layers, or dispersed cells can be utilized. Although the much debated question regarding the cellular source of ovarian sex steroid hormones is still not settled, an *in vitro* method involving the separation of the follicular components has facilitated investigations of the detailed mechanism of the production of two major follicular steroid hormones (17β-estradiol and 17α,20β-dihydroxy-4-pregnen-3-one). As a result of the usage of this technique, a two cell-type model involving thecal and granulosa cell layers has been proposed for the production of these two steroids for the first time in lower vertebrates. With further refinements this well characterized incubation procedure should provide an excellent system for studying the molecular basis of the mechanism of gonadotropin action on follicular steroidogenesis. The issue of whether the postovulatory follicle of teleosts is steroidogenic has now been resolved in one species, the amago salmon. The young, but not old postovulatory follicle of this species, produces steroids of unknown function in response to gonadotropin. The young postovulatory follicle of the rainbow trout, which spawns more than once is also capable of steroid production, as is the postovulatory follicle of the goldfish. It seems doubtful that steroidogenic postovulatory follicles are an unique feature of salmonid species, because 3β-HSD activity and other signs of steroidogenesis have been reported in the postovulatory follicle of diverse teleosts. The functional significance of postovulatory steroid production is presently unknown. The application of *in vitro* methods to the controversial question of the function of atretic follicles should similarly shed new light on an old problem.

The somewhat confused and conflicting terminology which has been

employed in studies of the testes is now gaining some uniformity, largely because of the recent histochemical and ultrastructural identification of steroid-producing sites. Although the interstitial cells are now established as the principal source of testicular androgens in teleosts, the possibility remains that Sertoli cells secrete steroids. Furthermore, it is worth speculating that an interaction similar to that observed in the ovarian follicular tissues of teleosts may occur in steroidogenic testicular tissues, probably between the interstitial cells and Sertoli cells. Sertoli cells of teleosts may play an important, but as yet undefined role in germ cell development. It is now understood in mammals that many of the hormonal effects on spermatogenesis are mediated through Sertoli cells, which produce the androgen-binding protein. One of the major gaps in our knowledge of testicular function evident in this discussion is the virtually neglected process of spermiation. That this process is under endocrine control is beyond doubt. What is lacking is a detailed morphological and functional study of this most important transformation of nonmotile sperm into viable, swimming gametes. Continued use of electron microscopy, immunocytochemistry, biochemical procedures, including the use of radiochemical autoradiography, and sensitive methods of hormone measurement combined with *in vitro* studies on isolated gonadal tissues should augment the base of knowledge already provided by the more classical methods of investigation.

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THE GONADAL STEROIDS

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I. INTRODUCTION

Since the publication of "Fish Physiology", Volume 3 (Hoar and Randall, 1969), the literature devoted to fish steroids has expanded greatly. In addition to "Steroids in Nonmammalian Vertebrates" edited by Idler in 1972, there have been several more restricted reviews dealing specifically with gonadal steroids (Gottfried, 1964; Chieffi, 1967, 1972; Idler, 1969; Tamaoki *et al.*, 1970; Reinboth, 1972, 1979; Colombo *et al.*, 1972a; Dodd, 1972, 1975; de Vlaming, 1974; Lambert and van Oordt, 1974a; Chan *et al.*, 1975; Colombo and Colombo Belvédère, 1975, 1976a; Guraya, 1976a,b; Lance and Callard, 1978; Billard *et al.*, 1978, 1982; Sandor, 1979; Kime, 1979a, 1980a; Martin, 1980; Tamaoki, 1980; Grier, 1981; Nagahama *et al.*, 1982). The exhaustive bibliography from Bern and Chieffi (1968) is valuable for the earlier period.

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Agnathans and cartilaginous fish are treated in Chapters 1 and 2 of this volume; therefore, in this chapter, attention is given to publications on osteichthyes (mainly teleosts) which have appeared since 1969. The various data which demonstrated the gonads potentiality to produce steroids, and the regulation of these syntheses are discussed. The secretion of sexual steroids by other tissues is also considered. Once hormones are elaborated, several mechanisms may act to regulate their activities. These mechanisms are analyzed first. The actual physiological role of gonadal steroids in fish is discussed with emphasis on gametogenesis.

II. STEROIDOGENIC TISSUES AND STEROID IDENTIFICATION

Technical tools used for studying steroids in fish are similar to those used in other zoological groups (Heftmann, 1973), although the most current, such as ultracytochemistry (Kagawa and Nagahama, 1980) radioimmunoassay (Schreck, 1972; Sangalang and Freeman, 1977; Simpson and Wright, 1977, 1978; Fostier *et al.*, 1981a; Wright and Hunt, 1981; Lamba *et al.*, 1982) or mass spectrometry (Yano and Ishio, 1978a,b,c; Diederik *et al.*, 1982; Diederik and Lambert, 1982) have only been recently used to study fish. Before reviewing the literature on gonadal steroidogenesis and other sources of sex steroids, a few remarks on these methodologies are in order.

Concerning the morphological approach, observations of ultrastructure (an extensive smooth endoplasmic reticulum or tubular mitochondrial cristae) provide the first arguments for a steroidogenic potentiality (Christensen and Gillim, 1969). Histochemical studies confirm the data from this method. Nevertheless the presence of sudanophilic lipid droplets containing triglycerids, cholesterol, or phospholipids are not specific and should be considered cautiously (Lofts and Bern, 1972). The same caution holds true for enzymatic activities indirectly related to the steroid metabolism, e.g., glucose-6-phosphate dehydrogenase or 6-phosphogluconate dehydrogenase (Lambert, 1970a; Livni, 1971; Saidapur and Nadkarni, 1976; Van Den Hurk and Peute, 1979).

More specific is the presence of a hydroxysteroid dehydrogenase (HSD) activity in cells detected by the reduction of a soluble tetrazolium salt to a precipitated colored formazan, using a steroid as substrate (Wattenberg, 1958). It should be remembered that the intensity of the reaction is related to the nature of the substrate used (Bara, 1969, 1972, 1974; Wiebe, 1969; Lambert, 1970a; Iwasaki, 1973), the cofactor added (Bara, 1969, 1972, 1974; Khoo, 1975), and the final electron acceptor (Bara, 1965, 1969). Furthermore, the existence of an intact diaphorase system remains to be tested (Gresik *et al.*, 1973). Such limitations could explain the discrepancies some-

times found, on the one hand, between histochemical and ultrastructural results (Yamamoto and Onozato, 1968; Gresik *et al.*, 1973; Kagawa *et al.*, 1981; Ohta and Teranishi, 1982), and on the other, between histoenzymochemical and biochemical results (Colombo *et al.*, 1978a; Kagawa *et al.*, 1981; Nagahama *et al.*, 1982a).

Concerning the biochemical approach, the different methodologies used to identify and quantify steroids have been reviewed and evaluated by Sandor and Idler (1972). From a physiological viewpoint, the qualitative and quantitative analysis of the peripheral blood steroids yields valuable data on the hormones available for the peripheral target tissues. Blood analyses also demonstrate the endocrinological activity of the steroidogenic glands, but Sandor and Idler (1972) emphasize the necessity of estimating the conjugated forms and the protein-bound fractions. As in mammals, steroid binding to proteins in fish appears to reduce the biological activities of the steroids (Fostier and Breton, 1975). However such peripheral measurements should be completed by an analysis of the gonadal tissues where the target cells are close to the secreting ones, and by an exploration of the other target tissues able to metabolize steroids. Until very recently *in vitro* studies of steroid metabolism dealt with whole gonads, but first attempts to isolate cell categories have recently been reported (Nagahama *et al.*, 1982a; Kagawa *et al.*, 1982b). These studies provide information on the steroidogenic potentialities of a gonad under incubation conditions. Several pitfalls may be encountered such as (1) the choice of the medium and the cofactors (Eckstein, 1970; Colombo and Colombo Belvédère, 1976b), (2) the integrity of the tissue (Colombo and Colombo Belvédère, 1976b), (3) the unknown competition between endogenous steroids and the labeled substrate for the enzymes, (4) the isotopic effect of the precursor (Kelly *et al.*, 1979), and (5) the variation of the partition coefficient between the tissue and the medium according to the metabolite (Colombo and Colombo Belvédère, 1976b; Colombo *et al.*, 1978a). Extrapolation of the *in vitro* results to the *in vivo* physiological situation must be done cautiously (De Bruijn and Van Der Mollen, 1974). Definitive conclusions require further identifications *in situ* or in the effluent blood of the glandular tissue.

A. The Ovary

1. Ultrastructural and Histoenzymochemical Data

Steroidogenic activities have been localized in interstitial, thecal, and granulosa cells according to the species and the stage of oogenesis (Table I). A weak activity is sometimes also reported in the ooplasm. A functional duality is apparently the rule because two types of cells generally appear to

Table I

Steroidogenesis Sites in Teleost Ovary

Species	Histochemical activity or electron microscopy (EM)	Localization					Reference
		Interstitial cells	Theca cells	Granulosa cells	Oocyte	Postovulatory follicle	
Salmoniformes							
Oncorhynchus kisutch	EM		++[a]	(+)		+	Nagahama *et al.* (1978);
Oncorhynchus gorbuscha	EM		++	(+)		+	Hoar and Nagahama (1978);
Oncorhynchus rhodurus	EM–3β-HSD		+				Nagahama *et al.* (1982a)
Salvelinus leucomaenis	EM		++				Kagawa *et al.* (1981)
	3β-HSD		++	+		+	
Salmo gairdneri	EM	++					Upadhyay (1977); Upadhyay *et al.* (1978)
	EM and 3β-HSD	++	++	+		+	Van Den Hurk and Peute (1979)
	3α-HSD	+	+	+	+		
Oncorhynchus keta	3β-HSD		+	+	+	+	Sufi *et al.* (1980)
	17β-HSD	–	–	–	–	–	
Oncorhynchus masou	3β-HSD		+	+	+	+	Sufi *et al.* (1980)
	17β-HSD			(+)	(+)		
Cypriniformes							
Cyprinus carpio	3β-HSD		++				Livni (1971)
Carassius auratus	3β-HSD, 3α-HSD, 17α-HSD, 17β-HSD,		(+)	++		+	Khoo (1975)
	EM and 3β-HSD		++			+	Nagahama *et al.* (1976); Hoar and Nagahama (1978)
Brachydanio rerio	EM and 3β-HSD	+	++				Yamamoto and Onozato (1968)
	3β-HSD	+	+	++			Lambert *et al.* (1972); Lambert and van Ordt (1974a)
	3β-HSD	+	+				Van Ree (1977a,b)
Acanthobrama terraesanctae	3β-HSD			+			Yaron (1971)
Siluriformes							
Clarias lazera	3β-HSD	+	++	+		+	Van Den Hurk and Richter (1980)

Mystus cavasius	3β-HSD, 17β-HSD, 11β-HSD	++	+	(+)	(+)	+	Saidapur and Nadkarni (1976)
Atheriniformes							
Fundulus heteroclitus	EM			++			Wallace and Selman (1980)
Poecilia reticulata	3β-HSD			++			Lambert (1970a)
	3α-HSD	+		+	+		
	17β-HSD			+	+		
Poecilia latipinna	HSD	+	+	+			Van Den Hurk (1979)[b]
Xiphophorus helleri	3β-HSD	+		+			Lambert and van Oordt (1974a)
Xiphophorus maculatus	3β-HSD	+					Schreibman *et al.* (1982)
Oryzias latipes	EM			(+)		(+)	Iwamatsu and Ohta (1981)
	EM and 3β-HSD			++		+	Kagawa and Takano (1979)
	3β-HSD	++		+		+	Iwasaki (1973)
	EM and 3β-HSD	++		(+)			Yamamoto and Onozato (1968)
Perciformes							
Sarotherodon niloticus	3β-HSD		+	++			Yaron (1971)
Sarotherodon aureus	3β-HSD			+			Livni (1971)
Cichlasoma nigrofasciatum	EM		++	(+)		+	Nicholls and Maple (1972)
Haplochromis multicolor	EM		++	(+)			
Perca fluviatilis	EM–3β-HSD			++		+	Lang (1981a,b)
Mugil capito	3β-HSD			+			Livni (1971)
Trachurus mediterraneus	3β-HSD		++	+		+	
	3α-HSD		+		+		
	11β-HSD, 20α-HSD		+	+		+	Bara (1974)
	17β-HSD, 20β-HSD		+				
	11α-HSD, 17α-HSD	–	–	–	–	–	
Cymatogaster aggregata	3β-HSD	+	+	+			Wiebe (1969) (no distinction between TC and GC)
Scomber scomber	3β-HSD		++	+		+	Bara (1965)
Pleuronectiformes							
Microstomus kitt	3β-HSD			+			Simpson *et al.* (1969)
Symbranchiformes							
Monopterus albus	3β-HSD	+		+			Tang *et al.* (1974a, 1975)
	17β-HSD		–	–	–	–	

[a] ++, dominating activity. [b] Unpublished results, mentioned in Van Den Hurk and Peute (1979).

be steroidogenic, interstitial or thecal cells, on the one hand, and granulosa cells, on the other hand, with the exception of *Sarotherodon aureus*, the mullet, *Mugil capito*, the carp, *Cyprinus carpio* (Livni, 1971), *Acanthobrama terrae sanctae* (Yaron, 1971), the perch, *Perca fluviatilis* (Lang, 1981a), and the lemon sole, *Microstomus kitt* (Simpson *et al.*, 1969) (see Table I).

The arguments for a steroidogenic potentiality of particular granulosa cells come mainly from histoenzymochemical results. Electron microscopy gives equivocal pictures because of the presence of organelles typical of protein-secreting cells, together with a few mitochondria containing tubular cristae and a small amount of agranular endoplasmic reticulum (Yamamoto and Onozato, 1968; Nicholls and Maple, 1972; Nagahama *et al.*, 1976, 1978; Iwamatsu and Ohta, 1981). However, these specific characteristics may develop during the sexual cycle, that is, the increase in the amount of smooth reticulum (Nicholls and Maple, 1972) and the appearance of mitochondria with tubular cristae (Kagawa and Takano, 1979; Iwamatsu and Ohta, 1981). In certain species, particular thecal cells have a clearer steroidogenic ultrastructure (Yamamoto and Onozato, 1968; Nicholls and Maple, 1972; Nagahama *et al.*, 1976, 1978; Van Den Hurk and Peute, 1979). These cells are larger than the other thecal cells, and have been called "special theca cells" (Nagahama *et al.*, 1976). They are often found close to blood capillaries (Nagahama *et al.*, 1976, 1978; Nicholls and Maple, 1972; Yamamoto and Onozato, 1968) and grouped in clusters (Bara, 1965, 1974; Livni, 1971; Nagahama *et al.*, 1978). In fact, these cells could be of the same fibroblastic origin as the interstitial cells (Yamamoto and Onozato, 1968; Saidapur and Nadkarni, 1976; Lambert and van Oordt, 1974a; Van Den Hurk and Peute, 1979). According to Yamamoto and Onozato (1968) in the zebrafish, *Brachydanio rerio*, the special thecal cells are differentiated from ovarian interstitial cells at an early stage and later become located in the thecal layer. During the follicular atresia in the catfish, *Mystus cavasius*, Saidapur and Nadkarni (1976) found that the special thecal cells remain in the stroma to form the interstitial gland.

The differences between species must be considered with some reserve, because all the studies have not been performed at every stage of the ovarian development, and, in fact, variations throughout the reproductive cycle have been observed. In the immature rainbow trout, *Salmo gairdneri*, some interstitial cells already show steroidogenic ultrastructural characteristics (Upadhyay, 1977). In the adult trout, the interstitial and thecal cells exhibit a maximal 3β-HSD activity at the time of meiotic maturation and ovulation (Fig. 1A, Van Den Hurk and Peute, 1979), leading researchers to hypothesize that progestins and/or corticosteroids, hormones involved in the regulation of these processes, originate in these cells. In another salmonid, the

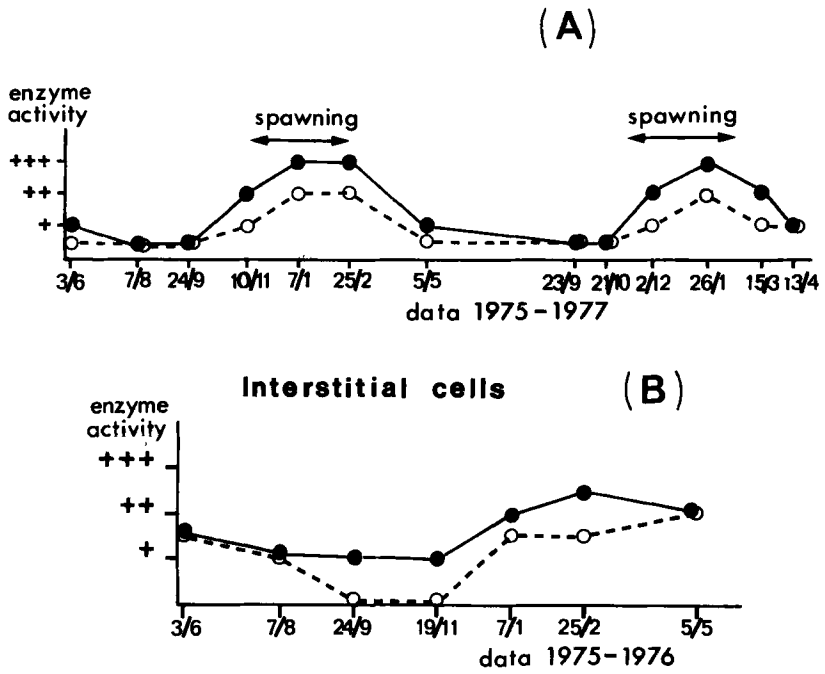

Fig. 1. The 3β-HSD (●) and 3α-HSD (○) activities in the somatic cells of the gonads of the rainbow trout, during the annual sexual cycle. The intensity of enzyme reactions was graded in arbitrary units: (A) the ovary (from Van Den Hurk and Peute, 1979); (B) the testis (from Van Den Hurk, *et al.*, 1978b).

white spotted char, *Salvelinus leucomaenis*, an intense 3β-HSD activity in the special thecal cells of the young postovulatory follicle was actually correlated with a peak of plasma progesterone (Kagawa *et al.*, 1981), but no activity was detectable before ovulation. The granulosa cells show a maximal 3β-HSD activity during vitellogenesis, especially at the exogenous phase, in *Poecilia reticulata* (Lambert, 1970a), the swordtail, *Xiphophorus helleri* (Lambert and van Oordt, 1974a), *Brachydanio rerio* (Lambert *et al.*, 1972; Lambert and van Oordt, 1974a), *Sarotherodon aureus*, the mullet, *Mugil capito* (Livni, 1971), *Trachurus mediterraneus* (Bara, 1974), and the trout, *Salmo gairdneri* (Van Den Hurk and Peute, 1979); therefore, it has been postulated that estrogens, hormones involved in vitellogenesis, originate in the granulosa cells. In *Brachydanio rerio*, Lambert (1978) has confirmed the concomitance between the 3β-HSD activity in the granulosa cells and the ability of the ovary to produce 17β-estradiol and estrone *in vitro*. In contrast to these observations, the granulosa cells appear active only during the spawning period in *Oryzias latipes* (Iwasaki, 1973) or after ovulation induced

with human chorionic gonadotropin (HCG) in *Clarias lazera* (Van Den Hurk and Richter, 1980; Lambert and Van Den Hurk, 1982). During vitellogenesis, the HSD activities are observed mostly in thecal or interstitial cells in *Scomber scomber* (Bara, 1974), or even exclusively in these cells in *Oryzias latipes* (Iwasaki, 1973), *Clarias lazera* (Van Den Hurk and Richter, 1980), or *Cyprinus carpio* (Livni, 1971); however, thecal and granulosa cells are both active in *Trachurus mediterraneus* (Bara, 1974) or in the rice-field eel, *Monopterus albus* (Tang *et al.*, 1974a). An intense reaction has also been observed in the loach, *Misgurnus anguillicaudatus*, micropolar cells (Ohta and Teranishi, 1982).

All these results must be considered in the light of the recent comparison of morphological observations with the *in vitro* analysis of steroid output from mechanically isolated thecal and granulosa cells of the salmon, *Oncorhynchus rhodurus*, ovary (Nagahama *et al.*, 1982a). The observed differences cast doubt on conclusions based solely on histochemical observations. Furthermore, these biochemical studies lead researchers to propose a "two cell-type" model both for the production of estrogens during vitellogenesis (Kagawa *et al.*, 1982b) and for the production of progestins during spawning (Nagahama *et al.*, 1983).

Special attention has been given to ovarian structures which could be homologous to the mammalian corpora lutea. In some species, the postovulatory follicles retain some steroidogenic potentialities (see Table I), but more often steroidogenesis is confined to the granulosa cells, although the thecal cells may also remain active (Bara, 1965, 1974; Van Den Hurk and Peute, 1979; Kagawa *et al.*, 1981). These enzymatic functions in the granulosa, often appear weak and transient (Nicholls and Maple, 1972; Lambert, 1978; Nagahama *et al.*, 1976; Van Den Hurk and Peute, 1979; Institute of Zoology, Academia Sinica and Yangtze Institute of Fisheries, 1978; Van Den Hurk and Richter, 1980). However, except for the histoenzymochemical observations of Saidapur and Nadkarni (1976) in the catfish, *Mystus cavasius*, and of Khoo (1975) in the goldfish, *Carassius auratus*, there is no direct argument for an endocrine role of the atretic preovulated follicle, invaded with granulosa (Bara, 1965; Lambert, 1970b; Yaron, 1971; Colombo *et al.*, 1978a; Kagawa and Takano, 1979; Lang, 1981b). Therefore, Lambert (1970b) has suggested calling it corpus atreticum.

2. Biochemical Data

The potentiality of the fish ovary for *in vitro* steroid synthesis from acetate has been demonstrated in *Sarotherodon aureus* by Eckstein (1970). From more elaborate precursors, ovaries from various species are able to synthesize progestins, corticosteroids, androgens, and estrogens (see Table

II), by the Δ-5 (17α-hydroxypregnenolone and dehydroepiandrosterone) or the Δ-4 (progesterone and 17α-hydroxyprogesterone) pathways. The Δ-5 pathway predominates in young fish such as the virgin *Poecilia reticulata* (Lambert and Pot, 1975), the immature *Jenynsia lineata* (Charreau and Tesone, 1974) and the silver eel, *Anguilla anguilla* (Colombo and Colombo Belvédère, 1976b). In the adult, steroid metabolism may change during the course of the sexual cycle. Therefore, in the rainbow trout, *Salmo gairdneri*, the ratio between the Δ-5 and Δ-4 steroids, yielded from pregnenolone, increased 500-fold during vitellogenesis (van Bohemen and Lambert, 1979; Lambert and van Bohemen, 1979). In *Brachydanio rerio* the Δ-5 pathway, related to the interstitial cell activity, was most active just after the oviposition, while the Δ-4, pathway, attributable to the granulosa cells, was dominant 4 days later, before the next oviposition (Lambert, 1978). There is also a third direct route from progesterone to testosterone, without androstenedione being an intermediate. This route has been proposed in *Sarotherodon aureus* after incubations where the use of both precursors [^{14}C]progesterone and [^{3}H]pregnenolone gave testosterone mainly labeled with ^{14}C and androstenedione mainly labeled with ^{3}H (Eckstein, 1970). However, Ozon (1972a) has emphasized that in this experiment, very different quantities of the two precursors were used. Such a metabolism could not be confirmed in *Poecilia reticulata* (Lambert and Pot, 1975).

a. Corticosteroids. The possibility that corticosteroidogenesis occurs in the ovary has been reported in many species: the sculpin, *Leptocottus armatus*; the Pacific tomcod, *Microgadus proximus*; the longjaw mudsucker, *Gillichtis mirabilis* (Colombo *et al.*, 1973); *Gobius jozo; Diplodus annularis*; the sole, *Soleae impar*, (Colombo and Colombo Belvédère, 1977); the sea bass, *Dicentrarchus labrax* (Colombo *et al.*, 1978b); *Salmo gairdneri* (van Bohemen and Lambert, 1979; Theofan, 1981); and *Jenynsia lineata* (Tesone and Charreau, 1980). However, this was not confirmed in the Indian catfish, *Heteropneustes fossilis* (Ungar *et al.*, 1977; Truscott *et al.*, 1978), thereby weakening the hypothesis that, in this species, 11-deoxycorticosteroids act as local inducers of maturation and ovulation of oocytes (Colombo *et al.*, 1973).

b. Progestins. Progesterone, 17α-hydroxyprogesterone, 20α- or 20β-dihydroprogesterone, 17α-hydroxy-20β-dihydroprogesterone are synthesized *in vitro* by fish ovaries (Table II). Progesterone was recognized in ovarian extracts of *Conger conger* (Lupo and Chieffi, 1963), the Pacific salmon, *Oncorhynchus nerka* (Botticelli and Hisaw, 1964), and carp, *Cyprinus carpio* (Horvath *et al.*, 1978). 17α-Hydroxy-20β-dihydroprogesterone has been identified in the plasma of *Oncorhynchus nerka* (Idler *et al.*, 1960a), *Salmo gairdneri* (Campbell *et al.*, 1980; Diederik and Lambert, 1982), *Pseudopleuronectes americanus* (Campbell *et al.*, 1976), and *Heteropneustes fossilis*

Table II

Steroid Biosynthesis *in Vitro* in the Ovary

Species	Sexual stages	Precursors	Corticosteroids	Progestins	Androgens	Estrogens	Methods[a]	Reference
Anguilliformes								
Anguilla anguilla	Silver stage immature	Pregnenolone		Progesterone 17α-Hydroxypregnenolone 17α-Hydroxyprogesterone	Dehydroepiandrosterone Androstenedione		TLC, Der Cry $C^3H/^{14}C$ with and without cof.	Colombo and Colombo Belvédère (1976b, 1977)
		Progesterone		17α-Hydroxyprogesterone	Androstenedione Testosterone 11β-Hydroxyandrostenedione Adrenosterone (water soluble derivatives)		Intact and Homo, (t = 7 min to 6 hr, θ = 16°C)	
	Silver stage immature	Progesterone			Androstenedione Testosterone 11β-Hydroxyandrostenedione 11β-Hydroxytestosterone 11-Ketotestosterone	—	C, PC, TLC Min.	Querat *et al.* (1982)
Salmoniformes								
Salvelinus fontinalis		Pregnenolone		(17α-Hydroxypregnenolone)	(Dehydroepiandrosterone)		TLC, Cry CSA Intact (t = 4 hr, θ = 12°C)	Theofan (1981)
		Progesterone	11-Deoxycorticosterone (11-Deoxycortisol)	Pregnanedione 17α-Hydroxyprogesterone (17α-Hydroxy-20β-dihydroprogesterone)	Androstenedione Testosterone	17β-Estradiol		
Salmo gairdneri	Immature	Pregnenolone		17α-Hydroxypregnenolone Progesterone	Androstenedione		TLC, Der, Cry CSA Cof. Homo. (t = 3 hr)	Van Den Hurk *et al.* (1982b)

				17α-Hydroxyprogesterone				
		Androstenedione			Testosterone	Estrone 17β-Estradiol	TLC, Der, Cry CSA Cof. Homo. (t = 3 hr)	
		Dehydroepiandrosterone			Androstenedione		TLC, Der, Cry CSA Cof. Homo (t = 3 hr)	
Salmo gairdneri	Various stages of the sexual cycle	Pregnenolone	Deoxycorticosterone (Deoxycortisol) (Cortisone)	Progesterone 17α-Hydroxyprogesterone 17α-Hydroxypregnenolone	Androstenedione Testosterone Dehydroepiandrosterone		TLC, Der, Cry CSA Cof., Homo (t = 2 hr, θ = 25°C)	van Bohemen and Lambert (1979, 1981), Lambert and van Bohemen (1979)
		Androstenedione				17β-Estradiol Estone 17β-Estradiol	TLC, Der, Cry CSA Cof. Homo (t = 2 hr, θ = 25°C)	
	Previtellogenesis and vitellogenesis	Androstenedione Testosterone			Testosterone Androstenedione 5α-Dihydrotestosterone (5α/5β-Androstanedione)	Estrone 17β-Estradiol Estrone	TLC, Der, Cry $C^3H/^{14}C$; Homo. Intact (perifusion) (t = 2 to 24 hr, θ = 14°C)	Sire and Depêche (1981)
Oncorhynchus rhodurus	Prespawning	17α-Hydroxyprogesterone		17α-Hydroxy-20β-dihydroprogesterone (17α-Hydroxypregnanedione)			TLC, Der, Cry CSA Cof. St; Homo. (t = 2 hr, θ = 20°C)	Suzuki *et al.* (1981b)
Plecoglossus altivelus	Prespawning	Progesterone		5β-Pregnan-3α, 17α-diol-20-one 17α-Hydroxyprogesterone 5β-Pregnan-3α-ol-20-one 17α-Hydroxypregnanedione 5β-Pregnan-17α,20β-diol-3-one 5β-Pregnan-3α,17α,20β-triol	Testosterone		TLC, Der, Cry CSA Cof. Homo., (t = 70 min, θ = 20°C)	Suzuki *et al.* (1981a) Suzuki *et al.* (1981a)

(*continued*)

Table II *Continued*

Species	Sexual stages	Precursors	Corticosteroids	Progestins	Androgens	Estrogens	Methods[a]	Reference
		17α-Hydroxyprogesterone		id. + 5β-Pregnan-3α, 17α-idiol-20-one				
		17α-Hydroxy-20β-dihydroprogesterone		5β-Pregnan-17α, 20β-diol-3-one				Suzuki *et al.* (1981a)
				5β-Pregnan-3α, 17α, 20β-triol				
		Progesterone		id. than without stimulation + 17α-Hydroxy-20β-dihydroprogesterone			+ St	Suzuki *et al.* (1981a)
		17α-Hydroxyprogesterone		id. than without stimulation + 17α-Hydroxy-20β-dihydroprogesterone			+ St	Suzuki *et al.* (1981a)
				5β-pregnan-3β, 17α, 20β-triol				
		17α-Hydroxy-20β-dihydroprogesterone		id. than without stimulation + 5β-Pregnan-3β, 17α, 20β-triol			+ St	Suzuki *et al.* (1981a)
Cypriniformes								
Brachydanio rerio		Pregnenolone	—	(Progesterone)	Androstenedione		GLC, Der Cof., Homo.	Lambert and van Oordt (1974b)
					Testosterone			
	Various stages of the ovogenesis	17α-Hydroxypregnenolone		17α-Hydroxyprogesterone			Intact and Homo.	Lambert (1978)
		Dehydroepiandrosterone			Androstenedione			Lambert (1978)
			—					Lambert (1978)
		Progesterone		17α-Hydroxyprogesterone	Androstenedione			
				5α-Pregnanedione	Testosterone			
		Androstenedione				17β-Estradiol		
		Testosterone				17β-Estradiol		
							Intact and Homo.	Lambert (1978)
								Lambert (1978)
								Lambert (1978)

Cyprinus carpio	Prespawning	20α-Dihydroprogesterone		5α-Pregnan-3α, 20α-diol			TLC, Der, GLC Cof. Homo. (t = 20 min, θ = 37°C)	Eckstein and Azoury (1979)
				5α-Pregnan-20α-ol-3-one				
		Androstenedione			Testosterone		TLC, Der, GLC Cof., Homo. (t = 20 min, θ = 37°C)	Eckstein and Azoury (1979)
	Postvitellogenic	Pregnenolone	—	Progesterone	Androstenedione	17β-Estradiol	TLC, Der, Cry $C^3H/^{14}C$ Intact (t = 6 hr, θ = 10° and 20°C)	Colombo *et al.* (1982a)
				17α-Hydroxyprogesterone	Testosterone			
		Progesterone		5α-Pregnan-3,20-dione				
		Androstenedione		5α-Androstan-3,17-dione				
Siluriformes								
Heteropneustes fossilis	Gravid	Pregnenolone	—	5β-Pregnan-3α-ol-20one (5β-Pregnan-3α, 20α,—diol)			TLC, Cry CSA Cof.; St; Homo. (t = 30 min, θ = 25°C)	Ungar *et al.* (1977)
Ictalurus punctatus		Pregnenolone	—	—	—		TLC, Cry CSA Cof.; St; Homo. (t = 30 min, θ = 25°C)	Ungar *et al.* (1977)
Clarias lazera		Pregnenolone		17α-Hydroxypregnenolone	Androstenedione Testosterone		St.	Lambert and Van Den Hurk (1982)
				17α-Hydroxyprogesterone				
				17α-Hydroxy-20β-dihydroprogesterone				
		Androstenedione				17β-Estradiol Estrone		
Gadiformes								
Microgadus proximus	Prespawning	Progesterone	11-Deoxycortisol 11-Deoxycorticosterone	17α-Hydroxyprogesterone	Androstenedione Testosterone		TLC, Der, Cry $C^3H/^{14}C$; Min. (t = 7 min to 6 hr, θ = 15°C)	Colombo *et al.* (1973)

(*continued*)

Table II *Continued*

Species	Sexual stages	Precursors	Corticosteroids	Progestins	Androgens	Estrogens	Methods[a]	Reference
Zoarces viviparus	Various stages of the sexual cycle	Pregnenolone		17α-Hydroxypregnenolone Progesterone			TLC, (Cry, CSA) Slices (t = 1 hr, θ = 15°C)	Kristofferson *et al.* (1976)
		17α-Hydroxyprogesterone			Androstenedione		TLC, (Cry, CSA) Slices (t = 1 hr, θ = 15°C)	Kristofferson *et al.* (1976)
		Progesterone		17α-Hydroxyprogesterone			TLC, (Cry, CSA) Slices (t = 1 hr, θ = 15°C)	Kristofferson *et al.* (1976)
Atheriniformes								
Poecilia reticulata	Virgin, 3 months old	Androstenedione Testosterone				17β-Estradiol 17β-Estradiol	PC, TLC, Der, Cry CSA, Cry $C^3H/^{14}C$ Cof.; Homo. (t = 2.5 hr, θ = 25°C)	Lambert *et al.* (1970, 1971)
	Virgin, 4 months old	[^{3}H]Pregnenolone + [^{14}C]Progesterone		17α-Hydroxyprogesterone (17α-Hydroxypregnenolone)	Dehydroepiandrosterone Androstenedione Testosterone		PC, TLC, Der Cry, CAS Cof.	Lambert and Pot (1975)
	Virgin, 12 months old	Androstenedione			11-Ketotestosterone 11β-Hydroxytestosterone (11β-Hydroxyandrostenedione) (Adrenosterone	17β-Estradiol (Estrone)	Cof., Cry $C^3H/^{14}C$ Homo. (t = 1.5 hr, θ = 25°C)	Lambert and Pot (1975)
Jenynsia lineata	Immature	Pregnenolone or Progesterone or Testosterone				17β-Estradiol (Estrone)	TLC, Cry CSA	Charreau and Tesone (1974)
	Immature	Pregnenolone	11-Deoxycortisol	Progesterone 17α-Hydroxyprogesterone	Dehydroepiandrosterone Androstenedione Testosterone		TLC, Der, Cry CSA Intact (t = 2hr, θ = 20°C)	Tesone and Charreau (1980)
		Progesterone	11-Deoxycortisol 11-Deoxycorticosterone	17α-Hydroxyprogesterone	Testosterone Androstenedione		TLC, Der, Cry CSA Intact (t = 2hr, θ = 20°C)	Tesone and Charreau (1980)
		Testosterone			Androstenedione 11β-Hydroxyandrostenedione		TLC, Der, Cry CSA Intact (t = 2hr, θ = 20°C)	Tesone and Charreau (1980)

Perciformes								
Gillichthys mirabilis	Prespawning	Progesterone	11-Deoxycortisol 11-Deoxycorticosterone	17α-Hydroxyprogesterone	Androstenedione Testosterone		TLC, Der, Cry $C^3H/^{14}C$ Min. (t = 7 min to 6 hr, θ = 15°C)	Colombo *et al.* (1973)
Gobius jozo	Vitellogenic oocytes	Pregnenolone	11-Deoxycorticosterone	Progesterone 17α-Hydroxyprogesterone	Androstenedione Testosterone	17β-Estradiol Estrone	TLC, Der, Cry $C^3H/^{14}C$ Min. (t = 7 min to 6 hr, θ = 16°C)	Colombo and Colombo Belvédère (1977)
		Progesterone	11-Deoxycorticosterone	17α-Hydroxyprogesterone 20α-Dihydroprogesterone 20β-Dihydroprogesterone	Androstenedione Testosterone		TLC, Der, Cry $C^3H/^{14}C$ Min. (t = 7 min to 6 hr, θ = 16°C)	Colombo and Colombo Belvédère (1977)
Pagellus acarne	Vitellogenesis	Progesterone	—	17α-Hydroxyprogesterone			PC, TLC, Der, Cry, CSA	Reinboth (1974, 1979)
Diplodus annularis	End of vitellogenesis	Pregnenolone or progesterone	11-Deoxycorticosterone 11-Deoxycortisol	Progesterone (17α-Hydroxyprogesterone)	Androstenedione Testosterone	17β-Estradiol Estrone	TLC, Der, Cry $C^3H/^{14}C$ Min (t = 7 min to 6 hr, θ = 18°C)	Colombo and Colombo Belvédère (1977)
Sparus aurata	Sex reversal	Pregnenolone		Progesterone 17α-Hydroxyprogesterone	Androstenedione Testosterone	17β-Estradiol Estrone	TLC, Der, Cry $C^3H/^{14}C$, Cof.; Min (t = 2 hr, θ = 15°C)	Colombo *et al.* (1972b)
	Beginning of the breeding season	Androstenedione			Testosterone 11-Ketotestosterone 11β-Hydroxytestosterone		TLC, GLC, Der Min (t = 10 to 80 min, θ = 25°C)	Eckstein *et al.* (1978)
Perca flavescens		Pregnenolone	(11-Deoxycortisol)	5α-Pregnanedione 17α-Hydroxyprogesterone 20α-Hydroxyprogesterone (17α-Hydroxy-20β-dihydroprogesterone) (17α-Hydroxy-20α-dihydroprogesterone)	Androstenedione Testosterone		TLC, Cry CSA Intact (t = 4 hr, θ = 10°C)	Theofan (1981)
		Progesterone	id.	id.	id.			

(*continued*)

Table II *Continued*

Species	Sexual stages	Precursors	Corticosteroids	Progestins	Androgens	Estrogens	Methods[a]	Reference
Coris julis		Progesterone	—	5α-Pregnan-3,20-dione 5α-Pregnan-3β-ol-20-one			PC, TLC, Der Cry CSA	Reinboth (1974, 1979)
		Testosterone			11β-Hydroxytestosterone			
Sarotherodon aureus	Prespawning	Acetate		Cholesterol Pregnenolone	Dehydroepiandrosterone		CC, TLC, Der, Cry CSA, GLC, Cof. Homo. (t = 2 hr, θ = 20–22°C)	Eckstein (1970)
		[^{3}H]Pregnenolone + [^{14}C]Progesterone			[^{3}H]Dehydroepiandrosterone Androstenedione [^{14}C]Testosterone		CC, TLC, Der, Cry CSA, GLC, Cof. Homo. (t = 2 hr, θ = 20–22°C)	Eckstein (1970)
		17α-Hydroxyprogesterone			Testosterone Androstenedione	—	CC, TLC, Der, Cry CSA, GLC, Cof. Homo. (t = 2 hr, θ = 20–22°C)	Eckstein (1970)
		Androstenedione			(Testosterone) 11-Ketotestosterone		CC, TLC, Der, Cry CSA, GLC, Cof. Homo. (t = 2 hr, θ = 20–22°C)	Eckstein (1970)
	Pre- and post-spawning	Pregnenolone		Progesterone	Androstenedione Dehydroepiandrosterone Testosterone 11β-Hydroxytestosterone 11-Ketotestosterone	—	TLC, CC, Der, Cry CSA Min. (t = 5 hr, θ = 25°C)	Eckstein and Katz (1971)
Mugil capito	Prespawning females from two biotopes	Androstenedione			Testosterone 11-Ketotestosterone		TLC, Der, Cry CSA Cof. Homo. (t = 2 hr, θ = 20–22°C)	Eckstein and Eylath (1970, 1969)
		Pregnenolone			Dehydroepiandrosterone			Eckstein (1975)
Mugil cephalus	Prespawning	20α-Dihydroprogesterone		5α-Pregnan-3α, 20α-diol Progesterone			TLC, Der, GLC, Cof. Homo. (t = 20 min, θ = 37°C)	Eckstein and Azoury (1979)

	Various stages of the sexual cycle	Androstenedione		Androsterone Testosterone 11β-Hydroxytestosterone 11β-Hydroxyandrostenedione 11-Ketotestosterone 5α-Androstan-3α, 17β-diol (5α-Androstan-3α-ol-17-one)		TLC, Der, Cry CSA GLC Cof. Homo. (t = 2 hr, θ = 37°C)	Azoury and Eckstein (1980)
Spicara maena	Spawning season	Progesterone	5β-Pregnan-17α-ol-3, 20-dione 11β-Hydroxyprogesterone	5α-Androstan-3α-ol-17-one	17β-Estradiol	PC, TLC, Der Cry CSA	Reinboth (1979)
		Testosterone		5α-Androstan-3α, 17β-diol 5α-Androstan-3, 17-dione 5α-Androstan-3α-ol Androstenedione 11-Ketotestosterone 5β-Androstan-3α, 17β-diol			Reinboth (1979)
Centropristes striatus		Progesterone	Pregnanedione			PC, TLC, Der Min. (t = 6 hr, θ = 20–22°C)	Reinboth *et al.* (1966)
		Testosterone		5β-Androstan-3, 17-dione 5β-Androstan-17β-ol-3-one 5β-Androstan-3α, 17β-diol		PC, TLC, Der Min. (t = 6 hr, θ = 20–22°C)	Reinboth *et al.* (1966)
Dicentrarchus labrax	Atretic oocytes	Pregnenolone	Progesterone (Polar compounds)			TLC, Der, Cry CSA Intact (t = 6 hr, θ = 16°C)	Colombo *et al.* (1978a)
	Various stages of the sexual cycle	Pregnenolone or Progesterone	Progesterone 17α-Hydroxyprogesterone	Androstenedione Testosterone	17β-Estradiol	TLC, Der, Cry CSA Intact (t = 6 hr, θ = 16°C)	Colombo *et al.* (1978b)

(continued)

Table II *Continued*

Species	Sexual stages	Precursors	Corticosteroids	Progestins	Androgens	Estrogens	Methods[a]	Reference
Serranus cabrilla		Progesterone		5β-Pregnan-17α-ol-3,20-dione 5β-Pregnan-3α, 20α-diol 5β-Pregnan-3β-ol-20-one 5β-Pregnan-3α, 17α, 20α-triol 11β-Hydroxyprogesterone 4-Pregnen-11β, 17α-diol-3, 20-dione			PC, TLC, Der, Cry CSA	Reinboth (1975a, 1979)
		Testosterone		5β-Androstan-3, 17-dione 5β-Androstan-3α, 17β-diol 5β-Androstan-3β-17β-diol 5β-Androstan-3α-ol-17one 5β-Androstan-17β-ol-3one Androstenedione 11-Ketotestosterone 11β-Hydroxytestosterone			PC, TLC, Der, Cry CSA	Reinboth (1975a, 1979)

Scorpaeniformes								
Leptocottus armatus	Prespawning	Progesterone	11-Deoxycorticosterone	17α-Hydroxyprogesterone	Androstenedione Testosterone		TLC, Der, Cry $C^3H/^{14}C$; Min (t = 7 min to 6 hr, θ = 15°C)	Colombo *et al.* (1973)
Myoxocephalus octadecimspinosus	Mature	Androstenedione				Estrone	TLC, Cry CSA Homo. (t = 24 hr, θ = 22°C)	Callard *et al.* (1978a)
Pleuronectiformes								
Microstomus kitt	Mature	Androstenedione			Testosterone 5β-Androstenedione 5β-Androstan-3α-ol-17-one	17β-Estradiol Estrone	PC, TLC, Der Cof.; Homo.	Simpson *et al.* (1969)
		Pregnenolone		17α-Hydroxypregnenolone 17α-Hydroxyprogesterone	Dehydroepiandrosterone Androstenedione Testosterone	Estrone	PC, TLC, Der Cof.; Homo.	Simpson *et al.* (1969)
Solea impar	Ovulated	Pregnenolone or Progesterone	11-Deoxycorticosterone 11-Deoxycortisol	Progesterone (17α-Hydroxyprogesterone)	Androstenedione Testosterone	17β-Estradiol Estrone	TLC, Der, Cry $C^3H/^{14}C$ Min. (t = 7 min to 6 hr)	Colombo and Colombo Belvédère (1977)
Symbranchiformes								
Monopterus albus	Beginning of the breeding season	Pregnenolone		17α-Hydroxyprogesterone	Androstenedione Testosterone	17β-Estradiol Estrone	TLC, Der, Cry CSA Min. (t = 4 hr, θ = 27–30°C, θ = 18°C)	Chan and Phillips (1969)

[a]Abbreviations for the methods are as follows: PC, paper chormatography; CC, column chromatography; TLC, thin-layer chromatography; Der, derivatives formation; GLC, gas–liquid chromatography; Cry CSA, crystallization to constant specific activity; Cry $C^3H/^{14}C$, crystallization to constant isotope ratio; Cof, addition of cofactors in the incubation medium; St. stimulation with gonadotropin; Homo, tissue homogenate; min, minced tissue; t, incubation time; θ, temperature.

(Truscott *et al.*, 1978), and was also present among the metabolites from incubations with precursors of the ayu, *Plecoglossus altivelis*, the amago salmon, *Oncorhynchus rhodurus* (Suzuki *et al.*, 1981a,b), and the African catfish, *Clarias lazera*, ovaries (Lambert and Van Den Hurk, 1982).

Using a specific radioimmunoassay, Fostier and co-workers (1981a) were able to follow *in vitro* the output of 17α-hydroxy-20β-dihydroprogesterone from clusters of rainbow trout follicles during oocyte maturation induced with gonadotropin. More accurately isolated thecal layers from ovarian follicles of *Oncorhynchus rhodurus* are able to secrete progesterone (unpublished results, cited in Nagahama *et al.*, 1982a); however, 17α-hydroxy-20β-dihydroprogesterone is only found in granulosa layers (Nagahama *et al.*, 1983).

If these progestins are more or less active in oocyte maturation (see Chapter 3, Volume 9B, this series), then the physiological significance of other pregnane derivatives is not yet understood. The 5α and 5β reduced C-21 steroids have been identified in ovarian incubations of (1) *Centropristes striatus* (Reinboth *et al.*, 1966), (2) *Pagellus acarne*, (3) *Coris julis* (Reinboth, 1974), (4) *Serranus cabrilla*, (5) *Spicara maena* (Reinboth, 1979), (6) *Brachydanio rerio* (Lambert and van Oordt, 1974b), (7) *Heteropneustes fossilis* (Ungar *et al.*, 1977), (8) *Cyprinus carpio*, (9) *Mugil cephalus* (Eckstein and Azoury, 1979), (10) *Plecoglossus altivelis* (Suzuki *et al.*, 1981a), (11) *Salvelinus fontinalis*, and (12) *Perca flavescens* (Theofan, 1981). 11β-Hydroxyprogesterone was identified for the first time by Reinboth (1974) in *Coris julis* ovarian incubations. It was later found in another ambisexual fish, *Spicara maena* (Reinboth, 1979) and in rainbow trout plasma (Diederik and Lambert, 1982).

c. Androgens. The *in vitro* production of androgens in ovaries may be of importance because in *Sarotherodon aureus* two-thirds of the steroids produced from pregnenolone are androgens (Eckstein and Katz, 1971). Dehydroepiandrosterone, androstenedione, and testosterone may be considered to be estrogen precursors, and probably, in rainbow trout, the very low output of androgens from vitellogenic ovaries, perfused *in vitro*, is related to their use for aromatization (Zohar *et al.*, 1982b). This does not appear to be the case of 11-oxygenated androgens such as 11-ketotestosterone (Callard *et al.*, 1981a). This steroid, which is known as a potent androgen (see Section II,B,2) is synthesized *in vitro*, in the ovaries of a few species: *Mugil capito* (Eckstein and Eylath, 1970), *Mugil cephalus* (Azoury and Eckstein, 1980), *Sarotherodon aureus* (Eckstein and Katz, 1971), *Poecilia reticulata* (Lambert and Pot, 1975), and *Sparus aurata* (Eckstein *et al.*, 1978). In the grey mullet, *Mugil cephalus*, the ovarian production of 11-ketotestosterone increases with the development of vitellogenesis and then decreases after spawning

(Azoury and Eckstein, 1980). During the breeding season, higher yields from precursors of testosterone, 11-ketotestosterone (Eckstein and Eylath, 1970), and dehydroepiandrosterone (Eckstein, 1975) were recorded in ovaries from another species, *Mugil capito*, kept in fresh water (where ovulation is blocked), than from females living in seawater. The concentration of 11-ketotestosterone measured in the ovary is actually higher in mullets confined in fresh water (Eckstein and Eylath, 1970). The dehydroepiandrosterone accumulation in ovaries from mullets confined in fresh water (Eylath and Eckstein, 1969) has been explained by an inhibition of its conversion into androstenedione (Eckstein, 1975).

d. Estrogens. In vitro estrogen synthesis, mainly 17β-estradiol and/or estrone, has been found in most teleost species examined, except in *Sarotherodon aureus* during the breeding season (Eckstein, 1970), in sexually regressed *Ictalurus punctatus* (Ungar *et al.*, 1977), and in *Mugil cephalus* confined in fresh water (Eckstein, 1975) (Table II). More accurately, from *in vitro* studies of the two cellular components of the *Oncorhynchus rhodurus* ovarian follicles, it appeared that thecal cells synthesized testosterone which is aromatized in granulosa cells (Kagawa *et al.* 1982b). Analyzing the complete sexual cycle of *Salmo gairdneri*, van Bohemen and Lambert (1981) recorded the predominance of estrone compared to estradiol production during the exogenous vitellogenesis. However, contrary to Sire and Depêche (1981), they did not find a significant aromatase activity at the beginning of the cycle.

Ozon (1972a) reviewed literature on the isolation and the exploratory identification, according to the criteria defined by Sandor and Idler (1972), of estrogens (i.e., estradiol and estrone) in fish ovaries from nine teleosts: *Salmo irideus, Cyprinus carpio, Anguilla anguilla, Gadus callarias, Conger conger, Oncorhynchus nerka, Ictalurus punctatus, Serranus scriba,* and *Protopterus annectens.* Estrone and 17β-estradiol were also identified in *Sarotherodon aureus* ovaries (Katz *et al.*, 1971). Other types of experiments have confirmed ovarian estrogen production. Using radioimmunoassay, Yaron and co-workers (1977) observed a drastic decrease in the level of plasma 17β-estradiol after ovariectomy in *Sarotherodon aureus.* Furthermore, *in vitro* secretion of 17β-estradiol occurred from the isolated ovary (Bogomolnaya and Yaron, 1982). Comparable results were obtained in the rainbow trout (Figs. 2 and 3) (Zohar *et al.*, 1982b). In this species, plasma 17β-estradiol was no longer detected during vitellogenesis after ovariectomy, even when females were injected with gonadotropin (Fig. 4). Output of 17β-estradiol *in vitro* from ovarian follicles was also observed in *Pleuronectes platessa* (Yaron and Barton, 1980) and in *Oncorhynchus rhodurus* (Kagawa *et al.*, 1982a; Young *et al.*, 1982).

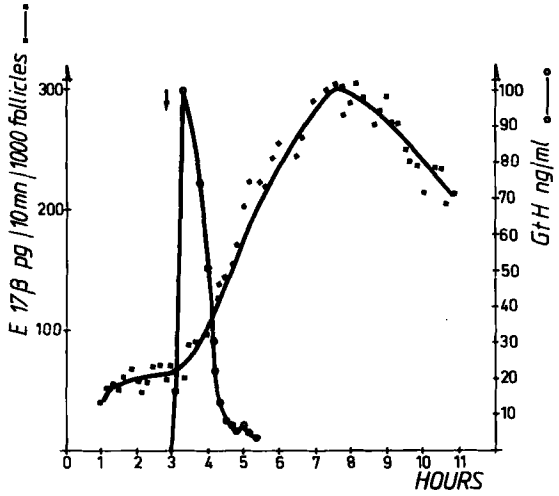

Fig. 2. *In vitro* 17β-estradiol output from a rainbow trout ovary undergoing endogenous and early exogenous vitellogenesis (March, GSI = 0.52). Ovarian fragments were cultured in an open perifusion system. The shape of the GTH pulse which was applied was determined according to previous *in vivo* analysis (from Y. Zohar, unpublished).

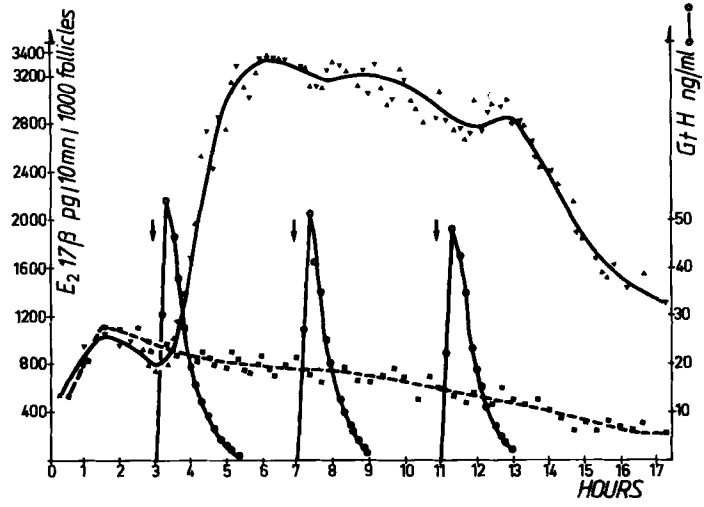

Fig. 3. *In vitro* 17β-estradiol output from a rainbow trout ovary undergoing exogenous vitellogenesis (June, GSI = 1.25). Ovarian fragments were cultured in an open superfusion system. One chamber (▲, ▼) received three GtH pulses (○) at 4-hr intervals corresponding to *in vivo* frequency (Zohar *et al.*, 1981), whereas the other (■) served as a control (from Zohar *et al.*, 1982b).

B. The Testis

1. Ultrastructural and Histoenzymochemical Data

Some interstitial cells are recognized as the main site of steroidogenesis in the testis according to either histoenzymochemical observations, as reported in Table III, or ultrastructural descriptions in *Salmo gairdneri* (Oota and Yamamoto, 1966; Van Den Hurk *et al.*, 1978a,b), in *Oncorhynchus kisutch* and *Oncorhynchus gorbuscha* (Nagahama *et al.*, 1978), in *Poecilia reticulata* (Follenius and Porte, 1960), in *Poecilia latipina* (Van Den Hurk *et al.*, 1974), in *Oryzias latipes* (Gresik *et al.*, 1973; Satoh, 1974), in *Gobius*

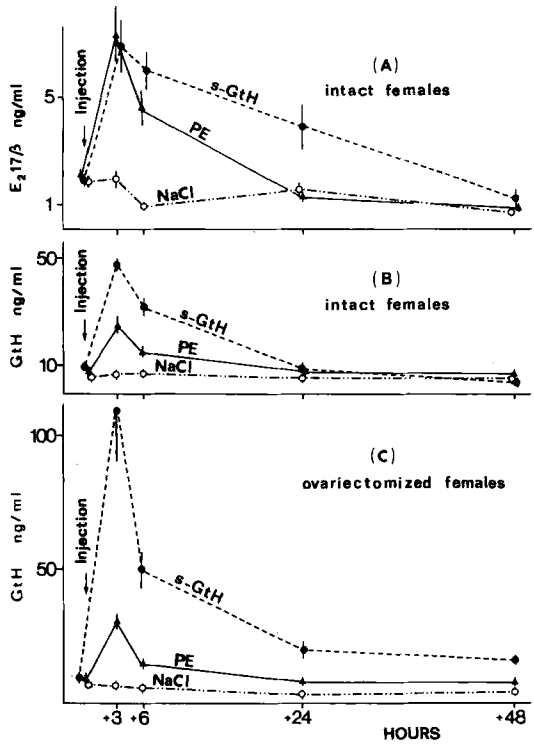

Fig. 4. Plasma GtH and 17β-estradiol in vitellogenic rainbow trout (August) after one intracardiac injection of either pure salmon gonadotropin (s-GtH), a crude pituitary extract in saline (PE), or saline (means of 5 females ± SE). Fish were either intact (A and B) or had been ovariectomized (C) 6 months previously. The quantity of injected PE was chosen to contain the same quantity of GtH as that injected, i.e., 5 μg/kg body weight (A. Fostier, B. Breton, and R. Billard, unpublished).

jozo (Colombo and Burighel, 1974), in *Spicara chryselis* (Carrillo and Zanuy, 1977), in *Cichlasoma nigrofasciatum* (Nicholls and Graham, 1972), and in *Gasterosteus aculeatus* (Follenius, 1968). In *Anguilla japonica*, Sugimoto and Takahashi (1979) observed the appearance of classical steroidogenic features in distinct interstitial cells after stimulation of the testis with human chorionic gonadotropin (HCG). In *Oryzias latipes*, Gresik and co-workers found neither 3β-HSD activity in these cells, in opposition to Takahashi and Iwasaki (1973b), nor smooth endoplasmic reticulum; however, they did find vesicular nuclei and rod-shaped mitochondria with tubular cristae (Gresik *et al.*, 1973; Gresik, 1975). Few histoenzymochemical studies of these cells have been conducted throughout the various stages of the sexual cycle. In *Fundulus heteroclitus* (Bara, 1969), the 3β-HSD activity is detectable from stage II (secondary spermatogonia and primary spermatocytes), and reaches a maximum at stage VI (spermiation), especially between the lobules containing spermatozoa. The 11β-HSD activity and a weak 17β-HSD activity are restricted to the latter period. In *Cymatogaster aggregata* (Wiebe, 1969), the main 3β-HSD activity is found during the breeding season. In *Salmo gairdneri* (Van Den Hurk *et al.*, 1978a,b), the maximal 3β-HSD reaction is also reported during full spermiation (Fig. 1B). At the end of this period, steroidogenic cells are seen in seminiferous tubules containing secondary spermatogonia. Maximal activities have also been reported in the fully mature testis of *Oncorhynchus nerka* and *Oncorhynchus masou* (Sufi *et al.*, 1980).

These steroidogenic interstitial cells may originate from the fibroblast-like connective tissue elements present in the interstitium (Nicholls and Graham, 1972). They occur either individually or in small clusters (Bara, 1969, 1972; Funk and Donaldson, 1972; Takahashi and Iwasaki, 1973a,b; Carrillo and Zanuy, 1977; Nagahama *et al.*, 1978), and are sometimes in close association with blood capillaries (Follenius and Porte, 1960; Delrio *et al.*, 1965; Nicholls and Graham, 1972). In the genus *Gobius*, these cells form a glandular mass distinct from the seminiferous tissue (Stanley *et al.*, 1965; Moiseyeva and Ponomareva, 1973; Belsare, 1973; Colombo and Burighel, 1974; Bonnin, 1975). They may also be located between efferent ducts (Bara, 1969; Gresik *et al.*, 1973; Schreibman *et al.*, 1982a).

Another type of steroidogenic cell has been described (Fig. 5). Originally, Marshall and Lofts (1956) found that cells localized within the lobule possessed round sudanophilic lipid droplets, although these presumed characteristics of steroidogenesis were absent in interlobular space. They called these cells "lobule boundary cells", but Grier (1981) argues that the term is uncorrect because the true teleost boundary cells reside outside the tubule basement membrane. Furthermore, classical steroidogenic ultrastructural

Table III

Histoenzymochemical Studies of the Testis

Species	Localization		References
	Intralobular	Interstitial	
Salmoniformes			
Salmo gairdneri	+	++[a]	Van Den Hurk *et al.* (1978a,b)
Salmo salar	+	—	O'Halloran and Idler (1970)
Oncorhynchus gorbuscha	—	+	Funk and Donaldson (1972)
Oncorhynchus kisutch	—	+	Chestnut (1970), cited in Funk and Donaldson (1972)
Oncorhynchus keta	+	++	Sufi *et al.* (1980)
Oncorhynchus masou	+	++	Sufi *et al.* (1980)
Cypriniformes			
Anoptichthys jordani	—	+	Van Den Hurk (1973)
Barbus schuberti	—	+	Van Den Hurk (1973)
Barbus tetrazoni	—	+	Van Den Hurk (1973)
Brachydanio rerio	—	+	Van Den Hurk (1973)
Tinca tinca	—	+	Delrio *et al.* (1965)
Carassius auratus	—	+	Yamazaki and Donaldson (1969)
Siluriformes			
Clarias batrachus	+	++	Satyanarayana *et al.* (1979)
Gadiformes			
Zoarces viviparus	—	+	Van Den Hurk (1973)
Atheriniformes			
Belone belone	—	+	Delrio *et al.* (1965)
Dermogenys pusillus	—	+	Van Den Hurk (1973)
Fundulus heteroclitus	—	+	Pickford *et al.* (1972)
	+	+	Bara (1969)
Poecilia reticulata	—	+	Van Den Hurk (1973) Takahashi and Iwasaki (1973a)
Poecilia latipinna	—	+	Van Den Hurk (1973)
Xiphophorus helleri	—	+	Van Den Hurk (1973)
Xiphophorus maculatus	—	+	Van Den Hurk (1973) Schreibman *et al.* (1982a)
Oryzias latipes	—	+	Takahashi and Iwasaki (1973b)
Gasterosteiformes			
Gasterosteus aculeatus	—	+	Delrio *et al.* (1965)
Perciformes			
Cichlasoma biocellatum	—	+	Van Den Hurk (1973)

(*continued*)

Table III (*continued*)

Species	Localization		References
	Intralobular	Interstitial	
Sarotherodon mossambicus	+	+	Yaron (1966)
Coris julis	—	+	Delrio *et al.* (1965)
Blennius pavo	—	+	Chieffi and Botte (1964)
Blennius tentacularis	—	+	Chieffi and Botte (1964)
Blennius sanguinolentus	—	+	Chieffi and Botte (1964)
Blennius ocellatus	—	+	Chieffi and Botte (1964)
Gobius paganellus	—	+	Stanley *et al.* (1965)
Cymatogaster aggregata	+	+	Wiebe (1968, 1969)
Pleuronectiformes			
Solea solea	—	+	Delrio *et al.* (1965)
Pseudopleuronectes americanus	+	++	Bara (1972)
Microstomus kitt	+	—	Simpson *et al.* (1969)
Symbranchiformes			
Monopterus albus	—	+	Tang *et al.* (1974b, 1975)

[a]The symbol ++ indicates dominating activity.

features were later observed in interstitial cells of pike, *Esox lucius* (Billard *et al.*, 1971; Grier and Linton, 1977), one of the species studied by Marshall and Lofts, in spite of the absence of lipids in these cells (Grier, 1981). However, from histoenzymochemical observations, steroidogenic activities

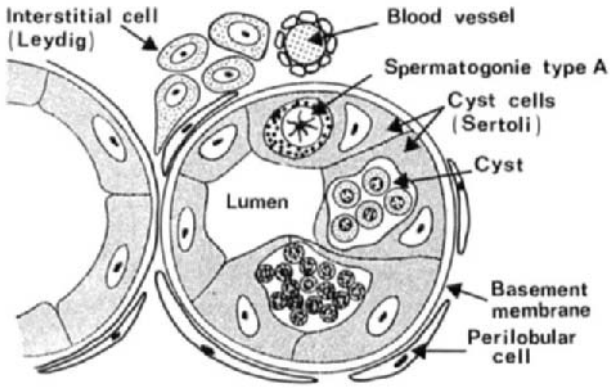

Fig. 5. Schematic representation of a cross section of a testicular lobule showing steroid synthesizing cells (from Billard *et al.*, 1982).

have been described in intralobular cells, either alone in the *Salmo salar* testis (O'Halloran and Idler, 1970) or with the activity of interstitial cells in other species (see Table III). It is of interest to note that in all these cases, and even when the studies have been performed throughout the whole of the sexual cycle (Wiebe, 1968; Bara, 1969; Van Den Hurk *et al.*, 1978a,b), the intralobular steroidogenic activity is found when spermatozoa are present in the testis. Otherwise, the intralobular cells also possess ultrastructural features comparable to the mammalian Sertoli cells (Billard *et al.*, 1972; Gresik, 1975; Nagahama *et al.*, 1978; Grier and Linton, 1977), but even if they are homologous it may be better to reserve the term "Sertoli cell" for a very specific mammalian type of cell and to use the term "cyst cell" for teleosts (Roosen-Runge, 1977; Billard *et al.*, 1982).

Finally, a third site of steroidogenesis has been found in the epithelial cells of the efferent duct, which may reveal a 3α-HSD activity in *Fundulus heteroclitus* (Bara, 1969), *Xiphophorus helleri*, *Xiphophorus maculatus*, *Dermogenys pusillus*, and *Poecilia reticulata* (Van Den Hurk, 1973), in *Poecilia latipina* (Van Den Hurk, 1974), and in *Salmo gairdneri* (Van Den Hurk *et al.*, 1978a).

2. Biochemical Data

a. Androgens. Testosterone has been isolated and identified from testes of *Salmo irideus*, *Cyprinus carpio* (Galzigna, 1961), *Tilapia leucosticta* (Hyder and Kirschner, 1969), and *Gasterosteus aculeatus* (Gottlied and Van Mullen, 1967). In the latter species, androstenedione and dehydroepiandrosterone were also found. Ozon (1972b) has reviewed the various criteria used for these identifications. Concerning the metabolic pathways, the isolation of 17α-hydroxypregnenolone and/or dehydroepiandrosterone, on the one hand, and the isolation of 17α-hydroxyprogesterone and/or androstenedione, on the other hand, indicate that both Δ-5 and Δ-4 pathways exist in fish testes.

Otherwise, attention has been focused on 11-oxygenated androgens because 11-ketotestosterone, which was found to be a potent androgen (Idler *et al.*, 1961a,c; Arai, 1967; Yamazaki and Donaldson, 1969; Hishida and Kawamoto, 1970; Takahashi, 1975; de Ruiter, 1981; Nakamura, 1981), was first identified in the peripheral plasma of the salmon, *Oncorhynchus nerka* (Idler *et al.*, 1960b), then in the testicular blood of *Salmo salar* (Idler *et al.*, 1971). Generally, when investigated, the synthesis of 11-oxygenated androgens (i.e., 11-hydroxytestosterone or 11-ketotestosterone and 11-hydroxyandrostenedione) has been found in teleost testes (see Table IV). However, 11β-hydroxyandrostenedione, but not 11-hydroxytestosterone or 11-ketotestosterone, was found in testis incubates of *Jenynsia lineata* (Tesone

Table IV

Steroid Biosynthesis *in Vitro* in the Testis

Species	Sexual stages	Precursors	Corticosteroids	Progestins	Androgens	Estrogens	Methods[a]	References
Anguilliformes								
Anguilla anguilla	Silver stage	Progesterone		17α-Hydroxyprogesterone	Androstenedione 11β-Hydroxyandrostenedione Adrenosterone			Colombo and Pesavento, mentioned in Colombo *et al.* (1972a)
		Androstenedione			11β-Hydroxyandrostenedione Adrenosterone Testosterone			Colombo and Pesavento, mentioned in Colombo *et al.* (1972a)
		Progesterone			Testosterone Androstenedione 11β-Hydroxytestosterone 11-Ketotestosterone Glu. derivatives[b]		PC, Der β-glucuronidase with or without St; Min. (t = 2 hr, θ = 22°C)	Leloup-Hatey *et al.* (1982)
	Silver stage immature	Progesterone			5β-Androstan-17β-ol-3-one 5β-Androstan-3α, 11β-diol-17-one 11β-Hydroxyandrostenedione		CC, TLC, Der Glc, Cry CSA Cof. Min. (t = 2 hr, θ = 25°C)	Eckstein *et al.* (1982)
		Androstenedione			id.			
	"Well developed testis"	Progesterone			id. + 5β-Androstan-3, 17-dione Adrenosterone 11β-Hydroxytestosterone		St, CC, TLC, Der Glc, Cry CSA Cof. Min. (t = 2 hr, θ = 25°C)	Eckstein *et al.* (1982)
		Androstenedione			id.		CC, TLC, Der Glc, Cry CSA Cof. Min. (t = 2 hr, θ = 25°C), St	Eckstein *et al.* (1982)

Salmoniformes							
Salmo gairdneri							
	Immature	Pregnenolone		17α-Hydroxypregnenolone Progesterone 17α-hydroxyprogesterone	Androstenedione 11β-Hydroxyandrostenedione	TLC, Der, Cry CSA Cof. Homo. (t = 3 hr)	Van Den Hurk *et al.* (1982b)
		Testosterone			Testosterone 11β-Hydroxyandrostenedione	TLC, Der, Cry CSA Cof. Homo. (t = 3 hr)	Van Den Hurk *et al.* (1982b)
		Dehydroepiandrosterone			Androstenedione	TLC, Der, Cry CSA Cof. Homo. (t = 3 hr)	
	Beginning of spermiation	Pregnenolone		17α-Hydroxy-20β-dihydroprogesterone	11β-Hydroxytestosterone 11-Ketotestosterone Testosterone–Glu	PC, TLC, Der, Cry $C^3H/^{14}C$ Min. (t = 3 hr, θ – 22°C) β-glucuronidase acid hydrolysis	Hews and Kime (1978) Kime (1979b)
	Beginning of spermiation	Progesterone			Testosterone Testosterone–Glu		Hews and Kime (1978)
	Breeding season	Progesterone		17α-Hydroxyprogesterone 17α-Hydroxy-20β-dihydroprogesterone	Androstenedione Testosterone	TLC, Der, Cry CSA Cof. Homo. (t = 30 min; θ = 16°C)	Arai and Tamaoki (1967a,b)
	Various stages of the sexual cycle	Progesterone		5α/5β-Pregnanedione 17α-Hydroxyprogesterone 17α-Hydroxy-20β-dihydroprogesterone	Androstenedione 11β-Hydroxyandrostenedione Testosterone 11β-Hydroxytestosterone Adrenosterone 11-Ketotestosterone	TLC, Der Min (t = 3 hr, θ = 14°C)	Depêche and Sire (1982)
		17α-Hydroxyprogesterone	Cortisol	17α-Hydroxy-20β-dihydroprogesterone	id.	TLC, Der Min (t = 3 hr, θ = 14°C)	Depêche and Sire (1982)
	Breeding season	Androstenedione			Testosterone 11α-Hydroxytestosterone	TLC, Der, Cry CSA Cof.; Homo. (t = 30 min, θ = 16°C)	Arai and Tamaoki (1967a,b)

(*continued*)

Table IV *Continued*

Species	Sexual stages	Precursors	Corticosteroids	Progestins	Androgens	Estrogens	Methods[a]	References
					11-Ketotestosterone			
	Breeding season	Androstenedione			Testosterone		TLC, Der, Cry CSA Cof. cellular fractions	Suzuki and Tamaoki (1972)
	Beginning of spermiation	Adrenosterone			11-Ketotestosterone 11-Ketotestosterone–Glu		TLC, PC, Der Cry CSA; Min. (t = 3 hr, θ = 1–37°C)	Kime (1979b)
	Beginning of spermiation	Testosterone			11β-Hydroxytestosterone 11-Ketotestosterone Testosterone–Glu		TLC, PC, Der Cry CSA; Min. (t = 3 hr, θ = 1–37°C)	Kime (1979b)
	Breeding season	Testosterone			11β-Hydroxytestosterone Androstenedione		TLC, Der, Cry, CSA Cof. cellular fractions (t = 1 hr, θ = 16°C)	Suzuki and Tamaoki (1972)
	Breeding season	11-Deoxycorticosterone	Corticosterone 11-Deoxycortisol				TLC, Der, Cry, CSA Cof. cellular fractions (t = 1 hr, θ = 16°C)	Suzuki and Tamaoki (1972)
	Breeding season	11-Deoxycortisol	Cortisol Cortisone					
Salmo salar	Spermiation	[^{14}C]Testosterone and [^{3}H]Adrenosterone			[^{14}C]11β-Hydroxytestosterone [^{3}H]11-Ketotestosterone		TLC, Der, Cry C^{3}H/^{14}C Cof. Min. (t = 5 hr, θ = 20°C)	Idler and Macnab (1967)
		[^{14}C]Androstenedione and [^{3}H]Dehydroepiandrosterone			Testosterone 11β-Hydroxyandrostenedione 11β-Hydroxytestosterone 11-Ketotestosterone 5-Androstenediol		TLC, Der, Cry C^{3}H/^{14}C Cof. (t = 1 hr)	Idler *et al.* (1968)
Salvelinus fontinalis	Beginning of spermiation and	Pregnenolone			11β-Hydroxytestosterone		TLC, Der, Cry C^{3}H/^{14}C	Sangalang and O'Halloran (1973)

	spermiation			11-Ketotestosterone Testosterone	Cof.; Min. (t = 4.5 hr, θ = 8–10°C)	
Esox lucius	Breeding season	Pregnenolone	Progesterone	Dehydroepiandrosterone	GLC, Der, Cof. Acetonic powder	Lupo di Prisco *et al.* (1970)
	Breeding season	Pregnenolone		Testosterone 11β-Hydroxytestosterone 11-Ketotestosterone	TLC, Der, PC Cry CSA or Cry $C^3H/^{14}C$ Min. (t = 3 hr, θ = 20°C)	Kime and Hews (1978)
	Breeding season	Progesterone	17α-Hydroxyprogesterone 11β-Hydroxyprogesterone	Testosterone 11β-Hydroxytestosterone 11-Ketotestosterone	TLC, Der, PC Cry CSA or Cry $C^3H/^{14}C$ Min. (t = 3 hr, θ = 20°C)	Kime and Hews (1978)
	Breeding season	Testosterone		11β-Hydroxytestosterone 11-Ketotestosterone	TLC, Der, PC Cry CSA or Cry $C^3H/^{14}C$ Min. (t = 3 hr, θ = 20°C)	Kime and Hews (1978)
	Breeding season	Dehydroepiandrosterone		Testosterone 5-Androstenediol	GLC, Der Cof. Acetonic powder (t = 0.5–3 hr, θ = 37°C)	Lupo di Prisco *et al.* (1970)
	Breeding season	5-Androstenediol		Dehydroepiandrosterone Testosterone	GLC, Der Cof. Acetonic powder (t = 0.5–3 hr, θ = 37°C)	Lupo di Prisco *et al.* (1970)
	Breeding season	Androstenedione		Testosterone		
Cypriniformes						
Carassius auratus	Breeding season	Pregnenolone	(Pregnenolone–Glu)	Testosterone 11-Ketotestosterone	PC, TLC, Der	Kime (1980b)
			4-Pregnen-17α, 20ζ-diol-3-11-dione	Testosterone–Glu 11-Ketotestosterone–Glu	Cry CSA, Cry $C^3H/^{14}C$ Min. (t = 3 hr, θ = 1–46°C)	

(*continued*)

Table IV *Continued*

Species	Sexual stages	Precursors	Corticosteroids	Progestins	Androgens	Estrogens	Methods[a]	References
	Breeding season	Adrenosterone			11-Ketotestosterone 11-Ketotestosterone–Glu		PC, TLC, Der Cry CSA, Cry $C^3H/^{14}C$ Min. (t = 3 hr, θ = 1–46°C)	Kime (1980b)
	Breeding season	Testosterone			Testosterone–Glu 11-Ketotestosterone 11-Ketotestosterone–Glu Androstenedione		PC, TLC, Der Cry CSA, Cry $C^3H/^{14}C$ Min. (t = 3 hr, θ = 1–46°C)	Kime (1980b)
Tribolodon hakonensis	Anadromous spawning migration	Progesterone		17α-Hydroxyprogesterone	Androstenedione		PC, Der, Cry CSA Cof. Min. (t = 20 hr, θ = 18°C)	Arai *et al.* (1964)
Atheriniformes								
Jenynsia lineata	Immature	Pregnenolone	11-Deoxycortisol	17α-Hydroxypregnenolone Progesterone	Androstenedione 11β-Hydroxyandrostenedione		TLC, PC, Der, Cry CSA Min. (t = 4 hr, θ = 20°C)	Tesone and Charreau (1980)
				17α-Hydroxyprogesterone	Testosterone			
	Immature	Progesterone	11-Deoxycortisol	5α-Pregnanedione	11β-Hydroxyandrostenedione		TLC, PC, Der, Cry CSA Min. (t = 4 hr, θ = 20°C)	Tesone and Charreau (1980)
			11-Deoxycorticosterone	17α-Hydroxyprogesterone	Testosterone Androstenedione			
Poecilia latipinna		Androstenedione			Testosterone Androsterone 11β-Hydroxytestosterone 11β-Hydroxyandrostenedione 11-Ketotestosterone Adrenosterone		TLC, Cry CSA Cry $C^3H/^{14}C$ Homo. (t = 3 hr, θ = 26°C)	Van Den Hurk and Lambert (1977)

Perciformes							
Dicentrarchus labrax	Spermiation	Pregnenolone	21-Deoxycortisol	Progesterone 17α-Hydroxyprogesterone 11-Ketoprogesterone	Androstenedione Testosterone 11β-Hydroxyandrostenedione 11β-Hydroxytestosterone 11-Ketotestosterone Adrenosterone	TLC, Der, Cry $C^3H/^{14}C$ Intact (t = 6 hr, θ = 16°C)	Colombo *et al.* (1978a)
Roccus saxatilis	Spermiation	Progesterone	21-Deoxycortisol	17α-Hydroxyprogesterone	Androstenedione Testosterone 11β-Hydroxyandrostenedione	TLC, Der, Cry $C^3H/^{14}C$ Cry CSA Min. (t = 7 min–6 hr, θ = 15°C)	Colombo and Colombo Belvédère (1977)
Serranus cabrilla		Progesterone		5α-Pregnanedione 5β-Pregnanedione 5β-Pregnan-3α, 20α-diol 5α-Pregnan-3β-ol-20-one 5β-Pregnan-3α-ol-20-one 5β-Pregnan-17α-ol-3, 20-dione 11β,17α-Dihydroxyprogesterone 11β-Hydroxyprogesterone 17α-Hydroxyprogesterone		PC, TLC, Der, Cry CSA	Reinboth (1979)
		Testosterone			5α-Androstan-3β, 17β-diol 5α-Androstan-3, 17-dione 5α-Androstan-17β-ol-3-one 11β-Hydroxytestosterone Androstenedione	PC, TLC, Der, Cry CSA	Reinboth (1979)

(*continued*)

Table IV *Continued*

Species	Sexual stages	Precursors	Corticosteroids	Progestins	Androgens	Estrogens	Methods[a]	References
					11β-Hydroxyandrostenedione 11-Ketotestosterone			
Sparus aurata	Mature	Pregnenolone		Progesterone 17α-Hydroxyprogesterone	Testosterone Androstenedione		TLC, Der, Cry CSA $^3H/^{14}C$, Cof. (t = 5 min–2 hr, θ = 15°C)	Colombo *et al.* (1972b)
	Beginning of the breeding season	Androstenedione			Testosterone 11β-Hydroxytestosterone 11-Ketotestosterone		TLC, Der, GLC St.; Min. (t = 10–80 min, θ = 25°C)	Eckstein *et al.* (1978)
Perca fluviatilis	Breeding season	Pregnenolone or Progesterone		—	—		TLC, Der, PC, Cry CSA or Cry $C^3H/^{14}C$	Kime and Hews (1978)
		Testosterone			11β-Hydroxytestosterone 11-Ketotestosterone		Min. (t = 3 hr, θ = 20°C)	Kime and Hews (1978)
Pagellus acarne	Breeding season	Testosterone			5β-Androstan-3α-17β-diol 5β-Androstan-3β-17β-diol 5β-Androstan-3α-ol-17-one 11β-Hydroxytestosterone 11β-Hydroxyandrostenedione Androstenedione 5β-Androstan-3, 17-dione 5β-Androstan-3, 17-dione 11-Ketotestosterone		PC, TLC, Der Cry CSA	Reinboth (1974, 1975a, 1979, 1982) Latz (1981)
		Androstenedione			5β-Androstan-3α-ol-17-one		PC, TLC, Der Cry CSA	Reinboth (1974, 1975a, 1979, 1982)

				5β-Androstan-17β-ol-3-one 11β-Hydroxytestosterone 11β-Hydroxyandrostenedione Testosterone			Latz (1981)
Mugil cephalus	Spermiation	Pregnenolone	Progesterone 17α-Hydroxyprogesterone	Dehydroepiandrosterone Androstenedione Testosterone	—	CC, PC, Der, Cry CSA Cof. Homo. (t = 22 hr, θ = 22°C)	Eckstein and Eylath (1968)
		Progesterone	17α-Hydroxyprogesterone	Testosterone Androstenedione		CC, PC, Der, Cry CSA Cof. Homo. (t = 22 hr, θ = 22°C)	Eckstein and Eylath (1968)
Spicara maena		Progesterone	5β-Pregnan-3α-ol-20-one 5β-Pregnan-17α-ol-3, 20-dione 17α-Hydroxyprogesterone			PC, TLC, Der Cry CSA	Reinboth (1979)
		Testosterone		5β-Androstan-3α, 17β-diol 11β-Hydroxytestosterone Androstenedione		PC, TLC, Der Cry CSA	Reinboth (1979)
Coris julis		Progesterone	5β-Pregnan-3α-ol-20-one 5β-Pregnan-3β-ol-20-one 5β-Pregnan-17α-ol-3, 20-dione 17α-Hydroxyprogesterone 11β-Hydroxyprogesterone	Testosterone Androstenedione		PC, TLC, Der Cry CSA	Reinboth (1975 1979)
		Testosterone		5β-Androstan-3α,17β-diol			

(*continued*)

Table IV *Continued*

Species	Sexual stages	Precursors	Corticosteroids	Progestins	Androgens	Estrogens	Methods[a]	References
					5β-Androstan-17β-ol-3-one 11β-Hydroxytestosterone			
Gobius jozo	Breeding season	Progesterone		17α-Hydroxyprogesterone	Androstenedione Testosterone 11β-Hydroxyandrostenedione 5β-Androstenedione 5β-Androstan-3α-ol-17-one 5β-Androstan-3α-ol-17-one–Glu		TLC, Cry CSA, Cry $C^3H/^{14}C$ Cof Min. (t = 7 min–6 hr, θ = 15°C)	Colombo *et al.* (1977)
		Pregnenolone		Progesterone 17α-Hydroxypregnenolone 17α-Hydroxyprogesterone Pregnenolone–Glu	id. Dehydroepiandrosterone		TLC, Cry CSA, Cry $C^3H/^{14}C$ Cof. Min. (t = 7 min–6 hr, θ = 15°C)	Colombo *et al.* (1977)
Gobius paganellus	Breeding season	Pregnenolone		Progesterone 17α-Hydroxyprogesterone 17α-Hydroxypregnenolone	Androstenedione Testosterone Dehydroepiandrosterone Glu, Sul[c]		TLC, Der, Cry CSA Min. (t = 0.5–4 hr, θ = 25°C)	Colombo *et al.* (1970)

Gillichthys mirabilis	Breeding season	Progesterone	21-Deoxycortisol	17α-Hydroxyprogesterone (11β-Hydroxyprogesterone)	11β-Hydroxyandrostenedione Androstenedione Testosterone		TLC, Der, Cry $C^3H/^{14}C$ Cry CSA Min. (t = 7 min–6 hr, θ = 15°C)	Colombo and Colombo Belvédère (1977)
Pleuronectiformes								
Microstomus kitt	Mature	Progesterone		17α-Hydroxyprogesterone	Androstenedione Testosterone (3β-Hydroxy-5β-reduced compounds)		PC, TLC, Der Cof.; Homo.	Simpson *et al.* (1969)
		Androstenedione or Testosterone			11β-Hydroxyandrostenedione Adrenosterone 11β-Hydroxytestosterone 11-Ketotestosterone		PC, TLC, Der Cof.; Homo.	Simpson *et al.* (1969)
Symbranchiformes								
Monopterus albus	Beginning of the breeding season	Pregnenolone		Progesterone 17α-Hydroxyprogesterone	Androstenedione Testosterone	17β-Estradiol Estrone	TLC, Der, Cry CSA; Min (t = 4 hr, θ = 27–30°C)	Chan and Phillips (1969)

[a]Abbreviations for the methods are as follows: PC, paper chromatography; CC, column chromatography; TLC, thin-layer chromatography; Der, derivatives formation; GLC, Gas–liquid chromatography; Cry CSA, crystallization to constant specific activity; Cry $C^3H/^{14}C$, crystallization to constant isotope ratio; Cof., addition of cofactors in the incubation medium; St., stimulation with gonadotropin; Homo., tissue homogenate; Min., minced tissue; t, incubation time; θ, temperature.

[b]Glucuronides.

[c]Sulfates.

and Charreau, 1980), *Gobius jozo* (Colombo *et al.*, 1977), *Roccus saxatilis*, and *Gillichthus mirabilis* (Colombo and Colombo Belvédère, 1977). Furthermore, Idler and co-workers (1976) detected and identified 11β-hydroxytestosterone, but not 11-ketotestosterone, in the blood of males of four ambisexual species sampled at various stages of the sexual cycle: *Diplodus sargus, Pagellus erythrinus, Pagellus acarne*, and *Serranus cabrilla*. However, 11-ketotestosterone was recognized as a metabolite of testosterone in the two latter species (Reinboth, 1975a, 1979) and of androstenedione in *Sparus aurata*, another ambisexual fish (Eckstein *et al.*, 1978).

Several hypotheses have been proposed for the metabolic pathway of the biosynthesis of 11-oxygenated androgens. From a double-isotopic technique, using [^{14}C]testosterone and [^{3}H]adrenosterone simultaneously as precursors, Idler and Macnab (1967) concluded that, in *Salmo salar* testis, 11β-hydroxytestosterone, mainly labeled with ^{14}C, is produced via the 11β-hydroxylation of testosterone, and that 11-ketotestosterone, mainly labeled with ^{3}H, is produced via the 17β-reduction of adrenosterone. Therefore, when adrenosterone was incubated with testicular tissue, 86% was converted to 11-ketotestosterone in 3 hr, and only 10% of 11β-hydroxytestosterone was converted to 11-ketotestosterone in the same time (Idler *et al.*, 1968). In *Salmo gairdneri*, Kime (1979b) obtained 11-ketotestosterone with both testosterone and adrenosterone as precursors, but the latter was found to be more efficiently used. In fact, adrenosterone has been identified in *Oncorhynchus nerka* plasma (Idler *et al.*, 1961b). 11β-Hydroxyandrostenedione, a possible precursor for adrenosterone has been found in incubations of Atlantic salmon testes with androstenedione as precursor (Idler *et al.*, 1968). Finally, 11β-hydroxyandrostenedione, adrenosterone, and 11-ketotestosterone have been recognized in incubations of testes from *Microstomus kitt* (Simpson *et al.*, 1969), from *Poecilia latipina* (Van Den Hurk and Lambert, 1977), and from *Salmo gairdneri* (Depêche and Sire, 1982). However, Arai and Tamaoki (1967a,b) claimed that 11-ketotestosterone is produced from androstenedione through testosterone and 11β-hydroxytestosterone because they have detected neither 11β-hydroxyandrostenedione, nor adrenosterone in incubations of rainbow trout testis with labeled progesterone. Working on subcellular fractions, Suzuki and Tamaoki (1972) confirmed that the 11β-hydroxylation of androstenedione did not occur. In the same study, Suzuki and Tamaoki localized the 11β-hydroxylase activity in the mitochondrial fraction, and both the 17β-hydroxysteroid dehydrogenase and 17α-hydroxylase activities in the microsomal fractions. Another metabolic route, whereby a C-21 steroid is first 11β-hydroxylated, has been proposed by Colombo and Colombo Belvédère (1977) from studies on *Gillichthys mirabilis* and *Roccus saxatilis* testes, namely, progesterone → 17α-hydroxyprogesterone → 21-deoxycortisol → 11β-hydroxyandrostene-

dione. The possibility that 11β-hydroxylase in the testes metabolizes C-21 steroids in *Coris julis* (Reinboth, 1975a), *Dicentrarchus labrax* (Colombo *et al.*, 1978a), *Esox lucius* (Kime and Hews, 1978), and *Salmo gairdneri* (Suzuki and Tamaoki, 1972), and even, in this latter species, prefers 11-deoxycorticosterone or 11-deoxycortisol as substrate rather than testosterone, gives substance to this hypothesis. Finally, Idler and co-workers (1968) have suggested a fourth Δ-5-pathway, in *Salmo salar* testis, where dehydroepiandrosterone is converted to 11β-hydroxytestosterone via 5-androstenediol, and to 11-ketotestosterone via 5-androstenetriol. Such a pathway was also proposed for the synthesis of testosterone in *Esox lucius* testis (Lupo di Prisco *et al.*, 1970).

b. Conjugates. A special characteristic of the endocrine testis of the fish is the formation of conjugates; glucuronidation appears to dominate (Grajcer and Idler, 1963; Colombo *et al.*, 1970; Idler *et al.*, 1971; Colombo and Colombo Belvédère, 1977; Hews and Kime, 1978; Bonnin, 1977; Kime and Hews, 1978; Kime, 1979b, 1980b). However, using different experimental conditions for *in vitro* incubations but comparable temperatures, Depêche and Sire (1982) found less glucuronidation in rainbow trout testis than Kime (1979b) found. Although the conjugation is generally considered as a deactivation mechanism, the recent finding of the possible role of conjugates as sexual attractant (Van Den Hurk *et al.*, 1982a; Colombo *et al.*, 1982b) raises, once more, the question of their biological activity.

c. 5α- and 5β-Reductase. Activity of 5α- or 5β-reductase on pregnen or androsten cycles has been found in the testis of several teleosts (see Table IV). This metabolism has been compared to the state of puberty in the mammal (Azoury and Eckstein, 1980), but it is not clear if this is simply a regulating mechanism or if the reduced compounds have some specific biological actions.

d. Progestins and Estrogens. The production of 17α-hydroxy-20β-dihydroprogesterone by the rainbow trout testis is more puzzling (Arai and Tamaoki, 1967a; Kime, 1979b; Depêche and Sire, 1982). The hormone level in male rainbow trout plasma, sampled during spermiation, was too low for a definitive identification (< 9 ng/ml, Campbell *et al.*, 1980), but, using RIA, Scott and Baynes (1982) found increasing plasma levels of the progestin with sperm production, and it has also been reported to reach high levels in sockeye salmon (Schmidt and Idler, 1962). A role has been hypothesized for 17α-hydroxy-20β-dihydroprogesterone in the process of androgen biosynthesis (Kime, 1979b) or in the stimulation of mitoses of germ cells (Depêche and Sire, 1982). Concerning estrogens, biosynthesis *in vitro* was only reported in the testes of an ambisexual species (Chan and Phillips, 1969).

When investigated in other species, the aromatization was never detected in testis (Eckstein and Eylath, 1968; Colombo *et al.*, 1978a; Callard *et al.*, 1978a; Depêche and Sire, 1982). These results raise the question of the origin of the low estrogen level found in male plasma by the use of RIA (see Section IV,C).

e. Spermatozoa. Activities of 17β-HSD resulting in conversion of 17β-estradiol into estrone, testosterone into androstenedione (Hathaway, 1965), and adrenosterone into 11-ketotestosterone (Idler and Macnab, 1967) have been associated with rainbow trout sperm.

C. Peripheral Sources of Sex Steroids

Glands other than the gonads may participate in the production of sexual steroids, either by synthesizing active steroids by themselves, or by secreting precursors used by the gonads, or by metabolizing some gonadal steroids into other active compounds.

1. Interrenal

Pregnenolone, 17α-hydroxypregnenolone, progesterone, 17α-hydroxyprogesterone, and 11β-hydroxyprogesterone, which are among metabolites of *in vitro* interrenal incubations (see Idler and Truscott, 1972), may be precursors of sexual hormone elaborated in the gonads, if they are released in plasma. Some of them are even active by themselves on oocyte maturation (see Chapter 3, Volume 9B, this series).

Cortisol is metabolized into precursors of androgens in the liver of some species and Kime (1978) has proposed the existence of an interrenal–liver–gonad axis for the synthesis of androgens. Furthermore, during *in vitro* incubations of rainbow trout interrenal tissue, 17α-hydroxyprogesterone is transformed into androstenedione, and androstenedione is transformed into testosterone or 11β-hydroxyandrostenedione (Arai *et al.*, 1969). In the Atlantic salmon, interrenal testosterone is metabolized into 11β-hydroxytestosterone, adrenosterone into 11-ketotestosterone (Idler and Macnab, 1967), and androstenedione or dehydroepiandrosterone into 11β-hydroxyandrostenedione (Idler *et al.*, 1968). Therefore, the interrenal tissue could itself elaborate active androgens.

2. Corpuscles of Stannius

A steroidogenic potentiality has been proposed for the corpuscles of Stannius (Idler and Truscott, 1972). Using chromatographic and fluorimetric methods, Cedard and Fontaine (1963) discovered the presence of androgens

and estrogens in extracts of corpuscles of Stannius from Atlantic salmon. This still must be confirmed by other methods.

3. Liver

As indicated previously, livers from the brown trout, *Salmo trutta*, the pike, *Esox lucius*, and the perch, *Perca fluviatilis*, are able to transform cortisol into cortisone, androstenedione, and 11β-hydroxyandrostenedione, which could be further precursors for the synthesis of 11-oxygenated androgens in the testis (Kime, 1978). However, Truscott (1979) did not find such C-19 steroids in the bile of trout after *in vivo* injection of labeled cortisol. Given the possibility that 17-hydroxy-C-21-steroids contribute to the gonadal production of active androgens, Hansson and Gustafsson (1981a) proposed that the increase in 17β-HSD activity, observed only in the male trout liver during the spawning season, could have a regulatory role in this production. However, these results have been drawn from the *in vitro*, 17-hydrogenation of a C-19 cycle (androstenedione), and no information on the possible production of 17-hydroxy-C-21 steroids in the liver is available.

4. Brain

The detection of aromatase activity in the fish brain (Callard *et al.*, 1978a,b, 1981a; Lambert and van Bohemen, 1980; Lambert and van Oordt, 1982) has raised the question of its contribution to the maintenance of estrogen levels in the plasma, especially for species such as *Myoxocephalus octadecimspinosus* where gonadal aromatization appears negligible in comparison to that of the brain (Callard *et al.*, 1978a). However, in rainbow trout, 17β-estradiol is not detectable in plasma after ovariectomy (Fig. 4). Because no 3β-HSD activity was found in the brain (Lambert and van Bohemen, 1980), estrogen production in the brain is dependent on exogenous precursors).

III. REGULATION OF STEROIDOGENESIS AND STEROID ACTIVITY

The nature, shape, and intensity of a hormonal signal, ready to be received by a target cell, is the result of an intricate series of positive and negative regulations. In the case of hormonal steroids in fish, only some aspects of this complex have been considered. Furthermore, at each step of this regulation consideration must be given to the modulatory role which could be played by external factors, such as temperature and photoperiod. Most of the studies are concerned with the genesis of steroids. Attention has

been focused particularly on the role of gonadotropin(s), but almost nothing is known about the possible direct action of other hypophysial hormones, or about short feedbacks of steroids. In other respects, few data are available on the changes in the gonadal receptivity to gonadotropin, in terms of steroidogenesis. When the gonad does not respond to the stimulation, further investigations will be needed to understand what the limiting step is, i.e., whether it is attributable to either a lack of receptors for gonadotropin, or a lack of a specific activated enzyme involved in steroidogenesis, or a lack of precursor.

Once a steroid is secreted, several mechanisms may inactivate it before it reaches its target. Little is known of catabolism of sexual steroids in teleosts. Most available data are concerned with the total radioactivity found in tissues after fish are fed labeled steroid. The biological significance of glucuronidation or sulfonation remains to be explored. Although the conjugated steroids are usually considered to be inactive, recent studies attribute a pheromonal role to glucuronides (Van Den Hurk *et al.*, 1982a; Colombo *et al.*, 1982b). In other respects, the binding to plasma proteins may lead to a reversible inactivation (Martin, 1980), although, in mammals, it has been suggested that steroid secretion may be enhanced by the presence of serum steroid-binding proteins (Condon and Pate, 1981). Finally, the conversion of plasma steroids into biologically active metabolites can occur in some target tissues.

A. Regulation of Steroidogenesis

1. Role of the Pituitary

Following hypophysectomy, the inhibition of the development of secondary sex characters, known to be induced with sexual steroids, has been an indicator of the presence of hypophysial factors active on steroidogenesis (Pickford and Atz, 1957). In fact, hypophysectomy of *Fundulus heteroclitus* reduced the size and the 3β-HSD activity of the interstitial cells in the testis (Pickford *et al.*, 1972).

Various experimental methods have been used to study the role of hypophysial hormones on steroidogenesis, but each of them has its limitations. One of the most serious criticisms is the use of mammalian hormone. Gonadotropin hormones are known to possess a zoological specificity (Fontaine *et al.*, 1972; Breton *et al.*, 1973a; Bonna Gallo and Licht, 1981; Fontaine-Bertrand *et al.*, 1981), and the biological efficiency of mammalian hormone in fish is poor. There are some exceptions with HCG (Harvey and Hoar, 1980), although the difference between physiological and pharmacological actions is difficult to evaluate. A second criticism is related to the

treatment of nonsurgically hypophysectomized fish. In such experiments, because of the possibility of indirect actions via the pituitary, the receptivity of steroidogenic structures, rather than the nature of the active hormone, should be considered. The use of "chemical hypophysectomy" may be an alternative tool. Methallibure treatments are known to reduce the activity of the gonadotrophs (Hoar *et al.*, 1967), possibly blocking the synthesis of gonadotropin (Breton *et al.*, 1973b), and they probably reduce the release of other pituitary hormones (Van Ree, 1976a). However, some doubts have been raised as to the total efficiency of methallibure because spermatogenesis in the guppy was not inhibited to the same extent as with chirurgical hypophysectomy (Billard *et al.*, 1970). In Atlantic salmon parr, methallibure reduced but did not suppress plasma 11-ketotestosterone (Murphy, 1980). *In vitro* studies have important advantages and are now being developed. Nevertheless, various aspects of the methodology are still being discussed: the complexity of the incubation medium, the possible addition of precursors, and the choice between static or dynamic incubations. Therefore, negative results can never be conclusive, because they may reflect a lack of a limiting factor. Finally, for both *in vivo* and *in vitro* experiments, the method of exogenous hormone administration must be carefully chosen, especially considering the existence *in vivo* of physiological fluctuations of the hormonal concentrations during short time periods (Zohar *et al.*, 1982a). Moreover, measurements of the actual gonadotropin levels induced by the various treatments have rarely been taken.

a. Action of Crude Pituitary Extracts. A crude pituitary extract, injected at the end of the vitellogenic stage, stimulated *in vivo* the 3β-HSD activity in the ovary of the catfish, *Clarias lazera* (Van Den Hurk and Richter, 1980). It raised plasma levels of 17β-estradiol in carp (Fostier *et al.*, 1979; Weil *et al.*, 1980), or of 17α-hydroxy-20β-dihydroprogesterone in rainbow trout (Scott *et al.*, 1982). A stimulation of estradiol secretion was also shown during vitellogenesis in brown trout, *Salmo trutta* (Crim and Idler, 1978), and in adult (Fig. 4; Billard *et al.*, 1978) and immature rainbow trout (Idler and Campbell, 1980). *In vitro* studies confirmed these results. The output of 17β-estradiol from the ovarian fragments of plaice, *Pleuronectes platessa,* (Yaron and Barton, 1980) or of *Sarotherodon aureus* (Bogomolnaya and Yaron, 1982), and the output of testosterone from the testicular fragments of plaice (Duggan and Bolton, 1982) were stimulated *in vitro* by homologous pituitary extracts. However, in the experiment by Duggan and Bolton, 11-ketotestosterone production by the spermiating testis tissue is reduced. Furthermore, in culture of testicular interstitial tissue from *Gobius niger*, pituitary explants increased the production of conjugates more than the production of free testosterone (Bonnin, 1977). All these data suggest an

influence of the whole pituitary which includes not only gonadotropin, but also other factors.

b. Effects of Methallibure Treatments. The administration of methallibure for 2–6 weeks, decreased the 3β-HSD activity in the testis of *Cymatogaster aggregata* (Wiebe, 1968, 1969) and *Poecilia latipinna* (Van Den Hurk and Testerink, 1975), in the ovary of *Brachydanio rerio* (Van Ree, 1976b), and in both sex gonads of *Cyprinus carpio* (Kapur and Toor, 1978). A 14-week treatment with 250 ppm methallibure in the diet lowered plasma 11-ketotestosterone levels in the male parr of Atlantic salmon (10.1 ng/ml to 3.5 ng/ml) (Murphy, 1980). Therefore, methallibure, possibly by decreasing the plasma gonadotropin levels, reduced steroidogenic activities.

c. Action of Mammalian Gonadotropins. Prolonged treatment with HCG stimulated the steroidogenic function of the testicular interstitial cells of the silver Japanese eel, *Anguilla japonica,* according to ultrastructural features (Sugimoto and Takahashi, 1979), and stimulated the androgen metabolism in the testis of the silver European eel, *Anguilla anguilla* (Eckstein *et al.*, 1982). Human chorionic gonadotropin enhanced the 3β-, 16β-, and 17β-HSD activities in the ovaries of *Mugil capito* (Blanc-Livni *et al.*, 1969) and increased the level of plasma testosterone in the male *Tilapia leucosticta* in correlation with an enlargement of the interstitial cells (Hyder *et al.*, 1970). After administering a single injection of HCG *in vivo* (400 IU/kg by weight), Eckstein and co-workers (1978) analyzed *in vitro* the metabolism of [^{3}H]androstenedione in the *Sparus aurata* gonads. The treatment increased the production of 11-ketotestosterone in both sexes, and, in the case of the ovary, only the production of 11β-hydroxytestosterone and testosterone increased; testosterone biosynthesis is inhibited in the testis. Eckstein and coworkers discussed these facts in relation to the sex reversal which occurs in this species. However, Yaron and Barton (1980) did not detect any *in vitro* effect of HCG (25 IU/ml) on 17β-estradiol output from ovarian fragments of *Pleuronectes platessa.*

Ovine luteinizing hormone (oLH) stimulated the 3β-HSD activity in the ovary of the intact *Monopterus albus* (Tang *et al.*, 1974a), and the 17β-HSD activity in the ovary of *Anabas testudineus* (Bhattacharya *et al.*, 1983). A single oLH injection (200 μg/fish 80–100g) applied to mature *Heteropneustes fossilis* induced a drastic rise in the level of the plasma testosterone (Truscott *et al.*, 1978). Although there were possible indirect actions in the case of nonhypophysectomized fish (Van Ree, 1976a,b), oLH, even at a low dose (0.02 μg/g by weight, 3 times per week for 8 weeks), restored the 3β-HSD activity in the interstitial cells of hypophysectomized male *Fundulus heteroclitus* (Pickford *et al.*, 1972). Injected simultaneously with methallibure treatment, oLH prevented the decrease of the 3α- and 3β-HSD

activities of the *Brachydanio rerio* ovaries (Van Ree, 1976b). *In vitro*, the mammalian hormone stimulated the 3β-HSD activity in the testis of *Cymatogaster aggregata* (Wiebe, 1969), and increased the 17β-estradiol output from *Sarotherodon* ovaries (Bogomolnaya and Yaron, 1982), or, to a lesser extent, the androgen output from carp ovaries (Huang and Chang, 1980). Ovine follicle stimulating hormone (FSH) had a weaker activity in both assays. In their comparative study of the gonadotropin specificity in the *in vitro* stimulation of testosterone secretion by fish testis, Bonna-Gallo and Licht (1981) were unable to draw phylogenic conclusions, and they concluded that there is a high degree of interspecific variability in response to FSH and LH.

d. Action of Fish Gonadotropin(s). Steroid production in fish has never been used as a bioassay during purification of fish gonadotropin (Burzawa-Gérard, 1981), but it is apparent that some of the bioassays involve such a step. Direct observations, *a posterieuri*, of the steroidogenic response to a glycoprotein gonadotropin stimulation were positive (Ng and Idler, 1980; Idler and Campbell, 1980; Huang and Chang, 1980; Fostier, *et al.*, 1981a; Bogomolnaya and Yaron, 1982). Cyclic adenosine 5′-monophosphate (cAMP) is probably a mediator of this action (Fontaine *et al.*, 1972; Huang and Chang, 1980; Bogomolnaya and Yaron, 1982; Chang and Huang, 1982). Prostaglandins can also stimulate the *in vitro* production of androgens by the carp testis (Chang and Huang, 1982).

According to Idler and co-workers, two types of gonadotropins are present in fish: a vitellogenic hormone [unadsorbed on Concanavalin A (Con A)–Sepharose] and a glycoprotein maturational hormone (adsorbed on Con A) (see Chapter 5, this volume). From studies using both hormones, and inhibition attempts with antisera raised against each of them, Idler and coworkers arrived at the conclusion that steroidogenic activity was restricted to the maturational gonadotropin (Con AII, GtH) (Ng and Idler, 1980). Some earlier contradictory results (Idler and Ng, 1979), mainly related to the effect of carp gonadotropins in the hypophysectomized male winter flounder, were attributed to a light contamination of the vitellogenic hormone with the maturational hormone or to a nonzoological specificity of action.

Long treatments *in vivo* with a piscine glycoprotein gonadotropin at an immature stage, stimulated testicular development and steroidogenesis: the salmon SG-G100, which may be contaminated with nonglycoproteins (Pierce *et al.*, 1976), enhanced 3β-HSD activity of interstitial cells of the testis in *Oncorhynchus nerka* (Funk and Donaldson, 1972); the carp gonadotropin (c-GtH) in *Anguilla anguilla* (Leloup Hatey *et al.*, 1983), and the salmon gonadotropin (s-GtH) in *Salmo gairdneri* (Magri *et al.*, 1982) increased androgen secretion; finally, in the hypophysectomized *Carassius*

auratus, SG-G100 restored the 3β-HSD activity of the testis (Yamazaki and Donaldson, 1969).

Quicker responses can occur after *in vivo* injections, as suggested by the secretion of 17β-estradiol in the vitellogenic rainbow trout (Billard *et al.*, 1978), or the secretion of androgens in the immature trout, male and female, or in the hypophysectomized male winter flounder (Ng and Idler, 1980). Such injections also stimulated the steroid metabolism in the ovaries of the catfish, *Heteropneustes fossilis* (Ungar *et al.*, 1977) and of the ayu, *Plecoglossus altivelis* (Suzuki *et al.*, 1981a). The stimulation of 3β-HSD activity in the carp ovary (Institute of Zoology, Academia Sinica and Yangtze Institute of Fisheries, 1978) and of the increase of plasma estradiol levels in the same species (Weil *et al.*, 1980) or in the brown trout (Crim and Idler, 1978) after *in vivo* LHRH treatments was also interpreted as an action resulting from the stimulation by the endogenous GtH. Nevertheless, at early stages of the sexual cycle in rainbow trout, 17β-estradiol is not stimulated *in vivo* with GtH (Breton *et al.*, 1983b).

In vitro, various fish glycoprotein gonadotropins are able to enhance the 3β-HSD activity in the ovary of *Brachydanio rerio* (Van Ree, 1977b) and the 17β-HSD activity in the ovary of *Anabas testudineus* (Bhattacharya *et al.*, 1983). Gonadotropins also stimulate the 17β-estradiol production of the ovary of *Sarotherodon aureus* (Bogomolnaya and Yaron, 1982), and the androgen production of the testis of *Cyprinus carpio* (Huang and Chang, 1980), *Gillichthys mirabilis* (Bonna Gallo and Licht, 1981), and *Pleuronectes platessa* (Duggan and Bolton, 1982). The SG-G100 induced a free-cholesterol depletion from *Channa punctatus* ovarian tissue incubated *in vitro* (Mukherjee and Bhattacharya, 1981). Complementary results, obtained *in vivo* with hypophysectomized fish, have shown that this mobilization arises mainly from the free cholesterol and not from the esterified fraction (Mukherjee and Bhattacharya, 1982). Using an *in vitro* perifusion technique, it has been demonstrated by Zohar and co-workers (unpublished) that a transient peak of s-GtH can induce, in *Salmo gairdneri*, a quick output of 17β-estradiol from a vitellogenic ovarian fragment (Fig. 2). Mimicking the plasma pulses found *in vivo* during exogenous vitellogenesis, the maintenance of a "plateau" was observed (Fig. 3) (Zohar, 1982; Zohar *et al.*, 1982b). However, in closely related conditions, but with a closed system at earlier stages and even at the beginning of exogenous vitellogenesis (March), Sire and Depêche (1981) induced an inhibition of the aromatase activity with GtH (50–300 ng/ml), measured from labeled androstenedione or testosterone transformation. From results obtained in *Oncorhynchus rhodurus* on thecal and granulosa fractions isolated from ovarian vitellogenic follicles, it appears that the granulosa cells aromatize androgens elaborated in the thecal layers (Kagawa *et al.*, 1982b). A partially purified chinook salmon gonadotropin

(SG-G100) stimulated testosterone production by the thecal layers, but did not enhance the production of 17β-estradiol in incubations of granulosa layers with exogenous testosterone. Therefore, estrogen production appears to be regulated by the availability of androgen for aromatization. Furthermore, a specific inhibitor of 3β-HSD (cyanoketone) prevents the stimulation by SG-G100 of 17β-estradiol production in salmon follicles (Young *et al.*, 1982). Returning to the results obtained by Sire and Depêche, one might expect that the endogenous testosterone production, stimulated with GtH, was in competition with the exogenous labeled precursor. Even when a high concentration of exogenous precursor in the medium is used as a control, one cannot be certain that the isotopic equilibrium between the medium and the granulosa cells of ovarian fragments has been reached. Nevertheless, during the preovulatory period of trout, the *in vitro* stimulation of 17β-estradiol output by s-GtH is brief and followed by an important temporary elevation of androgen output (Zohar *et al.*, 1982b). Therefore, before ovulation [a stage where a negative correlation was found *in vivo* between plasma levels of GtH and 17β-estradiol (Fostier *et al.*, 1978)], aromatization may become the limiting step of estrogen production. At the same stage GtH could initiate *in vitro* the secretion of 17α-hydroxy-20β-dihydroprogesterone, when the hormone application was continuous (Fostier *et al.*, 1981a; Zohar, 1982; Zohar *et al.*, 1982b).

e. Other Hypophysial Factors. As reported previously, *in vivo* treatment with an antiserum raised against GtH lowers the plasma level of estradiol in the female landlocked Atlantic salmon (Ng *et al.*, 1980) and the level of testosterone in the female winter flounder (Ng and Idler, 1980), but, does not abolish them completely. Considering the treatment time (6 weeks) and the fact that antiserum was used in excess, one could suspect the existence of other GtH-independant regulatory mechanisms. There are few studies of regulation of gonadal steroidogenesis by hypophysial factors other than gonadotropin(s). In contrast to SG-G100, ACTH has no effect on the pregnenolone metabolism in the ovary of *Heteropneustes fossilis* (Ungar *et al.*, 1977), or does ACTH increase the testosterone level in the plasma of this catfish, although cortisol secretion was stimulated. Furthermore, the restoration with bovine growth hormone of the 3β-HSD activity in the testis of hypophysectomized *Fundulus heteroclitus* was attributed to an LH contamination (Pickford *et al.*, 1972). Ovine prolactin also induced a steroidogenic response in nonhypophysectomized *Aequidens pulcher* (Blüm and Weber, 1968) and *Gobius niger* (Bonnin, 1981).

f. Gonad Sensitivity. In *Salmo gairdneri*, the steroidogenic response of the ovary to a GtH stimulation is related to the stage of oocyte development (Fostier *et al.*, 1981a; Breton *et al.*, 1983b; Zohar *et al.*, 1982b). Although a

thiourea treatment, unlike a methallibure treatment, has no effect on the 3β-HSD activity of the testis in *Cymatogaster aggregata* (Wiebe, 1968), thyroxine amplified the *in vitro* cholesterol depletion by the *Anabas testudineus* ovary stimulated with gonadotropin (Sen and Bhattacharya, 1981). Other indirect arguments agree with a sensitizing effect of thyroid hormones (Hurlburt, 1977; Dettlaf and Davydova, 1979; Bhattacharya *et al.*, 1982). However, in the smolt of the amago salmon, *Oncorhynchus rhodurus*, an inverse relationship between the levels of plasma sex steroids and thyroxine have been reported (Nagahama *et al.*, 1982b).

2. Role of Steroids

Steroids may direct their own metabolism toward a specific hormonal production, either by acting as precursors, or by regulating enzyme activities. Therefore, in male trout, pike, or perch interrenalian cortisol may be transformed, in the liver, into precursors for testicular 11-oxygenated androgen production (Kime, 1978). Considering the fact that androgen may activate the interrenal function (Fagerlund and Donaldson, 1969), Kime (1978) proposed an interrenal–liver–testis–interrenal positive feedback loop. This hypothesis may explain the great increase of the plasma 11-ket-

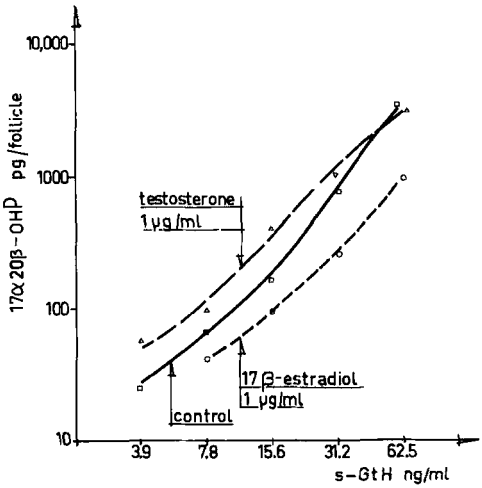

Fig. 6. Action of 17β-estradiol or testosterone (1 µg/ml) on the *in vitro* output of 17α-hydroxy-20β-dihydroprogesterone from rainbow trout ovarian follicles sampled before natural oocyte maturation and stimulated with increasing doses of pure salmon maturational GtH. The differences between treatments are statistically significant (E_2-17β–control: $p < 0.01$; testosterone–control: $p < 0.05$).

otestosterone level during spermiation in trout, in spite of the drop in GtH concentration (Sanchez-Rodriguez *et al.*, 1978; Fostier *et al.*, 1982). In this case, GtH could initiate the loop for 11-ketotestosterone production.

Furthermore, 17β-estradiol in the hypophysectomized or intact juvenile trout (Hansson and Gustafsson, 1981b) and androgens in the intact juvenile trout (Hansson, 1982) modified the metabolism of androstenedione by liver microsomes, in particular by decreasing 6β-hydroxylase activity. In the ovary of *Brachydanio rerio* deoxycorticosterone acetate (DOCA) reduced *in vitro* the 3β-HSD activity (Van Ree, 1977b). In trout, testosterone magnified and 17β-estradiol depressed the positive effect of GtH on the output of 17α-hydroxy-20β-dihydroprogesterone during the intrafollicular oocyte maturation (Fig. 6). These latter observations agree well with the previous analysis of synergetic actions of steroids on intrafollicular oocyte maturation induced with GtH (Jalabert, 1975).

B. Regulation of Steroid Activity

1. Catabolism

Studies of the distribution of radioactivity in the body after administration of labeled steroid (Schreck, 1973; Fagerlund and McBride, 1978; Fagerlund and Dye, 1979; Lone and Matty, 1981), or measurements of steroid metabolism (Lisboa and Breuer, 1966; Colombo *et al.*, 1972a; Truscott, 1979; Hansson *et al.*, 1979; Nienstedt *et al.*, 1981; Hansson and Gustafsson, 1981a) indicate that steroid excretion is mainly via the enterohepatic route. However, almost no information is available for the clearance rate of sexual steroids in teleosts under physiological conditions, excepted in the immature eel, *Anguilla anguilla*, where long half-lives have been reported for testosterone (11.5 hr) and 17β-estradiol (50.6 hr) (Querat *et al.*, 1982), and in the female rainbow trout, in which the metabolic clearance rate of 17β-estradiol (18–45 ml/hr/kg) increases with ovarian development (Zohar, 1982).

Conjugation of steroids is considered a deactivation mechanism prior to excretion (Yano and Ishio, 1978a,b,c). This is puzzling in view of the high potentiality for the fish testis to glucuronate androgens (see Section II,B,2). Nevertheless, it has been recently suggested that glucuronates could play a pheromonal role in fish sexual behavior. Therefore, in *Brachydanio rerio*, a steroid–glucuronid fraction isolated from female ovaries appears to contain the attractant for males (Van Den Hurk *et al.*, 1982a), whereas in *Gobius jozo* the prespawned female is attracted by etiocholanolone-3-glucuronide produced by the mesorchial gland of the male (Colombo *et al.*, 1982b).

Table V

Comparison of the 17α-Hydroxy-20β-dihydroprogesterone Activities on the Maturation of Trout Oocytes Incubated *in Vitro* Either in Balanced Salt Medium or in Blood Plasma[a]

	Incubation medium				
	Balance salt MED[c] of	Undiluted plasma[b]		1/10 Diluted plasma[b]	
Fish	17α,20β-OH-P[c] (ng/ml)	MED[c] of 17α,20β-OH-P[c] (ng/ml)	Concentration of unbound steroid at the MED[c] level (ng/ml)[d]	MED[c] of 17α,20β-OH-P (ng/ml)	Concentration of unbound steroid at the MED[c] level (ng/ml)[d]
Number 1	25.1	214.5	23	—	—
Number 2	12.2	122	5	21.3	2
Number 3	7.9	120	—	—	—

[a]From Fostier and Breton (1975).

[b]Plasma was treated with charcoal to eliminate the endogenous steroids.

[c]Median Efficient Dose of 17α,20β-OH-P on complete oocyte maturation (Jalabert *et al.*, 1974).

[d]The unbound fraction was estimated by gel filtration on a small Sephadex G-25 column of an aliquot of plasma (undiluted or diluted) used in the experiment, incubated with tritiated 17α,20β-OH-P and unlabeled 17α,20β-OH-P at the same concentration as that of the medium.

Testicular glucuronidation may be regulated by hypophysial factors (Bonnin, 1977) or directly by temperature (Kime, 1979b, 1980b). Such a temperature dependance was also reported for hepatic glucuronidation in trout and goldfish (Kime and Saksena, 1980).

2. Sex Steroid Protein Binding

The specific binding of steroids by plasma proteins in fish has recently been reviewed by Martin (1980). Androgens were bound in the plasma of the cod, *Gadus morhua*, the Atlantic salmon, *Salmo salar* (Freeman and Idler, 1971), the carp, *Cyprinus carpio* (Corvol and Bardin, 1973), the haddock, *Melanogrammus aeglefinus* (Idler and Freeman, 1973), the female trout, *Salmo gairdneri* (Fostier and Breton, 1975), and the winter flounder, *Pseudopleuronectes americanus* (Ng and Idler, 1980). A sex steroid binding protein with a high affinity for testosterone and estradiol has been recognized in the female rainbow trout (Fostier and Breton, 1975). As in other vertebrates (Martin, 1980; Wingfield, 1980), the reversible binding with plasma proteins may reduce the biological activity of steroids. Therefore, the activity of the maturation-inducing steroid for trout oocyte (i.e., 17α-hydroxy-20β-dihydroprogesterone) was reduced when *in vitro* incubations were performed in plasma instead of balanced salt medium (Fostier and Breton, 1975) (Table V). In other respects, the regulation of these proteins appears to be gonadotropin(s) dependant, because androgen-binding protein, which disappeared from male winter flounder plasma after hypophysectomy, was restored by both maturational or vitellogenic gonadotropin, the latter being apparently more efficient (Ng and Idler, 1980).

3. Metabolism in Target Tissues

A circulating steroid, elaborated in gonadal tissues, may be transformed into a more active metabolite(s) at the level of the target tissue. Therefore, in addition to the aromatization (see Section II,C,4), a 2-hydroxylase activity has been detected recently in the trout brain (Lambert and Van Oordt, 1982). An aromatase and 17β-HSD activity has also been found in the pituitary (Callard *et al.*, 1981b). These results are reinforced by the finding of estradiol binding in the teleost brain and pituitary (Davis *et al.*, 1977; Kim *et al.*, 1978, 1979a,b; Myers and Avila, 1980; Schreibman *et al.*, 1982b).

The transformation of testosterone into 5α-dihydrotestosterone, an active androgen in mammals, has been demonstrated in the skin and the skeletal muscle of rainbow trout (Hay *et al.*, 1976). The production of water-soluble metabolites (conjugates) has also been reported in the skin of brown trout, *Salmo trutta lacustris* (Soivio *et al.*, 1982).

C. Modulation by External Factors

1. Temperature

The effects of temperature, although important in poikilothermic animals, have been little studied in steroidogenesis. The stimulation of *in vitro* testosterone in *Gillichtys mirabilis* by a sturgeon-gonadotropin-enriched fraction was slightly enhanced between 10°C and 30°C (Bonna-Gallo and Licht, 1981). In *Salmo gairdneri* and *Carassius auratus*, the free 11-ketotestosterone *in vitro* production by the testis, from precursors, was slowly activated with increasing temperature, but this only occurred in the lower range (up to 21°C for the trout and 31°C for the goldfish). The production of androgen conjugates rose quickly even in the higher temperature range; the production of 17α-hydroxy-20ξ-dihydroprogesterone from pregnenolone was also maximal at the highest temperatures (Kime, 1979b, 1980b). Such a temperature dependance has also been reported for hepatic glucuronidation (Kime and Saksena, 1980). Therefore, it has been proposed that temperature could act directly on steroidogenic structures as a regulating factor (Kime, 1982).

The physiological response to steroids may also be temperature dependant. When injected into intact *Gillichtys mirabilis* with regressed testes, testosterone accelerated spermatogenesis at low temperature (10°C) but not at high temperature (27°C) (de Vlaming, 1972). *In vitro*, the response of goldfish oocytes to the maturational action of 17α-hydroxy-20β-dihydroprogesterone decreased with the increasing temperature (13°–35°C) (Gillet *et al.*, 1977). Finally, the vitellogenic response of the liver to *in vivo* estradiol injections has been shown to be related to the rearing temperature of *Mirogrex terraesanctae* (Yaron *et al.*, 1980) and *Heteropneustes fossilis* (Dasmahapatra *et al.*, 1981).

2. Photoperiod

The central nervous system (CNS)–hypophysis axis is probably the relay for the photoperiodic modulation of plasma estradiol in the female *Salmo gairdneri* (Whitehead *et al.*, 1978b; Bromage *et al.*, 1982) or *Mirogrex terrae sanctae* (Salzer and Yaron, 1979) and of plasma testosterone in the male trout (Whitehead *et al.*, 1979). The decrease in plasma estradiol of female *Carassius auratus* following section of the optic tract remains to be further investigated (Delahunty *et al.*, 1979), because pinealectomy or exposure to continuous darkness has little effect. The discovery of nerve terminals ending on interstitial cells of the testis of *Poecilia reticulata* (Follénius, 1964), *Poecilia latipinna* (Van Den Hurk *et al.*, 1974), *Oryzias latipes* (Gresik,

1973), and *Salmo gairdneri* (Van Den Hurk *et al.*, 1982b) gives credence to the hypothesis of direct CNS control.

3. Pollutants

Pollutants may disturb steroid regulation because plasma estrogen and androgen levels in trout or carp treated with polychlorinated biphenyls decreased significantly after 3–4 weeks, perhaps because of a stimulation of the hepatic catabolism (Sivarajah *et al.*, 1978). Sangalang and O'Halloran (1973) also observed negative direct effects of cadmium on the *in vitro* testicular synthesis of androgens in *Salvelinus fontinalis*, although *in vivo* plasma testosterone and 11-ketotestosterone levels were higher in treated fish (Sangalang and Freeman, 1974). Sangalang and Freeman suggested an impairment in the clearance and/or utilization of these hormones. Pollutants have also been shown to modify the hepatic metabolism of androstenedione in trout (Hansson, 1981).

4. Stress

Stress factors may disturb steroidogenesis. Stress, caused by repeated sampling in rainbow trout, depressed plasma androgen levels in male fish (Schreck *et al.*, 1972a), but did not affect estradiol levels (Schreck *et al.*, 1973). Repeated handling for saline injections decreased the plasma estradiol level in immature rainbow trout, in comparison with another control group which was not handled (Magri *et al.*, 1982). Furthermore, the effect of pollutants on hepatic metabolism of androstenedione, reported previously (Hansson, 1981), is mimicked by cortisol implants, indicating that, instead of acting directly, pollutants may disturb steroid metabolism via a stress reaction. The disturbance of gonadal steroidogenesis might be related, at least partly, to stress-induced ascorbic acid depletion (Wedemeyer, 1969; Seymour, 1981).

IV. PHYSIOLOGICAL ROLE OF GONADAL STEROIDS IN REPRODUCTION

Sex steroids function at various levels. They play a role in the genesis of the gonad both in the differentiation and maintenance of somatic tissues, mainly the gonadal ducts, and in gametogenesis. Steroidogenesis develops in immature fish when the endocrinological regulation of the future adult is developing, and then participate in this regulation in the adult. When gametes are ready for fertilization, steroids act to bring the sexes together,

stimulating the development of morphological characteristics and modulating sexual behavior (Yamazaki and Watanabe, 1979). Both these actions may be retained after fecundation in species which care for their brood. The role of gonadal steroids in the differentiation and development of secondary sexual characters has been intensively reviewed (Pickford and Atz, 1957; Dodd, 1960; Chester Jones *et al.*, 1972); reproductive behavior is reviewed in Chapter 1, Volume 9B, this series. Here, discussion mainly concerns the role of steroids in gametogenesis.

Direct actions are mainly discussed, but consideration is also given to some indirect effects via gonadotropin secretion, but without detailing the mechanism of this regulation, such as feedback at the CNS level (Dixit, 1967; Viswanathan and Sundararaj, 1974; Olcese and de Vlaming, 1979; Weil, 1981). Briefly, gonadal steroids generally exert a negative control over the gonadotrophs in adults, in immature fish, positive effects have been observed. Nevertheless, one must remember that other indirect actions may be important. Steroids are known to influence hypophysial cells other than the gonadotrophs (Goos *et al.*, 1976; Olivereau and Olivereau, 1979a; Olivereau *et al.*, 1981), and, considering the somatotrophs, one might expect a relation between growth and reproduction (Pickford *et al.*, 1972; Higgs *et al.*, 1977). In other respects, thyroidal hormones appear to play a role in reproduction (Sage and Bromage, 1970; Leloup *et al.*, 1976; Hurlburt, 1977; Dettlaff and Davydova, 1979; Pickering and Christie, 1981), and androgens stimulate thyroidal activity (Nishikawa, 1976; Singh, 1968; Higgs *et al.*, 1977; Hunt and Eales, 1979a,b) perhaps by a direct action on the thyroid gland (Sage and Bromage, 1970; Singh, 1969), although negative results have been reported (Milne and Leatherland, 1980). Concerning the action of estradiol on the thyroid, contradictory results have been obtained (Singh, 1969; Olivereau *et al.*, 1981).

In the following discussion, the difficulty, which sometimes arises, in drawing a definitive conclusion about the action of a particular steroid on one of the stages of gametogenesis is apparent. Some contradictory results may be related to the diversity of the various species studied. However, there are definite limitations in methodologies. The sensitivity of classical assays and the number of samples that can be analyzed are also limited. Although more efficient, the specificity of radioimmunoassays is still a matter for discussion. From this view point, data on the strict validation of these assays are not always published, and, furthermore, detailed information on the gonadal stages is not always given. This restriction is also encountered in experimental studies where little attention is directed to the actual inducing concentration of exogenous steroid that is administered, and even less to the possible metabolization. Therefore, contradictory results could arise from unknown

differences in experimental factors. This is especially true in vertebrates which are very sensitive to variations in external factor.

A. Sexual Differentiation

Determination of the precise period of sexual differentiation is difficult because this depends on the precision of the criteria used to measure sexual differences. Although Bruslé (1980) identified spermatogonia and oogonia in mullet at the ultrastructural level, the lack of clear distinctive morphological features between these two germ cell categories has been stressed (Reinboth, 1980), and detection of the first discrete and polymorphic signs of differentiation is rather uncertain.

The localization of the germinal cells along the embryonic gonad and the number of these cells are apparently the earliest clear morphological evidence of a sex difference in trout (Lebrun *et al.*, 1982). These are followed by the appearance of the meiotic prophase after which more obvious morphological differences take place, e.g., ovigerous lamellae or somatic cell arrangement around the oogonia. Gonadal differentiation of the gonochoric species could be considered complete when the gonadal ducts are established.

Experimental studies have shown that the germ cells retain a bipotentiality during, at least, a part of the differentiation period. That steroids determine the final sex of the gonad during this period is not enough to prove that a natural sex-inducer appears at this moment, and that this inducer is a steroid. An indirect effect could occur, as is without doubt the case with feminization of the rainbow trout by dimethylformamid (Van Den Hurk and Slof, 1981). Furthermore, exogenous steroids may stimulate pituitary activity in very young fish, as demonstrated in rainbow trout in which methyltestosterone, progesterone, or 17α-hydroxyprogesterone, administered shortly after hatching, stimulated GtH-cell development (Van Den Hurk, 1982). Besides, from ultrastructural observations in *Oryzias latipes* gonads, Satoh (1974) did not find steroidogenic cells prior to sexual differentiation. Yamamoto (1969) has proposed a dual model whereby the androgens are the andro-inducers and the estrogens the gynoinducers. Numerous experiments support this hypothesis (see Chapter 5, Volume 9B, this series). Nevertheless, Satoh (1973) transplanted undifferentiated presumed testis of *Oryzias latipes* into the anterior eye-chamber of an adult female and observed a normal testicular development. Estradiol did not modify the evolution of spermatogonia in the testis of the adult goldfish cultured *in vitro* (Remacle, 1976); however, ovaries cultured in a hormone-free medium showed some

oogonial evolution toward the first stages of spermatogenesis (Remacle *et al.*, 1976). Testosterone isobutyrate appears to stimulate these abnormal differentiations, but is not necessary to initiate them. Paradoxal feminization effects have been obtained with methyltestosterone in various cichlid species (von Müller, 1969; Hackmann, 1974; Nakamura, 1975). Although a possible metabolism into estrogen has been postulated (Hackmann and Reinboth, 1974), no aromatase activity has been revealed in the undifferentiated gonad or the newly differentiated ovary of the rainbow trout fry (Van Den Hurk *et al.*, 1982b). Van Den Hurk and co-workers found a 3β-HSD, a Δ-5,4-isomerase, and a 17α-hydroxylase activity in the undifferentiated gonad and the newly differentiated ovary. Because in trout progesterone treatments have a feminizing effect, Van Den Hurk and Slof (1981) proposed that progestins may be the gyno-inducers possibly by blockage of the androgenic pathway. Nevertheless, the presence of 17α,20-desmolase and 11β-hydroxylase activities, permitting androgen synthesis in the young testis, is in agreement with Yamamoto's hypothesis concerning the male. The progestin biosynthesis potentiality in the undifferentiated gonads and the late occurrence of androgen synthesis at the onset of male differentiation, provide some explanations for the juvenile hermaphrodism sometimes reported in trout (Mrsic, 1923); the existence of this hermaphrodism has been questioned (Takashima *et al.*, 1980).

Natural sex inversion in ambisexual adult fish may also provide some information about sexual differentiation, even though synchronous hermaphrodism raises confusing questions. In *Monopterus albus* (Chan and Phillips, 1969) and *Sparus auratus* (Colombo *et al.*, 1972b), the male phase is characterized by a higher androgen to estrogen ratio, produced *in vitro*, than in the female phase. In the protandrous *Sparus auratus*, the biosynthesis of metabolites from [^{3}H]pregnenolone is minimal, during the inversion period; however, in the protogynous *Monopterus albus* the production of androgens increases with a detectable peak of testosterone in plasma (Chan *et al.*, 1975). However, in the latter species, androgens are not effective in bringing about a precocious sex inversion in the female, even after treatment with cyanoketone, which is a 3β-HSD inhibitor (Tang *et al.*, 1974b). However, they are effective in other protogynous fish (Reinboth, 1962, 1975b; Fishelson, 1975). It must be stressed that these experiments were performed using intact adult fish, and it is known that during the sex inversion of *Monopterus albus* the pituitary is active (Chan *et al.*, 1975), and that a mammalian gonadotropin (oLH) can stimulate the process (Tang *et al.*, 1974b).

The role of sex steroids in gonadal duct differentiation is better established. Steroidogenic activities are detectable before this differentiation in the testis of *Oryzias latipes* (Satoh, 1974), *Salmo gairdneri* (Upadhyay, 1977;

Van Den Hurk *et al.*, 1982b), *Poecilia reticulata* (Takahashi and Iwasaki, 1973a), *Poecilia latipinna* (Van Den Hurk, 1974), and in the ovary of *Salmo gairdneri* (Upadhyay, 1977; Van Den Hurk *et al.*, 1982b). In *Poecilia reticulata*, (Takahashi and Iwasaki, 1973a) and in *Poecilia latipinna* (Van Den Hurk, 1974), 3β-HSD activity in the testis increased with the development of the duct system. Furthermore, in *Poecillia* sp. a weak 3α-HSD activity was shown in the duct epithelium. Numerous publications have reported the positive effects of sex steroids on the gonad ducts and their accessories in intact fish (Hoar, 1969; Chester Jones *et al.*, 1972; Takahashi and Takano, 1971; Takahashi, 1972, 1974; Riehl, 1981). These results have been confirmed in the hypophysectomized juvenile guppy (Pandey, 1970), the hypophysectomized adult of *Poecilia reticulata* (Pandey, 1969b), *Fundulus heteroclitus* (Lofts *et al.*, 1966), *Heteropneustes fossilis* (Nayyar *et al.*, 1976), *Gillichthys mirabilis* (de Vlaming and Sundararaj, 1972), and the methallibure-treated *Poecilia latipinna* (Van Den Hurk and Testerink, 1975). A synergism with prolactin on the maintenance of seminal vesicle activity has been suggested in *Heteropneustes fossilis* (Sundarararay and Nayyar, 1969) and in *Gillichthys mirabilis* (de Vlaming and Sundararaj, 1972).

Other organs such as the liver (Ishi and Yamamoto, 1970; Hansson and Gustafsson, 1981b) or the brain (see Chapter 1, Volume 9B, this series) could also be under the sex-determining influence of gonadal steroids.

B. The Ovary

1. Development of the Young Immature Gonad

Few studies have concentrated on the initial stages of gonad development in the female teleost. Using electron microscopy, Satoh (1974) could not identify cells with steroidogenic features in the well differentiated ovary of the *Oryzias latipes* fry, but he emphasized the difficulty of locating them in a large gonad. The ovary was able to perform steroid conversions in older *Anguilla anguilla* at the silver stage (Colombo and Colombo Belvédère, 1976b). In 3- to 5-months-old trout, ovarian interstitial steroidogenic cells have been found (Upadhyay *et al.*, 1978) and the enzyme activities necessary to elaborate progestins have been detected (Van Den Hurk *et al.*, 1982b). Later, at age 7 months, an aromatase activity was detectable (Van Den Hurk *et al.*, 1982b). At this stage, very low levels of estrogens were measured using a radioimmunoassay (0.2 ng/ml in a pool of plasma), but higher levels were noted at age 13 months (Magri *et al.*, 1982). Surprisingly, repeated handling for GtH or saline injections depressed these levels in comparison with another control group which was not handled. Further investigations are required to analyze the possible repercussions of stress on sex steroid

regulation (see Section III,C,4). When a more rapid response was analyzed, the increase of androgens, mainly testosterone, and of 17β-estradiol levels occured in the plasma of juvenile female trout after two successive injections a glycoprotein GtH (Ng and Idler, 1980; Idler and Campbell, 1980). Therefore, the steroidogenic potentiality and the sensitivity to a GtH stimulation appeared early in the juvenile ovary.

17β-Estradiol is known to stimulate the development of the gonadotroph cells in the pituitary of the female silver eel (Olivereau and Chambolle, 1978; Olivereau and Olivereau, 1979b) and to increase the content of pituitary GtH in the immature rainbow trout (Crim *et al.*, 1981). Nevertheless, there is no clear direct trophic action on the ovary. Although injections of estrogen appeared to stimulate oocyte growth slightly in the silver eel (Olivereau and Olivereau, 1979b), no effect was obtained in the juvenile trout (Upadhyay *et al.*, 1978; Yamazaki, 1976).

2. The Adult Sexual Cycle

a. Estrogens. Sire and Depêche (1981) found a low aromatase activity from the first days after ovulation in the rainbow trout ovary; thereafter, aromatase activity increased with ovarian development. However, Lambert and van Bohemen (1979a) did not detect aromatase activity at the beginning of the cycle (see also van Bohemen and Lambert, 1981). Low levels of plasma 17β-estradiol have been measured during the regressed stage of the sexual cycle of the plaice, *Pleuronectes platessa* (Wingfield and Grimm, 1977), the striped mullet, *Mugil cephalus* (Dindo and MacGregor, 1981), *Sarotherodon aureus* (Terkatin-Shimony and Yaron, 1978; Yaron *et al.*, 1977), the brown trout, *Salmo trutta* (Crim and Idler, 1978; Billard *et al.*, 1978), and the rainbow trout (Whitehead *et al.*, 1978a; van Bohemen and Lambert, 1981). The physiological significance of estrogens during previtellogenesis remains to be explored. It has been suggested that they may induce an oogonial recrudescence (Khoo, 1975). Estradiol in the intact goldfish (Khoo, 1974) and estrone in the intact minnow, *Phoxinus laevis* (Bullough, 1942) and gudgeon, *Hypseleotris galii* (Mackay, 1973) causes a proliferation of oogonia. However, the inhibition of this effect with a methallibure treatment in the latter species indicates that it is probably an indirect action, or at least a synergic action with pituitary hormones. Furthermore, Remacle and coworkers (1976) did not detect any *in vitro* effect of estradiol monobenzoate on the germ cells of the goldfish ovary.

During vitellogenesis an increase in plasma estrogen levels, mainly 17β-estradiol, correlated with the growth of vitellogenic oocytes (Fig. 7) has been confirmed in many species: goldfish (Schreck and Hopwood, 1974), plaice (Wingfield and Grimm, 1976, 1977), *Sarotherodon aureus* (Yaron *et al.*,

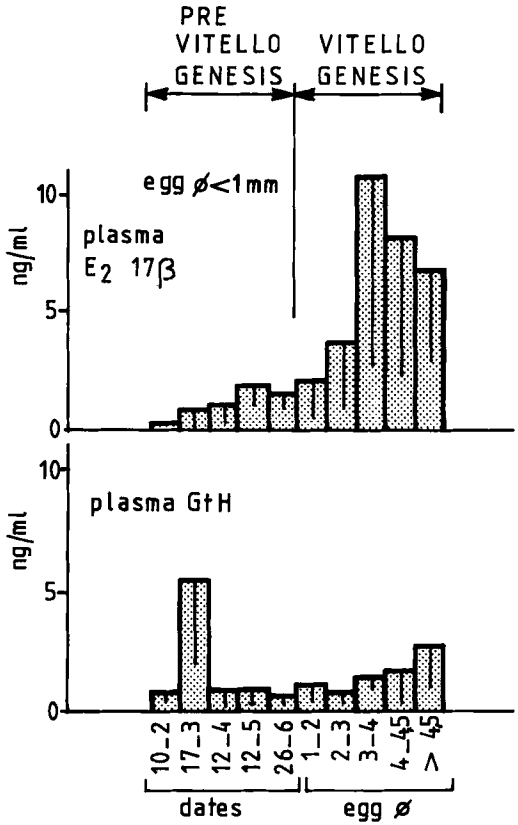

Fig. 7. Evolution of plasma GtH and 17β-estradiol during the previtellogenesis and vitellogenesis in the brown trout, *Salmo trutta* (Breton *et al.*, 1983) (means ± SD).

1977), brown trout (Crim and Idler, 1978; Billard *et al.*, 1978; Soivio *et al.*, 1982), rainbow trout (Breton *et al.*, 1975a, 1983a; Whitehead *et al.*, 1978a,b; Lambert *et al.*, 1978; Billard *et al.*, 1978; Scott, *et al.*, 1980b; van Bohemen and Lambert, 1981; Bromage *et al.*, 1982), Atlantic salmon (Idler *et al.*, 1981), striped mullet (Dindo and MacGregor, 1981), and *Pomatus saltator* (MacGregor *et al.*, 1981). Estrone levels have been found to be lower than estradiol ones (van Bohemen and Lambert, 1981; Soivio *et al.*, 1982). At this stage the best known action of estrogens is related to the hepatic synthesis of vitellogenin, a lipophosphoprotein precursor of oocyte vitellus (Amirante, 1972; Korsgaard-Emmersen and Petersen, 1976; Emmersen *et al.*, 1979; Roach and Davies, 1980; Sundararaj and Nath, 1981; see Chapter 8, this volume). In the rainbow trout, estrone might have a priming role for the

estradiol stimulation of the liver (van Bohemen *et al.*, 1982). However, the uptake of vitellogenin by oocytes is gonadotropin dependent (Idler and Ng, 1979; Sundararaj *et al.*, 1982a; Burzawa-Gérard, 1981, see Chapter 5, this volume). In addition to participation in the synthesis of vitellogenin, estrogens regulate carbohydrate and lipid metabolism (Plack and Pritchard, 1968; de Vlaming *et al.*, 1977a,b; Korsgaard and Petersen, 1979; Sand *et al.*, 1980; Dasmahapatrae and Medda, 1982). They probably control the lipid mobilization from fat stores (de Vlaming *et al.*, 1977a) and the calcium mobilization from scales (Mugiya and Watabe, 1977; Mugiya and Ichii, 1981). Concerning a direct action of estrogens on the vitellogenic ovary, a partial maintenance of the yolky oocytes with 17β-estradiol has been observed in the hypophysectomized catfish, *Heteropneustes fossilis* (Anand and Sundararaj, 1974; Sundararaj and Goswami, 1968). Estradiol, estrone, or estriol induce the formation of yolk vesicles (composed of mucopolysaccharides and not lipoprotein) in the hypophysectomized goldfish (Khoo, 1979). However, in the latter species, estradiol monobenzoate has no effect *in vitro* on vitellogenic oocytes (Remacle *et al.*, 1976, 1977).

The inhibitory or atretic action of estradiol, administered in intact fish, on vitellogenesis has been attributed to a negative feedback at the pituitary level. Several facts support this hypothesis. 17β-Estradiol or estradiol monobenzoate supress hypertrophy of pituitary gonadotrophs caused by ovariectomy in *Serranus scriba* and *Serranus cabrilla* (Febvre and Lafaurie, 1971), and *Oncorhynchus nerka* (Van Overbeeke and McBride, 1971). Furthermore, the compensatory hypertrophy of the remaining ovary after unilateral ovariectomy in *Heteropneustes fossilis*, attributed to an increase of pituitary secretion, was prevented by estrogen (Goswami and Sundararaj, 1968). Tamoxifen and Clomiphene, which are considered as antiestrogens from studies performed in mammals, cause a rise in serum GtH levels of goldfish, when implanted in the nucleus lateral tuberis or in the pituitary (Billard and Peter, 1977). Clomiphene, when injected intraperitoneally in carps, induced GtH discharges (Breton *et al.*, 1975b). Nevertheless, at the end of vitellogenesis, intraperitoneal injections of 17β-estradiol (200 μg/kg by weight, 2 times per week) did not prevent the increase of the plasma GtH level after ovariectomy in the rainbow trout (Bommelaer *et al.*, 1981).

At the end of the sexual cycle, especially prior to maturation and ovulation, a drop in plasma estradiol levels of the trout was observed (Fostier *et al.*, 1978; Fig. 8). Low levels of estrogens at the time of spawning have been confirmed in rainbow trout (Scott *et al.*, 1980b) and in other species: carp (Elefthériou *et al.*, 1968), plaice (Wingfield and Grimm, 1977), the striped mullet (Dindo and McGregor, 1981), the king mackerel, *Scomberomus cavalla* (McGregor *et al.*, 1981), and the white-spotted char (Kagawa *et al.*, 1981) and the coho salmon (Jalabert *et al.*, 1978; Sower and Schreck, 1982).

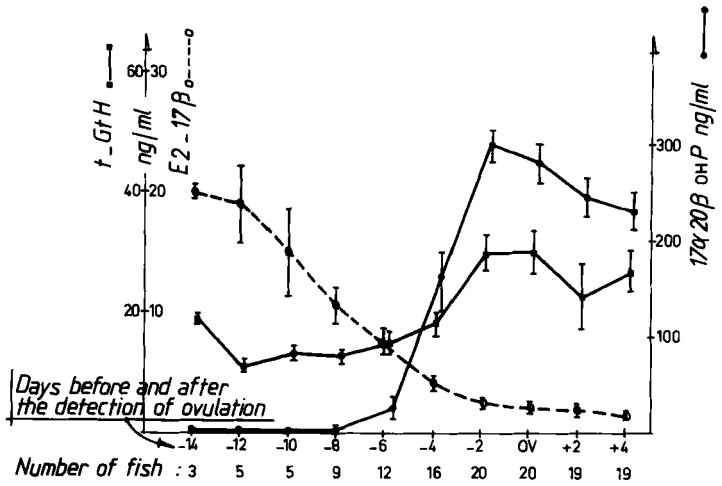

Fig. 8. Concomitant evolutions of plasma GtH, 17β-estradiol, and 17α-hydroxy-20β-dihydroprogesterone (measured with RIA) at the end of the cycle in the rainbow trout (Fostier and Jalabert, 1982) (means ± SE).

Fostier and co-workers suggested the removal of a negative feedback of estradiol on GtH secretion prior to ovulation. In fact, the increase of plasma GtH observed after ovariectomy at this stage was prevented in some trouts with estradiol treatment (Bommelaer *et al.*, 1981). A direct negative effect on ovarian sensitivity to maturational GtH also exists (Jalabert, 1975; Fig. 6).

b. Androgens. Testosterone, which may be found at higher levels in females than in males (Campbell *et al.*, 1976, 1980; Stuart-Kregor *et al.*, 1981; Scott *et al.*, 1980a,b), showed a maximum in the last part of vitellogenesis in the winter flounder (Campbell *et al.*, 1976), the rainbow trout (Scott *et al.*, 1980b; Fig. 9), and the plaice (Wingfield and Grimm, 1977). In the rainbow trout, a little peak of testosterone was detected in plasma just before ovulation (Fostier and Jalabert, 1982; Scott and Baynes, 1982; Scott *et al.*, 1983). In the *Sarotherodon aureus* female, the initiation of spawning by increasing the water temperature is followed by a rise of 11-ketotestosterone, 11β-hydroxytestosterone, and testosterone in the plasma (Katz and Eckstein, 1974).

Several hypotheses have been proposed for the role of androgens in the female. Androgens are the precursors for estrogens, and they are released into the plasma when no longer needed for aromatization (Campbell *et al.*, 1976). They might act for the maintenance of sexual behavior and/or for the increase of GtH secretion (Scott *et al.*, 1980b). A role has also been suggested for 11-oxygenated androgens in the sexual inversion of her-

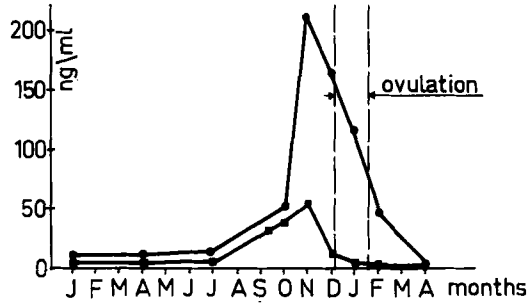

Fig. 9. Mean levels of testosterone (●) and 17β-estradiol (■) in plasma of the rainbow trout female over the period of their first spawning season (Scott *et al.*, 1980b).

maphrodite species such as *Coris julis* and *Pagellus acarne* (Reinboth, 1972), or aged *Poecilia reticulata* (Lambert and Pot, 1975). In *Anguilla anguilla*, androgens might be secreted by residual testicular components from the long period of intersexuality (Colombo and Colombo Belvédère, 1976b). Higher levels of 11-ketotestosterone found in *Mugil capito* confined in fresh water, compared to the fish in seawater, could explain the lack of normal ovulation (Eckstein and Eylath, 1970).

However, actual positive actions of androgens have been shown. They possess, at high doses, a vitellogenic potentiality on the liver of *Gobius niger* (Le Menn, 1979) and goldfish (Hori *et al.*, 1978), probably via the estrogen receptors (Le Menn *et al.*, 1980). Furthermore, a physiological action of androgens on the metabolism of the plasma free fatty acid could exist (Wiegand and Peter, 1980). Although the 11-ketotestosterone levels are low in females of Atlantic salmon, Idler and co-workers (1981) found a significant positive relationship with plasma vitellogenin level. At the end of the cycle, some effect of androgens has been found *in vitro* on the process of intrafollicular oocyte maturation, either directly (Goswami and Sundararaj, 1974; Goetz and Bergman, 1978; Iwamatsu, 1978) or by synergy with maturational gonadotropin (Jalabert, 1975; Fig. 6).

Finally, effects on central regulation, comparable to estrogen effects, could not be excluded because aromatase activities have been detected in the pituitary (Callard *et al.*, 1981b) and in the central nervous system (Callard *et al.*, 1978a,b, 1981a).

c. Other Steroids. Progestins (Schmidt and Idler, 1962; Campbell *et al.*, 1976, 1980; Sower and Schreck, 1982; Kagawa *et al.*, 1981; Fostier *et al.*, 1981b) (Fig. 8) and corticosteroids (Katz and Eckstein, 1974; Fuller *et al.*, 1976; Wingfield and Grimm, 1977; Cook *et al.*, 1980; Pickering and Christie, 1981) often reach their maximal concentrations during the spawning season.

Both types of hormones have been implicated in oocyte maturation and ovulation (Jalabert, 1976; Sundararaj and Goswami, 1971; see Chapter 3, Volume 9B, this series). Van Ree (1977b) has also observed *in vitro* a maintenance effect of deoxycorticosterone acetate on the development of zebrafish follicles and during vitellogenesis, cortisal enhances the estrogen-induced vitellogenin synthesis in the catfish Heteropneustes *fossilis* (Sundararaj *et al.*, 1982b).

After ovulation the large increase of GtH levels in rainbow trout has been related to the drop of 17α-hydroxy-20β-dihydroprogesterone concentrations in plasma, a progestin which is known to exert a negative feedback on GtH secretion at this stage (Jalabert *et al.*, 1976). Progestins could also be involved in preserving mechanisms for nonspawned ovules, as proposed in stickleback (Lam *et al.*, 1978, 1979), in the control of pregnancy in *Gambusia* (Chambolle, 1969), or of birth in *Zoarces viviparus* (Korsgaard and Petersen, 1979).

C. The Testis

1. Development of the Young Immature Gonad

As discussed previously, steroidogenic activities have been detected very early in the immature testis (Oota and Yamamoto, 1966; Satoh, 1974; Takahashi and Iwasaki, 1973a; Upadhyay, 1977; Van Den Hurk, 1974; Van Den Hurk *et al.*, 1982b; Nagahama *et al.*, 1978). The induction of precocious sexual maturity with gonadotropin injections stimulated gonadal 3β-HSD activity in *Oncorhynchus gorbuscha* (Funk and Donaldson, 1972) and caused development of interstitial cells identified by their ultrastructural characteristics in *Salmo gairdneri* (Upadhyay, 1977) and *Anguilla japonica* (Sugimoto and Takahashi, 1979). Androgen levels, increased in plasma during this process in trout (Magri *et al.*, 1982; Crim *et al.*, 1982; Gielen *et al.*, 1982). In the parr of *Salmo salar* the plasma level of testosterone and mostly 11-ketotestosterone rose with the natural precocious maturation, although remaining at lower levels than in adults (Dodd *et al.*, 1978; Stuart-Kregor *et al.*, 1981). Nevertheless, a reduction of the plasma 11-ketotestosterone level, by means of a methallibure or cyproterone acetate treatment, did not prevent this precocious maturation (Murphy, 1980).

Observations of the effect of exogenous androgens on the juvenile gonad appear somewhat contradictory. Hamaguchi (1979), using methyltestosterone or cyproterone acetate treatments, concluded that androgens have no effect on the first waves of germ cell proliferation in the male of the medaka, *Oryzias latipes*. Such a conclusion was also drawn from studies of the hypophysectomized juvenile guppy, *Poecilia reticulata*, by Pandey

(1969a). However, in the intact molly, *Poecilia latipina*, spermatogonial multiplication, which was not affected after methallibure treatment, was accelerated with methyltestosterone or 11-ketotestosterone (Van Den Hurk and Van de Kant, 1975). With respect to later stages, androgens did not stimulate spermatogenesis in the silver eel (Sokolowska *et al.*, 1978) or in the hypophysectomized guppy (Pandey, 1969b), and even inhibited it in the juvenile goldfish (Takahashi, 1972) and coho salmon, *Oncorhynchus kisutch*, (McBride and Fagerlund, 1973; Higgs *et al.*, 1977; Yu *et al.*, 1979). However, a stimulation has been observed in the intact *Poecilia reticulata* (Clemens *et al.*, 1966), *Poecilia latipinna* (Van Den Hurk and Van de Kant, 1975), *Salmo salar* (Crim and Peter, 1978; Dodd *et al.*, 1978), *Oncorhynchus tshawytscha* (Schreck and Fowler, 1982), and *Salmo gairdneri* (Crim and Evans, 1982). Crim and Evans (1979) suggested an indirect action at the pituitary level, knowing that androgen injections increased the GtH accumulation in the pituitary of the immature trout, probably via an *in situ* aromatisation (Crim *et al.*, 1981; Gielen *et al.*, 1982). This hypothesis is supported by the stimulation of the gonadotroph cells after estradiol injections in male silver eels (Olivereau and Chambolle, 1978; Olivereau and Olivereau, 1979b) and the aromatase activity found in the *Myoxocephalus* pituitary (Callard *et al.*, 1981b). Nevertheless, plasma GtH changes have only been reported in trout (Crim and Evans, 1982).

2. The Adult Sexual Cycle

a. Androgens. Androgens, mainly testosterone and/or 11-ketotestosterone, have been measured in plasma during the sexual cycle. Using a nonspecific radioimmunoassay, Schreck and Hopwood (1974) demonstrated that in *Carassius auratus* the highest level was reached during the breeding season (33 ng/ml) and constant lower levels were maintained during the resting period (9 ng/ml). More accurately, the rise in androgen levels occured with the appearance of spermatozoa and was then amplified during spermiation in *Salmo trutta* (Billard *et al.*, 1978). This finding was confirmed in *Salmo gairdneri* (Schreck *et al.*, 1972b; Sanchez-Rodriguez *et al.*, 1978). In carp, where spermatogonia type B and spermatozoa were present throughout the year, no clear androgen changes could be detected in monthly samples (3 ng/ml) (Billard *et al.*, 1978; Weil, 1981). However, other researchers have reported higher levels during the breeding season of carp (Sivarajah *et al.*, 1979).

i. Testosterone. Using more specific assays, Sangalang and Freeman (1974) noted a slight rise in plasma testosterone levels in *Salvelinus fontinalis* from the beginning of spermatozoa production (June, 0.45 ng/ml) up to a maximum (October, 2.5 ng/ml) at the onset of spermiation. Such a pattern

occurred in *Salmo trutta* (Soivio *et al.*, 1982; Kime and Manning, 1982) and in *Salmo gairdneri* (Scott *et al.*, 1980a) but in these species with higher levels. Nevertheless, in *Salmo gairdneri*, Whitehead and co-workers (1979) reported a constant increase from 4 ng/ml in April to 20 ng/ml in January of the next year; spermiation occurred in December. Scott and Baynes (1982) studying a strain of rainbow trout other than that one used in a previous study (Scott *et al.*, 1980a), found the highest levels of plasma testosterone during the active spermiation. In another salmonid, *Salmo salar*, Idler and co-workers (1971) reported an almost constant testosterone level in the peripheral or testicular plasma during the final stages of spermatogenesis and during spermiation, although Stuart-Kregor and co-workers (1981) found higher levels in males which have not begun their spermiation (22 ng/ml) than in "ripe" ones (5.2 ng/ml). In several other families, testosterone values increase during testicular recrudescence, with a maximum just prior to the full breeding season: *Pseudopleuronectes americanus* (Campbell *et al.*, 1976) and *Pleuronectes platessa* (Wingfield and Grimm, 1977). Two maxima (about 7 ng/ml) have been recorded in *Gobius niger*, one before and the other during the breeding season (Bonnin, 1979).

ii. 11-Ketotestosterone. When both testosterone and 11-ketotestosterone are measured during spermiation, 11-ketotestosterone is always quantitatively preponderant in *Oncorhynchus nerka* (Schmidt and Idler, 1962), in *Salmo salar* (Idler *et al.*, 1971; Stuart-Kregor *et al.*, 1981), in *Pseudopleuronectes americanus* (Campbell *et al.*, 1976), in *Salmo gairdneri* (Campbell *et al.*, 1980; Scott *et al.*, 1980a; Kime and Manning, 1982), and in *Salvelinus fontinalis* (Sangalang and Freeman, 1974). In the two latter species, testosterone reached its maximum before 11-ketotesterone. In *Salmo salar* (Idler *et al.*, 1981) and *Salmo trutta* (Kime and Manning, 1982), the variations of plasma 11β-hydroxytestosterone levels parallel those of 11-ketotestosterone, although they remain lower. Levels of 11-ketotestosterone are correlated with the male gonadosomatic index (Simpson and Wright, 1977; Sangalang and Freeman, 1977; Idler *et al.*, 1981), and rise slowly during spermatozoa production, increasing sharply at the end of the cycle (Idler *et al.*, 1971; Campbell *et al.*, 1976; Sangalang and Freeman, 1974; Scott *et al.*, 1980a; Fostier *et al.*, 1982). Therefore, 11-ketotestosterone has been used, with success, to identify the sexes in the maturing trout species (Sangalang and Freeman, 1977; Simpson and Wright, 1977) in cod and tuna (Sangalang *et al.*, 1978) and in the juvenile trout after one GtH injection (Le Bail *et al.*, 1983).

iii. Glucuronides. In Atlantic salmon, 11-ketotestosterone and 11β-hydroxytestosterone glucuronides (mainly present in testicular plasma) are found in higher levels in the more mature males. In *Gobius niger*, the

concentration of testosterone glucuronide is not always parallel to free testosterone, indicating a possible regulation of the glucuronidation. The highest concentrations are found during the spawning period, concomitantly with the second free testosterone peak (Bonnin, 1979). In the brown trout, plasma androgens glucuronides peak during spermiation, at the same period as 11-ketotestosterone (Kime and Manning, 1982).

iv. Role of Androgens in Spermatogenesis. The effects of androgens on spermatogenesis have been investigated in some species with variable results which were dependent on the stage of the sexual maturity, on the species, and on the methodology used. In intact *Cymatogaster aggregata*, Wiebe (1969) failed to stimulate spermatogenesis of the regressed testis by adding methyltestosterone to the aquarium water; indeed, some suppression of spermatogonial mitoses were noticed. Pycnosis of spermatogonia was also reported when male trout were injected for 1 month with 4-chlorotestosterone acetate during the sexually inactive season (Hirose and Hibiya, 1968). Oral administration (50 μg/g of food) of methyltestosterone has a degenerative effect on germ cells in *Oncorhynchus* species (Yamazaki, 1972). These results suggest inhibition of GtH secretion. Although intraperitoneal injections of testosterone in rainbow trout stimulated GtH secretion during sexual quiescence (in March), they depressed it at the beginning of spermatogenesis (June) (Billard, 1978). Only one injection of testosterone in carp was followed, 30 min later, by a decrease of plasma GtH level (Weil, 1981). However, neither detectable decrease of GtH pituitary content nor decrease of pituitary sensitivity to LHRH were observed. Prolonged testosterone treatments in *Misgurnus anguillicaudatus* (Ueda and Takahashi, 1980) and in *Poecilia latipinna* (Van Den Hurk and Testerink, 1975) resulted in gonadotroph inactivation. Moreover, a loss of anti-GtH binding to these cells was noted in *Poecilia latipinna* (Goos *et al.*, 1976). However, the inhibition of spermatogenesis in rainbow trout fed with methyltestosterone is not accompanied by a reduction of the plasma GtH level, suggesting that steroids could also have a direct inhibiting effect on the testis (Billard *et al.*, 1981). Besides, if antiandrogens (cyproterone acetate or oxymetholone) are given continuously in the diet of male rainbow trout before the initiation of spermatogenesis, the testicular growth is prevented (Billard, 1982).

Although inhibiting effects are obtained in intact fish, qualitative restoration of a complete spermatogenesis, with repetitive testosterone or methyltestosterone injections, has been reported in the hypophysectomized *Fundulus heteroclitus* (Burger, 1942; Pickford and Atz, 1957; Lofts *et al.*, 1966) and *Heteropneustes fossilis* (Sundararaj and Nayar, 1967; Sundararaj *et al.*, 1971). The restoration was limited to the spermatocyte stage in *Carassius auratus* after hypophysectomy during the breeding season (Pandey, 1969b),

and no effect was observed in methallibure-treated *Tilapia nigra* (Hyder *et al.*, 1974). Maintenance of the established spermatogenesis has also been demonstrated in hypophysectomized *Heteropneustes fossilis* (Nayyar *et al.*, 1976) and *Carassius auratus* (Billard, 1974); in these studies, high doses were often used and the efficiency of the treatment was poor. In a maintenance study of goldfish (Billard, 1974), it was necessary to use a high dose of testosterone propionate to approximate the testicular weight of the intact fish; quantitative analysis of spermatogenesis shows that the number of spermatogonia type B and spermatids is much lower than in the intact control. A low dose of testosterone (10 μg/g by weight) maintained only spermatogonia type B, and a higher dose (200 μg/g by weight) was necessary to maintain spermatocytes and spermatids (Billard, 1974). This may be because of the blood–testis barrier of meiotic cysts (Marcaillou and Szöllösi, 1980; Abraham *et al.*, 1980), particularly if one considers that "the spermatogenetic maturation of germ cells takes place in a milieu of high local androgenic concentration" (Lofts, 1980). Further evidence of the direct role of androgens in spermatogenesis was furnished by *in vitro* culture of testicular explants of *Carassius auratus*, in which maintenance and even initiation of a complete spermatogenesis were observed in the presence of testosterone isobutyrate crystals (Remacle *et al.*, 1976, 1977). Furthermore, testosterone stimulated the protein and RNA synthesis *in vitro* of the rainbow trout testis, suggesting a stimulation of cellular mitoses (Costa, 1972).

v. Role of Androgens in Spermiation. Late in the cycle, injections of 11-ketotestosterone in the sockeye salmon (Idler *et al.*, 1961c), of methyltestosterone in mullet (Shehadeh *et al.*, 1973), and testosterone, but not 11-ketotestosterone, in rainbow trout (Billard *et al.*, 1981) were efficient in stimulating spermiation in nonhypophysectomized fish, but androgen implants (in silastic) were inefficient in the rainbow trout (Billard *et al.*, 1982). The fact that, at this stage in rainbow trout, testosterone exerts negative feedback on GtH secretion (Billard, 1978) and furthermore stimulates spermiation in the hypophysectomized goldfish (Yamazaki and Donaldson, 1969; Billard, 1976) suggests a direct action of androgens on the testis. Moreover, 11-ketotestosterone levels, but not GtH levels, are related to the sperm production in rainbow trout (Fostier *et al.*, 1982).

b. Other Steroids. Measurements by RIA show only low levels or even traces of 17β-estradiol in males of *Carassius auratus* (Schreck and Hopwood, 1974), *Salmo gairdneri* (Schreck *et al.*, 1973; Billard *et al.*, 1978; Whitehead *et al.*, 1978a), *Salmo trutta* (Billard *et al.*, 1978; Soivio *et al.*, 1982), *Sarotherodon aureus* (Terkatin-Shimony and Yaron, 1978), and *Salmo salar* (Idler *et al.*, 1981), but in *Pleuronectes platessa* the levels were higher (Wingfield and Grimm, 1977). However, estradiol was ineffective on testis

explants from goldfish cultured *in vitro* (Remacle, 1976). Prolonged treatment with a low dose of estradiol administered via the diet (0.5 mg/kg) inhibited spermatogenesis without modifying plasma GtH levels (Billard *et al.*, 1981). Later, during spermiation, a transitory rise of GtH occurred with such a treatment, and there was no significant effect on sperm production. However, a higher dose (2.5 μg/g by weight), injected intraperitoneally, depressed GtH levels (Billard, 1978). The presence of aromatase activity in the brain and pituitary together with estradiol binding in these areas (see Section IV,B) are in accordance with a regulation of pituitary activity.

The significant production of progestins, especially 17α-hydroxy-20β-dihydroprogesterone reported in trout testis is puzzling (see Table IV). During the breeding season, low plasma levels of these hormones have been measured in the rainbow trout by Campbell and co-workers (1980), but higher levels were found by Scott and Baynes (1982) in the same species and by Schmidt and Idler (1962) in the sockeye salmon. Progesterone was the most efficient, in comparison with any other tested steroid, in stimulating spermiation of the hypophysectomized goldfish (Billard, 1976) or the intact pike, *Esox lucius* (De Montalembert *et al.*, 1978).

Cortisol, which may be high during the breeding season (Campbell *et al.*, 1976; Wingfield and Grimm, 1977; Pickering and Christie, 1981), stimulated spermiation in intact or hypophysectomized goldfish (Billard, 1976), but had no effect on the efferent duct system and spermatogonial multiplication in hypophysectomized *Fundulus heteroclitus* (Lofts *et al.*, 1968). To explain the high concentration found during gonad development in plaice, Wingfield and Grimm (1977) also hypothesized a role for cortisol in the metabolization of stored energy.

V. CONCLUDING REMARKS

The research on sex steroids in fish raises two categories of questions. One is concerned with the validity of analytical methodologies and the other with the nature of experimental procedure. Methodologies have been carefully discussed by Sandor and Idler (1979), and, according to their criteria, few definitive identifications of steroids have been made in fish species. As mentioned in the introduction, mass spectrometry could be an efficient tool for this purpose. However, this technique has been inadequate for measurements of numerous and small samples until now. For this purpose, the use of rapid resolutive separating techniques, i.e., high-performance liquid chromatography may be a useful tool, if associated with radioimmunoassays, or

sensitive enough physical detectors. With respect to experimental procedures, *in vitro* incubations of steroidogenic organs with labeled precursors contribute very important information. However, they have not been corroborated with analysis of the steroids actually present in the glandular tissue or in efferent blood. The measurements of steroid levels are almost always performed in the peripheral blood. These measurements are useful as an indicator of a steroidogenic secretion during a particular stage of the sexual cycle, and they are necessary to characterize the hormonal signal to which peripherial target tissues respond. Nevertheless, they give poor indications of the hormonal environment of target cells such as germ cells situated close to the glandular structure. An illustrative example could be the study of the gametogenesis regulation by steroids in simultaneous hermaphrodites induced in gonochoric species (Jalabert *et al.*, 1975). Recent results (McGregor, 1983; Zohar, 1982; Zohar *et al.*, 1982b) have shown that rhythms in plasma GtH may be associated with rhythms in plasma steroids, as already demonstrated in mammals (Rebar and Yen, 1979). These results add to the knowledge of the regulation of steroidogenesis and physiological steroid activity. However, they imply the need to develop new experimental methodologies (Zohar, 1980; Bry and Zohar, 1980). From a physiological viewpoint, many questions still remain without conclusive answers. Some are as follows:

1. In fry, if sex steroids are actually natural inducers of gonadal differentiation, are they the earliest signal?
2. Later in the immature fish, how is steroidogenesis regulated before the release of GtH in plasma?
3. Then in the adult fish, does only one glycoprotein, GtH, control the whole steroidogenesis process?
4. What is the role of other pituitary factors?
5. Are steroids involved in the stimulation of new waves of gonia mitoses?
6. Have they a direct effect on the previtellogenic ovarian follicle?
7. Are estrogen only working outside the ovary during vitellogenesis?
8. Is there any action on follicular cells development?
9. What is the role of the high levels of testosterone at this stage?
10. In males, 11-ketotestosterone has been shown to stimulate sex secondary characters, but what is its accurate role on germ cells maturation?
11. What is the significance of the testicular biosynthesis of 17α-hydroxy-20β-dihydroprogesterone?
12. Is glucuronidation only an inactivating process or do glucuronides possess biological activities?

If these few questions show the importance of efforts to investigate the field of gonadal steroids in fish, they also show the originality of the fish

physiology. Its study can bring progress in techniques for fish aquaculture, but also give substance for phylogenic considerations (Tamaoki, 1981) and contributions to general physiology.

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8

YOLK FORMATION AND DIFFERENTIATION IN TELEOST FISHES

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I. INTRODUCTION

Discussion in this chapter is restricted to teleost fishes, but a large base of knowledge has come from studies on other nonmammalian vertebrates, in particular the hen and *Xenopus*. Wallace and co-workers have made outstanding contributions to the regulation of oocyte growth, first in Xenopus and more recently in fish, and have published an excellent review of ovarian growth throughout the nonmammalian vertebrates (Wallace, 1978). Expression of the vitellogenin genes in eukaryotic organisms and the relationship between vitellogenin and egg yolk proteins has been discussed by Tata and Smith (1979), and there are recent reviews of the stages of ovarian

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growth and vitellogenesis in teleosts (Wallace and Selman, 1981; Wiegand, 1982).

II. YOLK PROTEINS

A. Purification

It is a well established fact that the ovaries of oviparous vertebrates, including fowl and amphibians, contain yolk proteins which are termed lipovitellin and phosvitin (see Follett and Redshaw, 1974). A number of investigators have attempted to isolate lipovitellinlike and phosvitinlike yolk proteins from the ovaries of a variety of teleost species such as the herring (Barman *et al.*, 1964), trout (Ando, 1965; Campbell and Idler, 1980; Ito *et al.*, 1966; Wallace *et al.*, 1966), Pacific salmon (Markert and Vanstone, 1968), Atlantic salmon (Idler *et al.*, 1979), and cod (Plack *et al.*, 1971). The yolk proteins are extracted from the entire ovaries with a concentrated inorganic salt solution, e.g., 0.5 *M* NaCl (Wallace *et al.*, 1966; Jared and Wallace, 1968; Markert and Vanstone, 1968; Plack *et al.*, 1971; Campbell and Idler, 1976, 1980; Idler *et al.*, 1979), 0.2 *M* NaCl (Mano and Lipmann, 1966a), or 1.2 *M* $MgSO_4$ (Barman *et al.*, 1964). Centrifugation of the extract at high speed is then performed to remove debris and fatty material. Alternatively, ovarian fluid and blood clots can be removed from the ovary by rinsing with 1% saline. The eggs are broken and the contents are collected (Markert and Vanstone, 1968). The yolk proteins are then precipitated as a lipovitellin–phosvitin complex by dialysis against, or dilution with, a large volume (5–7 volumes) of distilled water. Lipovitellin can be separated from phosvitin by addition of $(NH_4)_2SO_4$ to 100% saturation to a solution of yolk proteins at 0° C (Barman *et al.*, 1964; Schmidt *et al.*, 1965; Wallace *et al.*, 1966; Markert and Vanstone, 1968; Plack *et al.*, 1971; Campbell and Idler, 1980). Lipovitellin is precipitated and can be further purified by being redissolved in NaCl and reprecipitated by adding $(NH_4)_2SO_4$. Phosvitin remains in solution and can be further purified by dialysis against distilled water, precipitation by acidification, successive washes with 0.04 *M* HCl, alcohol–ether (3:1 v:v) and ether, air drying, followed by chromatography on diethylaminoethyl (DEAE)-cellulose using Tris-Cl–ethylenediaminetetracetate (EDTA) buffer as eluent. Alternatively, the crude phosvitin in solution can be further purified by dialysis against distilled water and precipitation by adding 5 m*M* $CaCl_2$, followed by a wash with water and ethanol and drying under vacuum (Barman *et al.*, 1964).

Phosvitin and lipovitellin can be further purified by gel filtration on Ultrogel AcA 22, Ultrogel AcA 54 or Sephadex G200 (Campbell and Idler, 1980). Livetins can be isolated from the fraction of the ovarian extract which is not precipitated on dilution with an excessive amount of distilled water (Markert and Vanstone, 1968; Jared and Wallace, 1968). They are not phosphoproteins, and are probably a group of serum-derived proteins with distinct electrophoretic mobilities. Their number and concentration vary from one species to another (Markert and Vanstone, 1968). The β-component is extractable with 0.5 *M* NaCl and is coprecipitated with lipovitellin when $(NH_4)_2SO_4$ is added to saturation. It can be separated from lipovitellin by exclusion chromatography in which β component behaves as a smaller molecule (Campbell and Idler, 1980).

B. Characteristics

1. Solubility

Besides being soluble in concentrated salt solutions, such as 0.5 *M* NaCl, the yolk proteins are also soluble in citric acid–Monol (2-amino-2-methyl-1-propanol) buffer (Jared and Wallace, 1968), which is used for triethylaminoethyl (TEAE)-cellulose chromatography. Trout phosvitin is distinctive in that it is not precipitated by 15% trichloracetic acid which precipitates contaminating proteins in the phosvitin preparation (Schmidt *et al.*, 1965; Campbell and Idler, 1980).

2. Chemical Characteristics

The yolk proteins can be separated by hydroxyapatite chromatography using phosphate buffer as eluent. Livetins are unadsorbed on the ion exchanger equilibrated and eluted with 0.2 *M* phosphate buffer. Lipovitellin and phosvitin are adsorbed and can be eluted from the column by increasing the ionic strength to 0.6 *M* and 2 *M*, respectively (Markert and Vanstone, 1968).

In general, lipovitellins are glycolipophosphoproteins with a very low content of alkali-labile phosphorus; phosvitins are rich in phosphorus but lacking in lipid and carbohydrate. However, some glucosamine is found in salmonid phosvitin (Mano and Yoshida, 1969). A lipid content of 25% and an alkali-labile phosphorus content of 0.007% are found for trout lipovitellin; phosvitin has an alkali-labile phosphorus content of 15.8% (Campbell and Idler, 1980). The lipid content in trout lipovitellin is similar to that estimated by other researchers (Plack *et al.*, 1971); phosvitin has a higher and lipovitellin has a lower alkali-labile phosphorus content than previously reported

values (Mano and Lipmann, 1966a,b; Wallace *et al.*, 1966). The discrepancy may have been caused by differences in the degree of purity of the various preparations (Campbell and Idler, 1980).

Trout lipovitellin (Campbell and Idler, 1980) is similar in amino acid composition to that of *Xenopus laevis* lipovitellin (Redshaw and Follett, 1971) except for its serine and valine contents. Trout lipovitellin has a molecular weight (MW) of about 300,000 according to Hara and Hirai (1978) and Campbell and Idler (1980), but Ando (1965) found a value of MW 240,000.

The molecular weight of trout phosvitin has been reported to be 43,000 (Campbell and Idler, 1980) and 19,000 (Schmidt *et al.*, 1965; Mano and Yoshida, 1969). The β component has a MW 21,000 (Campbell and Idler, 1980; Markert and Vanstone, 1971).

The presence of two types of lipovitellin in the cod ovary, which cannot be separated by Sephadex G200 chromatography but which can be partially resolved by TEAE-cellulose chromatography, is claimed by Plack and Fraser (1970). There are indications that trout lipovitellin is composed of two subunits (Ando, 1965). Mobility during sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis indicated two subunits for lipovitellin of goldfish with MW of 105,000–110,000 and 19,000–25,000 (de Vlaming *et al.*, 1980). The ayu lipovitellin binds calcium (Aida *et al.*, 1973a).

In salmonid phosvitins (Mano and Yoshida, 1969; Campbell and Idler, 1980) and ling phosvitin (Mano and Lipmann, 1966a), serine is the predominant amino acid accounting for as much as 42% of the amino acid content and it exists in the phosphorylated form, phosphoserine. Sulfur-containing amino acids such as half-cysteine and methionine, aromatic amino acids such as tyrosine and phenylalanine and histidine, and the amino acids valine and leucine, are either absent or present in very low amounts. These characteristics of teleost phosvitins are mostly common to toad and chicken phosvitins (Clark and Joubert, 1971; Redshaw and Follett, 1971). In the salmonid phosvitin, tyrosine is the NH-terminal residue and aspartic acid is the C-terminal residue (Mano and Yoshida, 1969).

Interestingly, Schmidt *et al.* (1965) found that *Salmo trutta* phosvitin resembled hen phosvitin more than *Salmo irideus* phosvitin isolated by Ito *et al.* (1966). Ovaries from winter flounder *Pseudopleuronectes americanus* extracted with 0.5 *M* NaCl–5 m*M* EDTA followed by precipitation on dialysis against a copious volume of 5 m*M* $CaCl_2$, when redissolved and fractionated by gel filtration, yielded a peak of MW 500,000 and a peak of MW 30,000 both containing alkali-labile phosphorus, corresponding to lipovitellin and phosvitin, respectively (Ng and Idler, 1979). Trout phosvitin loses its phosphorus in molar NaOH or Na_2CO_3 in 12 min (Barman *et al.*, 1964). Teleost phosvitins absorb ultraviolet (UV) light at 280 nm like other vertebrate phosvitins (Wallace *et al.*, 1966).

It is speculated that phosvitin represents a reservoir of phosphorus and is therefore an energy source for embryonic development (Barman *et al.*, 1964; Ando, 1965). Phosvitin is not found in eggs of some marine teleosts, and it appears that phosphorus needed for metabolic purposes is derived from the environment (Jared and Wallace, 1968).

Mano and Lipmann (1966a,b) observed that there are several forms of ling, cod, and flounder phosvitins with varying degrees of phosphorylation which can be separated by chromatography on DEAE-cellulose. These fractions have the same amino-acid composition and therefore they are probably produced by stepwise dephosphorylation and phosphorylation. However, salmon phosvitin yields a single peak on DEAE-cellulose.

At the beginning of vitellogenesis nonphosphorylated phosvitin is incorporated by the oocytes. This is then phosphorylated to yield the final form containing 10% phosphorus without passage through any intermediate forms. During the course of vitellogenesis three phosvitins with intermediate phosphorus contents appear and they are eventually phosphorylated to the final product. Phosphorylation of nonphosphorylated and the three partially phosphorylated phosvitin fractions to give the final fully phosphorylated form is accomplished by distinct protein kinases which have optimal activities at different levels of phosphorylation of the acceptor phosvitin molecule. However, the enzyme phosphoprotein phosphatase is not involved in the formation of these phostivin fractions with different degrees of phosphorylation. Accumulation of the final fully phosphorylated form of phosvitin provides a signal for the formation of the intermediate partially phosphorylated fractions (Mano, 1969, 1970).

Mano and Lipmann (1966b) observe that the reactivity of teleost phosvitin as an acceptor of phosphate from protein phosphokinase and adenosine triphosphate decreases after enzymatic dephosphorylation in contrast to the case of hen phosvitin. The arrangement of four consecutive phosphorylated serine residues appears to confer specificity on the phosphate acceptor.

Autoxidative degradation in the presence of ferrous salt leading to labilization and liberation of phosphate (Grant and Taborsky, 1966) may occur in fish phosvitin (Mano and Lipmann, 1966a,b), with the protein-bound phosphoserine undergoing α,β dehydrogenation to form the corresponding enol phosphate derivative.

The amino acid composition of trout β component is distinct from those of vitellogenin, lipovitellin, and phosvitin. However, it is related immunologically to vitellogenin and lipovitellin (Campbell and Idler, 1980). These characteristics of β component indicate that it is a protein different from vitellogenin, lipovitellin, and phosvitin. It may be a heterosynthetic or an autosynthetic yolk protein (Campbell and Idler, 1980).

Leger and associates (1981) provided evidence that essential fatty acids

"22:6 (n − 3)" are enriched in trout lipoproteins, vitellogenin, egg lipovitellin, and oil globules.

C. Differences from Other Vertebrates

The ovarian lipovitellin and phosvitin of most vertebrates are complexed together in the form of crystalline platelets. However, most teleost eggs contain a nongranular fluid yolk and the yolk proteins are present in fluid-filled spheres together with the other egg components. However, Yamamoto and Oota (1967) observed in the zebrafish that its ovarian yolk is deposited in a crystalline structure, although the crystalline pattern is distinct from the amphibian pattern. During the terminal stages of oocyte maturation the egg yolk coalesces and mixes with the other egg contents.

The teleost yolk proteins are more soluble in water than those from other vertebrates because of their lower level of phosphorylation. A lower protein kinase activity is detected in the acetone powder of some fish ovaries. Heterogeneity in the extent of phosphorylation is frequently observed among teleost yolk proteins (Jared and Wallace, 1968).

Some workers found that teleost phosvitin has a molecular weight which is lower than that of other vertebrate phosvitins (Schmidt *et al.*, 1965; Wallace *et al.*, 1966; Markert and Vanstone, 1971), and it may sometimes be absent from the ovarian yolk (Jared and Wallace, 1968). Teleost lipovitellin possesses a very low amount of phosphorus (Campbell and Idler, 1980). Some yolk components distinct from lipovitellin and phosvitin can be found in the teleost ovary, e.g., β component (Markert and Vanstone, 1971; Campbell and Idler, 1980). The interesting work of Sand and others on ovarian wax esters was reviewed by Wiegand, (1982).

III. VITELLOGENIN

Vitellogenin is a word coined by Pan *et al.* (1969). Changes in the plasma electrophoretic pattern occur parallel to the progress of vitellogenesis. The most conspicuous change observed is the appearance of a lipophosphoprotein identified as vitellogenin (Campbell and Idler, 1980; Emmersen and Petersen, 1976; Heesen and Engels, 1973; Thurston, 1967). Immunological methods have also been used to detect the presence of vitellogenin (Aida *et al.*, 1973a; Fine and Drilhon, 1963; Utter and Ridgeway, 1967). Because the occurrence of vitellogenin is specific to the female, vitellogenin is also known as the female-specific plasma protein (Aida *et al.*, 1973a). Its occurrence in plasma of maturing females coincides with the appearance of yolk granules beneath the vitelline membrane (Aida *et al.*, 1973a).

A. Isolation

Methods for isolation of vitellogenin from plasma of *Xenopus* have been reviewed by Wallace and co-workers (Wiley *et al.*, 1979). They no longer advocate the use of TEAE-cellulose because the product did not behave as did a former preparation. This is reminiscent of experiences with other preparations, e.g., XAD-2 (Bradlow, 1977). The TEAE-cellulose (Cellex T, Bio-Rad) was used to prepare salmon vitellogenin (Idler *et al.*, 1979). If Ca^{2+} is used for precipitating vitellogenin, the preparation should be checked for possible degradation by Ca^{2+}-activated protease (Wiley *et al.*, 1979). For *Xenopus* a combination of Mg^{2+} and EDTA was satisfactory for precipitating a relatively undegraded vitellogenin. However, for goldfish vitellogenin the combination was ineffective, possibly because of the relatively low phosphorus content of the glycolipophosphoprotein (de Vlaming *et al.*, 1980).

1. Atlantic Salmon

Blood from a mature female is treated with $CaCl_2$, diluted with distilled water, and then dialyzed against 5 m*M* $CaCl_2$. The precipitate is extracted with 0.5 *M* NaCl–5 m*M* EDTA. Chromatography of the yolk extract on TEAE-cellulose yields a single peak which is fractionated into three peaks on Bio-Gel with Tris-NaCl buffer. The middle peak is identified as vitellogenin by its immunoreactivity toward an antiserum raised against a fraction of ovarian yolk proteins retarded on Sephadex G200 (Idler *et al.*, 1979).

2. Rainbow Trout

The starting material is serum from male trout treated with estradiol. The serum is treated with $CaCl_2$ until a final concentration of 20 m*M* is attained. The serum is then added to 30 volumes of cold, distilled water saturated with phenylmethylsulfonyfluoride (PMSF), stirred overnight, and centrifuged. The precipitate is dissolved in 10 ml buffer (0.25 *M* NaCl, 20 m*M* $CaCl_2$, 50 m*M* Tris, pH 8.0). Cold, distilled water is then added to the solution to dilute the NaCl concentration to 5 m*M*. The resulting precipitate is redissolved in buffer and chromatographed on TEAE-cellulose which is eluted with a linear gradient of citric acid–Monol buffer. The protein is eluted as a single peak and can be further purified on Ultrogel AcA 22. However, TEAE-cellulose chromatography can be omitted to minimize losses attributable to the solubility problem (Campbell and Idler, 1980). The integrity of vitellogenin prepared by this method is attested by the parallel displacement given by serum of estrogenized fish to the vitellogenin standard used in radioimmunoassay (Campbell and Idler, 1980) and incorporation into oocytes (Campbell, 1978; Campbell and Jalabert, 1979). Vitello-

genin can also be isolated from the blood of estrogenized trout by gel filtration on Sepharose 6B followed by affinity chromatography on Con A–Sepharose (Sumpter, 1982).

Vitellogenin messenger ribonucleic acid (mRNA) has been isolated from liver of bullhead and rainbow trout (Roach and Davies, 1980; Chen *et al.*, 1982).

3. Catfish

Serum from estradiol-treated *Ictalurus nebulosus* is chromatographed on DEAE-cellulose equilibrated and eluted with dilute citric acid–Monol buffer. Vitellogenin is eluted with buffer of high ionic strength. Phenylmethylsulfonylfluoride is used to inhibit proteolysis during isolation. In the absence of the protease inhibitor, vitellogenin is nicked by proteolysis as judged by the presence of numerous bands in SDS–polyacrylamide gel electrophoresis (Hickey and Wallace, 1974).

Vitellogenin can also be prepared from the serum of mature female or estrogen-treated male flounder *Platichthys flesus* (Emmersen and Petersen, 1976) and catfish *Heteropneustes fossilis* (Nath and Sundararaj, 1981a) by gel filtration.

4. Goldfish

Vitellogenin has been isolated from goldfish blood by Hori *et al.*, (1979) and de Vlaming *et al.* (1980). The latter chromatographed on DEAE-cellulose and used PMSF to inhibit proteolysis.

B. Characteristics

Vitellogenin is inducible in immature and male teleosts by estrogen treatment (Aida *et al.*, 1973a; Emmersen and Petersen, 1976; Nath and Sundararaj, 1981a; Campbell and Idler, 1980). Its production can be greatly augmented in the females by estrogen treatment (Bailey, 1957; Campbell and Idler, 1976).

In oviparous vertebrates, vitellogenin undergoes posttranslational modification including lipidation, glycosylation, and phosphorylation before being secreted into the blood (Tata and Smith, 1979). The lipophosphoprotein nature of teleost vitellogenin has been demonstrated by Emmersen and Petersen (1976), Hori *et al.* (1979), Campbell and Idler (1980), and by Nath and Sundararaj (1981a). It contains carbohydrate and it binds calcium (Hori *et al.*, 1979). The calcium content of goldfish vitellogenin is only one-third of that of *Xenopus laevis* vitellogenin, probably because of losses during experi-

mental manipulation (Hori *et al.*, 1979). Rainbow trout vitellogenin resembles vitellogenins from *Xenopus laevis* (Redshaw and Follett, 1971) and *Gallus domesticus* (Cristmann *et al.*, 1977) in amino acid composition except for differences in serine and alanine contents. Similar chemical structure between vitellogenin and egg yolk protein has been demonstrated in the catfish by peptide mapping and SDS gel electrophoresis (Sundararaj, 1981). Analytical ultracentrifugation of trout vitellogenin gave peaks of 11.6 S, 18.9 S and 23.8 S (Campbell and Idler, 1980) suggesting that polymerization occurs during experimental manipulation, as in the case of toad vitellogenin (Redshaw and Follett, 1971). A range of molecular weights has been reported for teleost vitellogenins: 330,000 and 280,000 for goldfish (Hori *et al.*, 1979; de Vlaming *et al.*, 1980), 390,000 for Coho salmon (Markert and Vanstone, 1971), 400,000 for cod (Plack *et al.*, 1971), 470,000 for trout (Campbell and Idler, 1980), 550,000 for flounder (Emmersen and Petersen, 1976) and catfish (Nath and Sundararaj, 1981a), and 600,000 for trout (Hara and Hirai, 1978). Vitellogenin of the catfish *Amerieurus nebulosus* is reported to have a molecular weight for the delipidated monomer of 145,000 by SDS-Polyacrylamide gel electrophotesis PAGE (Roach and Davies, 1980).

Trout vitellogenin has a lipid content of 21.5% and an alkali-labile phosphorus content of 0.6% (Campbell and Idler, 1980).

Hen vitellogenin has a MW 235,000 (Christmann *et al.*, 1977) and *Xenopus laevis* vitellogenin has a MW 390,000 (Redshaw and Follett, 1971); therefore, some teleost vitellogenins (Campbell and Idler, 1980; Emmersen and Petersen, 1976) appear to be larger molecules than avian and amphibian vitellogenins. Van Bohemen *et al.* (1981) suggest that trout vitellogenin exists in forms with different degrees of phosphorylation. Campbell and Idler (1976) find that in sham-hypophysectomized winter flounder some dephosphorylation of vitellogenin occurs during incorporation into ovarian yolk.

C. Measurement

Various methods have been utilized to estimate plasma vitellogenin concentration. The level in the pike *Esox lucius* was estimated to be 1–48 mg/ml by quantitative immunoelectrophoresis (Goedmaker and Verbroom, 1974) and 32 mg/ml in maturing female cod by indirect semiquantitative immunodiffusion (Plack *et al.*, 1971). There were 9 mg/ml in sockeye salmon *Oncorhynchus nerka* by the difference in serum protein content between males and females (Ho and Vanstone, 1961). In flounder *Platichthys flesus*, by alkali-labile phosphorus determination (Emmersen and Petersen, 1976) 5–180 μg PO_4^{-3}/ml were found, and in catfish *Heteropneustes fossilis* (Nath

and Sundararaj, 1981a) values ranged to 900 μg PO_4^{-3}/ml. Vitellogenin levels of 0.1–13 mg/ml were detected in trout *Salmo gairdneri* by densitometric scanning following polyacrylamide gel electrophoresis (van Boheman *et al.*, 1981), and 32 mg/ml in goldfish *Carassius auratus* by densitometry following polyacrylamide gel electrophoresis (Hori *et al.*, 1979). It is not always clear whether alkali-labile phosphorus is expressed as P or PO_4^{-3}.

Vitellogenin-enriched serum from estradiol-treated goldfish forms a single connecting precipitin arc with ovarian extract and serum of mature goldfish (Hori *et al.*, 1979). Immunological relationship between vitellogenin and ovarian extract has also been demonstrated in other teleost species (Utter and Ridgeway, 1967; Plack *et al.*, 1971; Amirante, 1972; Aide *et al.*, 1973b; Hara and Hirai, 1978; Idler *et al.*, 1979). This fact has been used by Idler *et al.* (1979) and Campbell and Idler (1980) to develop radioimmunoassays for measurement of circulating vitellogenin in salmonids. The assay for vitellogenin of Atlantic salmon *Salmo* is set up, using an antiserum raised against the fractions of the ovarian yolk extract which are retarded on Sephadex G200, and the antigen as label and standard. A standard curve can be established from 0.05 to 10 ng. Despite a parallelism shown by a serial dilution of Atlantic salmon plasma to the standard curve, it requires 47-fold more vitellogenin than the ovarian yolk standard to give the same displacement. Analysis of a small plasma sample enables a male donor to be distinguished from a female donor (Idler *et al.*, 1979, 1981) and also spawners of the year from nonspawners several months in advance of spawning (Idler *et al.*, 1981). The plasma vitellogenin content rises from 5 μg/ml in fish caught 14 months away from spawning to a value of 6 mg/ml in animals 1–2 months prior to spawning. There is a significant correlation between the stage of gonadal development and the plasma vitellogenin concentration, the plasma estrogen level and the plasma vitellogenin level, the plasma estrogen titers and the gonadosomatic index, and the stage of gonadal development and the gonadosmatic index, indicating that estrogen and vitellogenin are involved in gonadal development and maturation and increase in gonadal size (Idler *et al.*, 1981). The validity of the salmon assay was questioned by de Vlaming *et al.* (1980), but the plasma levels found in the two sexes and in different physiological states (Idler *et al.*, 1981) confirm the utility of the assay. Ideally one would wish to use a completely homologous assay, but the failure to label vitellogenin with radioactive iodine with any method then available prevented this (see further discussion).

The assay for vitellogenin of trout *Salmo gairdneri* is set up using an antiserum against trout vitellogenin and labeled trout lipovitellin. Lipovitellin, vitellogenin β component, and serum give parallel displacement to the label; however, trout phosvitin does not displace the label. A similar

assay can be conducted using an antiserum against trout vitellogenin and labeled trout β component. In this assay system, trout vitellogenin, lipovitellin, and serum all give displacement parallel to the label. The three types of assay buffer investigated including barbital buffer, a low-salt buffer, and a buffer of 0.25 M NaCl, 20 m*M* $CaCl_2$, 50 m*M* Tris, pH 8.0, exert no influence on the sensitivity of the assay or the slope of the displacement curve. However, under nonequilibrium condition the sensitivity of the assay is increased sevenfold (Campbell and Idler, 1980).

In both the Atlantic salmon and the trout vitellogenin radioimmunoassays, difficulty has been experienced in iodinating vitellogenin with the conventional chloramine T method (Greenwood *et al.*, 1963), or with the microelectrolytic method (Donabedian *et al.*, 1972), but the yolk proteins, phosvitin in case of Atlantic salmon, and lipovitellin and β component in case of rainbow trout, can be easily iodinated (Idler *et al.*, 1979; Campbell and Idler, 1980).

Even in species as closely related as the Atlantic salmon and the rainbow trout, it is found that the vitellogenin radioimmunoassay is specific to each species. In the Atlantic salmon vitellogenin assay, no parallel displacement is given by trout vitellogenin or vitellogenic trout serum. In the trout vitellogenin assay, parallel displacement is given by serum samples from chinook (*Oncorhynchus tshawytscha*) and sockeye (*O. nerka*) salmon, but not by those from coho salmon (*O. kisutch*) and Atlantic salmon (*Salmo salar*) (Campbell and Idler, 1980). Species specificity is also a characteristic of the reptilian vitellogenin assay (Gapp *et al.*, 1979). Vitellogenin from other vertebrates cannot be measured with the hen vitellogenin radioimmunoassay (Redshaw and Follett, 1971).

Because of the possibility of loss of immunological potency of vitellogenin with purification (Redshaw and Follett, 1976), the results obtained from the vitellogenin assays (Campbell and Idler, 1980; Idler *et al.*, 1979) may not represent the absolute values but they permit valuable comparisons. Campbell and Idler (1980) estimate that the assay values are close to the absolute values because a plasma vitellogenin level greater than 5 mg/ml can achieve a daily increase in ovarian weight of about 1 g which can account for a gonadosomatic index of 15 at the end of 6 months of vitellogenic growth.

The advent of a very mild method for incorporating radioiodine into proteins and peptides "without damage" is a major contribution (Salacinski *et al.*, 1979); the reagent is Iodogen. Sumpter (1981) has used it to label rainbow trout vitellogenin and a homologous assay was developed. In this assay, immature female trout had low levels of vitellogenin. Some male trout also had low levels of vitellogenin, but the levels did not correlate with the degree of sexual maturity. In the maturing female, the level increased from a

value of 0.5 mg/ml in March to 50 mg/ml in December. Ng and Idler have labeled salmon and flounder vitellogenin with Iodogen and are now using a homologous assay for these species (Y. P. So and D. R. Idler, unpublished).

Van Bohemen and Lambert (1981) delineate the reproductive cycle of the rainbow trout into four periods: May–July, endogenous vitellogenesis; July–December, exogenous vitellogenesis; December, spawning time; December–March, postspawning and previtellogenesis. The plasma vitellogenin level is low during the period of endogenous vitellogenesis, rises to 0.5 mg/ml at the beginning of exogenous vitellogenesis and continues to increase throughout the period of exogenous vitellogenesis, attaining a level of 2 mg/ml during spawning. By March, the plasma vitellogenin titer increased to 5 mg/ml, probably because of a combination of continued hepatic synthesis and a lack of gonadal incorporation. There is a correlation between the plasma levels of estrogen and vitellogenin during vitellogenic growth of the ovary; during previtellogenesis and endogenous vitellogenesis the estrogen level is only 1 ng/ml, but it increases to a value of 17 ng/ml during exogenous vitellogenesis (van Bohemen and Lambert, 1981). In the catfish *Heteropneustes fossilis*, the plasma vitellogenin and estrogen titers increased with ovarian vitellogenic growth. The estrogen level declined to a basal level about 1 month before spawning and the vitellogenin concentration fell to an undetectable level after spawning (Lamba, 1981).

D. Mechanism of Vitellogenesis

Bailey (1957) proposed a hypothesis for the mechanism of vitellogenesis in the goldfish. Vitellogenin is synthesized in the liver, under stimulation of estrogens produced from the ovary, secreted into the circulation, and is transported to the ovary where it is taken up into the oocytes. The mechanism is analogous to that operating in the amphibian and fowl (see Follett and Redshaw, 1974). Evidence for such a mechanism was obtained in the zebrafish by Korfsmeier (1966). Injected labeled amino acids are first incorporated into hepatic proteins and the rate of incorporation is maximal 3 hr postinjection; subsequently the labels appear in ovarian proteins and the process of incorporation still proceeds after 24 hr. Heesen and Engels (1973) observe the presence in the zebrafish ovary of a major lipophosphoprotein component which can also be located in the serum and the liver. Sundararaj and Nath (1981) have demonstrated that radiophosphate administered into catfish gets incorporated into hepatic phosphoproteins as early as 1 hr postinjection, the incorporation rate peaks at 12 hr postinjection after which labeled vitellogenin starts to appear in the circulation. Yolk proteins are detected in the liver, but not in the gonad of sexually immature cod after

estrogen treatment, indicating the hepatic source of vitellogenin (Plack and Fraser, 1970). Estrogen treatment promotes the incorporation of [^{3}H]leucine into hepatic yolk proteins in immature female and male cod *in vitro* (Plack and Fraser, 1970; 1971). Campbell (1978) demonstrated that oocytes from immature trout incorporate labeled vitellogenin. Campbell and Idler (1976) observe that radioactive phosphate injected into the flounder *Pseudopleuronectes americanus* occurs as labeled yolk proteins in plasma and ovary; their finding has been confirmed by Ng and Idler (1978a).

Ultrastructural evidence for ovarian incorporation of exogenous yolk by micropinocytosis is provided by Droller and Roth (1966) in the guppy *Lebistes reticulatus*, by Anderson (1968) in the pipefish *Syngnathus fuscus*, and by Wegmann and Götting (1971) in the swordtail *Xiphophorus helleri*. Droller and Roth (1966) suggest that there are two types of micropinocytic structures which incorporate different exogenous yolk material.

In the amphibian, vitellogenin is converted into yolk proteins by proteolysis (Bergink and Wallace, 1974). Catfish vitellogenin is very susceptible to proteolytic attack, implying that a mechanism similar to that in the amphibian operates in the teleost (Hickey and Wallace, 1974). The β component of salmonid ovarian yolk is believed to be derived from the plasma of mature females (Markert and Vanstone, 1971). Teleost vitellogenin, lipovitellin, and phosvitin exhibit similarities in amino-acid composition to the corresponding yolk proteins from other vertebrate classes, further supporting the operation of a similar mechanism of vitellogenesis (Campbell and Idler, 1980). Trout lipovitellin manifests a marked resemblance in amino-acid composition and lipid content to those of trout vitellogenin (Campbell and Idler, 1980). Idler and Campbell (1980) find that following treatment of male trout with estradiol the serum vitellogenin concentration increases at the expense of other proteins, but there is no gonadal growth. Evidence for selectivity of incorporation of macromolecules into the teleost ovary is provided by Campbell (1978) and by Campbell and Jalabert (1979).

E. Metabolic Changes during Vitellogenesis

Sensitivity of the processes of vitellogenesis and oocyte maturation to the nutritional state of the animal has been suggested by Wallace and Selman (1980). Petersen and Emmersen (1977) studied the variation in the plasma titers of several metabolites throughout the reproductive cycle of the flounder *Platichthys flesus*, and there was a correlation between the serum phospholipid level and the serum vitellogenin level throughout the reproductive cycle. Phospholipid is necessary for the biosynthesis of yolk proteins and oocyte membranes. Large amounts of glucose and lipids occur in

the circulation before ovarian vitellogenic growth begins and plasma concentrations decline during the early phase of vitellogenesis and then increase to a maximum at spawning after which the levels drop again.

The liver glycogen content does not bear a distinct relation to either ovarian growth or plasma glucose level. Liver glycogen content is the net result of the activities of the enzymes glycogen synthetase and glycogen phosphorylase; the plasma glucose level represents a balance between nutrition, hepatic supply, and tissue uptake. Hepatic glycogen and lipid probably represent energy sources to meet the metabolic needs encountered in ovarian growth and spawning (Petersen and Emmersen, 1977). An increase in the plasma level of lipids during vitellogenesis has been demonstrated in the cod *Gadus morhua* (Plack and Woodhead, 1966). This phenomenon is probably attributable to the lipid mobilizing effect of estrogen as shown in the amphibian (Follett and Redshaw, 1974).

The metabolism of protein, RNA, and DNA also increases with the progress of vitellogenesis in the flounder (Emersen and Emmersen, 1976).

F. Microscopic Changes in Ovary and Liver during Vitellogenesis

In trout, during endogenous vitellogenesis, the hepatocytes contain moderately developed rough endoplasmic reticulum, small Golgi bodies containing no electron-dense material, and an increased amount of cytoplasmic glycogen and lipid. There is no difference in the ultrastructure of the liver in the two sexes during this stage. During exogenous vitellogenesis the rough endoplasmic reticulum is strongly developed, the Golgi bodies are much enlarged with electron-dense inclusions, the mitochondria possess densely packed and concentrically arranged membrane configurations, the cytoplasm is depleted of glycogen granules and lipid droplets, and the nucleus and nucleolus present a hypertrophied appearance. All these ultrastructural changes suggest that the endoplasmic reticulum is involved in protein synthesis, that the Golgi is responsible for the packaging of secretion of these proteins, and that the mitochondrion strives to meet the energy demands of these processes. Concomitant with the increased activity of the liver is the enhancement in the steroidogenic activity in the ovarian follicular envelope (Aida *et al.*, 1973b; Peute *et al.*, 1978; van Bohemen *et al.*, 1981). The dramatic structural changes undergone by the liver during exogenous vitellogenesis are similar to the changes induced by estrogen in the livers of male and immature fish. In contrast to the female liver, the male liver of the nonestrogen treated trout shows little changes in the ultrastructure with gonadal maturation (Aida *et al.*, 1973b).

G. Gonad

The microscopic structure of the vitellogenic teleost egg is described in detail by Guraya (1978). The zona pellucida is divisible into two layers, the layer immediately adjacent to the oocyte is termed the zona radiata, the layer further away from the oocyte is the zona pelucida proper. However, depending on species and the stage of oocyte growth, the zona pellucida can be divided into one, two, or three layers. Above the zona pellucida lie the follicle cells. These cells usually undergo mitosis to form a continuous epithelial layer. During oocyte growth the nucleus of the follicle cell assumes a spherical shape and the cell possesses an activated protein synthetic machinery. Microvilli arise from the surface of the oocyte and the follicle cells. Nutrients may reach the microvilli directly from the follicle cells when the microvilli pass through the zona radiata and the zona pellucida proper and come into contact with the follicle cells. It is believed that the follicle cells may secrete the zona pellucida; the zona radiata originates from the oocyte (Guraya, 1978). The zona radiata thickens and the microvilli widen as the oocyte grows in diameter. However, the thickness of the zona pellucida, the total area, and the number of microvilli initially increase and then decrease to a basal level with the progress of oocyte growth (Hurley and Fisher, 1966).

The sequence of endogenous and exogenous vitellogenesis has been observed in the zebrafish *Brachydanio rerio* (Ulrich, 1969), in the guppy *Lebistes reticularis* (Droller and Roth, 1966), in the killifish *Fundulus heteroclitus* and in the pipefish *Syngnathus fuscus* (Anderson, 1968), and in the goldfish *Carassius auratus* (Khoo, 1979). The polysaccharide nature of "endogenous yolk" has been demonstrated by a number of researchers (Aketa, 1954; Malone and Hisoaka, 1963; Guraya, 1965; Yamamoto, 1956; Yamazaki, 1965; Korfsmeier, 1966; Heesen and Engels, 1973; Khoo, 1979). Its origin is believed to be either the Golgi (Yamamoto and Onozato, 1965; Yamamoto and Oota, 1967; Droller and Roth, 1966; Anderson, 1968; Ulrich, 1969) or the endoplasmic reticulum (Droller and Roth, 1966; Anderson, 1968; Ulrich, 1969; Beams and Kessel, 1973). Endogenous yolk has also been described as yolk vesicles, cortical alveoli, intravesicular yolk, vacuoles, yolk globules, and yolk spheres by various researchers (see Khoo, 1979). According to Beams and Kessel (1973), the Balbiani body, which is a duplex body distinct from Golgi and mitochondria and which consists of a nonbasiphilic portion called the idiosome and a highly basiphilic portion called the pallial substance, may contain a yolk precursor.

At their first appearance, the yolk vesicles take up a peripheral position in the ooplasm and then spread toward the center as time goes on until the whole ooplasm is filled with them. They are revealed as vacuoles after hematoxylin and eosin staining, but stain light blue with Mallory trichome. Dur-

ing endogenous vitellogenesis the zona radiata is poorly developed, but toward the end of the stage it assumes a striated appearance. By this time the granulosa cells and the thecal cells in the follicular envelope have become fully developed. Yolk granules then begin to appear and they gradually replace the yolk vesicles. Although generally two types of yolk inclusion, yolk vesicles and yolk granules, are found, e.g., in zebrafish (Malone and Hisoaka, 1963), in *Liopsetta obscura* and in *Clupea pallasii* (Yamamoto, 1956), three types designated yolk vesicles, yolk globules, and lipid globules have been identified in *Hypomesus japonicus* (Yamamoto, 1956) and in *Channa marulius* (Guraya, 1965). Khoo's histochemical work (Khoo, 1979) reveals that yolk vesicles differ from yolk granules in a number of aspects. Yolk vesicles contain glycogen and complex acidic polysaccharides but no lipids are present. Yolk granules contain lipids (neutral fats and phospholipids) but no polysaccharides. The protein content in yolk vesicles is lower than that in yolk granules, and sulfhydryl groups are present in yolk vesicle proteins but not in yolk granule proteins. The zona radiata contains phospholipids but neither neutral fats nor polysaccharides.

A dramatic increase in oocyte diameter occurs during vitellogenic growth because of deposition of yolk. Hydration of the oocyte prior to ovulation also brings about an increase in oocyte size (Wallace and Selman, 1979).

H. Relation of Vitellogenesis to Oocyte Maturation

Ovarian incorporation of vitellogenin declines to a negligible level after germinal vesicle breakdown in both the landlocked Atlantic salmon *Salmo salar sebago* and the winter flounder *Pseudopleuronectes americanus*. The plasma level of vitellogenin in the landlocked salmon does not change appreciably after germinal vesicle breakdown. Therefore, the decrease in the incorporation of radioactivity into ovarian yolk is presumably attributable to a change in the ability of the oocyte to incorporate vitellogenin (Ng *et al.*, 1980b).

I. Hormonal Control of Vitellogenesis

1. Steroids

Estrogen elevates the plasma calcium concentration in the rainbow trout *Salmo gairdneri* (Elliot *et al.*, 1979), in the goldfish and snakehead fish (Oguri and Takada, 1967). The hypercalcemic effect is specific to estrogens. Other steroids tested including testosterone, progesterone, and hydrocortisone acetate are without effect. The plasma calcium level correlates well

with ovarian maturation (Oguri and Takada, 1967; Yaron *et al.*, 1977) but not with testicular maturation (Oguri and Takada, 1967). Estrogen elevates the serum content of nonultrafilterable calcium and phosphorus and proteins without affecting the content of ultrafilterable calcium and phosphorous. A part of the increase in nonultrafilterable calcium is attributable to the increase in the serum concentration of vitellogenin which is a calcium-binding protein. The increase in serum protein level can also be accounted for by the increase in serum vitellogenin (Aida *et al.*, 1973a).

A relationship exists between the plasma calcium level and vitellogenin level in the rainbow trout (Whitehead *et al.*, 1980), and between the plasma estrogen level and protein concentration in *Tilapia aurea* (Yaron *et al.*, 1977).

Estrogen elevates the plasma contents of calcium and proteins in the goldfish (Hori *et al.*, 1979) and in *Tilapia aurea* (Yaron *et al.*, 1977) and also the contents of lipids and protein-bound phosphorus in the goldfish (Hori *et al.*, 1979).

The effects of the sex steroids estrone, estradiol, progesterone, and testosterone on plasma lipids were studied in the goldfish *Carassius auratus* by Wiegand and Peter (1980b). In fish adapted at 12° C only estrone and estradiol elevated the plasma concentrations of total cholesterol and lipid phosphorus. Progesterone increased the plasma triglyceride level in maturing fish at 12° C; estrone had a similar action in regressed fish. Testosterone did not produce an effect on plasma lipids at the temperature used and the reproductive stages of the fish examined.

In the catfish *Heteropneustes fossilis* estrogen increases the hepatosomatic index, the hepatic protein and RNA contents, and the plasma protein content, but does not affect the muscle protein and RNA contents. Estrogen induced hepatic hypertrophy in all the teleost species studied to date: *Oryzias latipes* (Egami, 1955), flounder *Platichthys flesus* (Emmersen and Petersen, 1976), trout *Salmo gairdneri* (Campbell and Idler, 1980), winter flounder *Pseudopleuronectes americanus* (Campbell and Idler, 1976), *Misgurnus anguillicaudatus* (Kobayashi, 1953), and catfish *Heteropneustes fossilis* (Medda *et al.*, 1980; Nath and Sundararaj, 1981b). Estrogen also increased the hepatic RNA content in the male flounder (Emmersen *et al.*, 1979). However, estrogen does not affect the gonadosomatic index in female teleosts (Plack *et al.*, 1971; Campbell and Idler, 1976; Medda *et al.*, 1980). Most researchers are in favor of a concept of negative feedback at the level of the hypothalamus exerted by estrogen on pituitary gonadotropin secretion (Egami, 1955; Egami and Ishii, 1962; Sundararaj and Goswami, 1968). However, Olivereau and Olivereau (1979b) observed a positive feedback of estrogen on the pituitary gonadotrops in the silver eel. In the rainbow trout, estrogen synthetic capacity of the ovary increases gradually from May during

endogenous vitellogenesis to a maximum in October during exogenous vitellogenesis (Elliot *et al.*, 1979). The absence of a significant level of estrogen in the male teleost and in the immature teleost accounts for the virtual absence of vitellogenin from their circulation (Idler and Campbell, 1980).

The stimulatory action of estrogen on vitellogenin production is universal throughout the nonmammalian vertebrate classes: lamprey (Pickering, 1976), hagfish (Yu *et al.*, 1981), elasmobranch (Craik, 1978), amphibian (Wallace and Bergink, 1974; Wangh and Knowland, 1975), reptile (Hahn, 1967; Callard and Banks, 1970; Ho *et al.*, 1981), and bird (Heald and McLachlan, 1965).

The ability of estrogen to induce formation of vitellogenin in immature teleost and male teleost is well documented (Idler and Campbell, 1980; Emmersen *et al.*, 1979; Campbell and Idler, 1980; Hori *et al.*, 1979; Nath and Sundararaj, 1981a; Medda *et al.*, 1980; Plack and Fraser, 1970; Plack *et al.*, 1971).

Estrogen increases plasma opalescence in the eel *Anguilla anguilla* probably because of increased secretion of vitellogenin into the bloodstream (Olivereau and Olivereau, 1979a).

Sundararaj and Nath (1981) compare the activities of estradiol, estrone, and estriol in inducing vitellogenin in the catfish and discover that the relative potencies of estrone and estriol differ depending on dosage employed. Estradiol is the most active, but other steroids including testosterone, progesterone, and cortisol are ineffective. Estrone induces vitellogenin synthesis in the bass *Paralabrax clathratus* (Urist and Schjeide, 1961). Estrone and estradiol are implicated in the regulation of vitellogenin production in the rainbow trout (van Bohemen and Lambert, 1981). Estrone by itself has only 5–10% the potency of estradiol in stimulating hepatic vitellogenin production. Estrone treatment concurrent with estradiol administration or estrone pretreatment followed by estradiol treatment potentiates the action of estradiol (Lambert and van Bohemen, 1981).

Vitellogenin synthesis in the catfish can be amplified by three injections of estradiol given at intervals (Sundararaj and Nath, 1981). This phenomenon can be explained by a memory effect in which the first estrogen treatment primes the liver to respond to subsequent estrogen treatments, as in the case of the amphibian (Bergink *et al.*, 1974).

In the hypophysectomized goldfish estradiol, estrone and estriol induce the formation of yolk vesicles; however, pregnenolone induces the production of yolk granules (Khoo, 1979). Stimulation of yolk granule formation induced by pregnenolone differs from the normal physiological process in that the granules are formed without the prior appearance of yolk vesicles and also when the vitelline membrane of the oocyte is not yet fully developed.

In the goldfish, massive doses of the androgen methyltestosterone evoke in the liver the same response as that elicited by estrogen. In the hepatocytes there is an extensive proliferation of rough endoplasmic reticulum, hypertrophy of the Golgi body, a marked increase in the number of secretory granules, and a decrease in the number of glycogen granules: vitellogenin appears in the serum. Ethynyltestosterone and methylandrostenediol also elicit the same response but testosterone, dihydrotestosterone and methyldihydrotestosterone are much less potent; progesterone and methylprogesterone are totally inactive (Hori *et al.*, 1979). These results are explicable if testosterone is converted into estrogen. However, testosterone has no effect on vitellogenin production in the catfish *Heteropneustes fossilis* (Medda *et al.*, 1980).

The source of estrogen has been demonstrated to be the ovary. In *Tilapia aurea* the plasma calcium and protein levels are raised following estrogen treatment and decline after ovariectomy (Yaron *et al.*, 1977). The salmon gonadotropin preparation SG-G100 (Donaldson *et al.*, 1972) has no influence on the plasma vitellogenin level in ovariectomized catfish; however, estrogen elevates the level (Nath and Sundararaj, 1981b). There is no unanimity in opinion on the type of estrogen-producing cells in the ovary. In the theca cells of some fish 3β-hydroxysteroid dehydrogenase activity is most intense when vitellogenesis commences and the intensity of the enzyme activity decreases with the progress of follicular maturation (Guraya, 1978). In some species the theca cells appear to be the site of steroidogenesis; in others the granulosa cells are responsible for steroid production (Elliott *et al.*, 1979; Hoar and Nagahama, 1978). Nagahama *et al.* (1981, 1982) have shown that in the amago salmon ovary the theca cells produce androgens which are converted to estrogen in the granulosa layer.

Studies on the variation in the plasma levels of estrogen and testosterone throughout the reproductive cycle of the female plaice *Pleuronectes platessa* reveal that the curve for testosterone lags immediately behind the curve for estradiol, suggesting that testosterone may serve as a precursor of estrogen during vitellogenesis (Wingfield and Grimm, 1977). A similar opinion is held by Campbell *et al.* (1976) on the winter flounder *Pseudopleuronectes americanus* and by Lamba (1981) on the catfish *Heteropneustes fossilis*.

The effect of estrogen in inhibiting atresia of oocytes after hypophysectomy is controversial (Lam *et al.*, 1978).

2. Pituitary Hormones

Unilaterally ovariectomized *Tilapia aurea* exhibit the phenomenon of compensatory hypertrophy of the remaining ovary. Microscopic examination reveals changes in the zona radiata and the granulosa cells of the follicles compatible with enhanced vitellogenesis (Dadzie and Hyder, 1976). Hypo-

physectomy results in a decrease in gonadosomatic index and a diminished incorporation of yolk into the gonad (Campbell and Idler, 1976). This finding confirms the observation of many investigators that vitellogenesis is arrested by ablation of the pituitary, e.g., Vivien (1941) on *Gobius paganellus*, Barr (1963) on plaice *Pleuronectes platessa*, Yamazaki (1965) on goldfish *Carassius auratus*, and Sundararaj and Goswami (1968) on catfish *Heteropneustes fossilis*.

Salmon gonadotropin SG-G100 (Donaldson *et al.*, 1972) increases the plasma concentrations of triglycerides and total cholesterol in goldfish with small ovaries. The lipid mobilization may be mediated by estrogen produced by the ovary under gonadotropic stimulation. The same treatment produces a decrease in the plasma concentrations of lipids in fish with big ovaries, probably because of gonadotropin-stimulated uptake of the lipids into ovary (Wiegand and Peter, 1980a).

Oogonial multiplication during the postspawning period may be under the control of a pituitary factor other than gonadotropin, and the primary growth phase of the nonvitellogenic oocytes may occur independently of the pituitary. The secondary growth phase or vitellogenesis is pituitary-dependent (Dodd, 1972). The pituitary control of folliculogensis is not clear. There is also some evidence that gonadotropin directly or indirectly regulates oogonial mitosis through steroidogenesis; however, there is also evidence to the contrary (see Tokarz, 1978; Lam *et al.*, 1978).

Hurlburt (1977) suggests that thyroxine may synergize with gonadotropin in vitellogenesis.

Vitellogenic activity in the teleost gonadotropin preparation SG-G100 (Donaldson *et al.*, 1972) and carp gonadotropic hormone (GtH) (Burzawa-Gérard, 1971) has been noted by Burzawa-Gérard (1974), Funk *et al.* (1973), and Sundararaj *et al.* (1972). A similar gonadotropin preparation has been shown to stimulate vitellogenesis in the eel *Anguilla anguilla* (Fontaine *et al.*, 1976). Nath and Sundararaj (1981b) report that SG-G100 and carp GtH stimulate vitellogenin and yolk granule production; however, the mammalian gonadotropins including luteinizing hormone, pregnant mare serum gonadotropin, human chorionic gonadotropin, and the subunits of luteinizing hormone stimulate vitellogenin production without yolk granule production. The activities of follicle stimulating hormone, thyrotropin, growth hormone, and prolactin on vitellogenin production are attributable to contamination with luteinizing hormone.

Campbell and Idler (1976) discovered that the Con AI (unadsorbed on Concanavalin A–Sepharose) fraction of the pituitary extract of American plaice (*Hippoglossoides platessoides*) stimulated incorporation of radiophosphate labeled plasma yolk into the ovary of winter flounder *Pseudopleuronectes americanus*. Idler and Ng (1979) and Ng and Idler (1978a,b, 1979)

confirmed this finding by extending the study to the Con AI fractions of winter flounder, chum salmon (*Oncorhynchus nerka*) and carp (*Cyprinus carpio*) pituitaries. Campbell (1978) demonstrated that chinook salmon (*O. tshawytscha*) pituitary Con AI fraction stimulated incorporation of vitellogenin into immature trout oocytes *in vitro*. The vitellogenic activity of chinook salmon pituitary Con AI fraction was also inferred by the finding of Upadhyay *et al.* (1978): salmon maturational hormone (Breton *et al.*, 1976) was incapable of inducing vitellogenin incorporation in the immature trout; however, the whole pituitary extract produced ultrastructural changes in the ovary characteristic of exogenous vitellogenesis.

Vitellogenic hormone* administered to estrogenized or nonestrogenized flounder, hypophysectomized when they were actively undergoing vitellogenesis (in November–January), stimulates incorporation of vitellogenin into the ovary (Idler and Ng, 1979; Ng and Idler, 1978a,b, 1979), but is not able to do so in nonestrogenized flounder at an earlier phase of vitellogenesis in September (Ng *et al.*, 1980b). Presumably the discrepancy in results is attributable to the low plasma titer of vitellogenin in fish hypophysectomized at the earlier phase with the consequence that the stimulatory effect of vitellogenic hormone on vitellogenin incorporation is not obvious. Fish actively undergoing vitellogenesis before hypophysectomy would be expected to continue producing vitellogenin at an appreciable, albeit decreased, rate after hypophysectomy.

Dependence of ovarian estrogen production on the pituitary has been established by various investigators. *In vitro* evidence was obtained by Yaron and Barton (1980) using plaice ovaries and homologous pituitary extract and by Yaron and Bogomolnaya (1981) using *Sarotherodon aureus* ovarian fragments and pituitary extracts of fish representing different taxonomical groups. Nagahama *et al.* (1981) showed that SG-G100 enhances the output of estrogen by isolated follicle cells. *In vivo* evidence was obtained by Crim and Idler (1978) by administering salmon pituitary extract to trout, and by Fostier *et al.* (1979) by using the homologous carp system. Bhattacharya *et al.* (1981) find in the freshwater perch *Anabas testudineus* that the activity of ovarian 17β-hydroxysteroid dehydrogenase, the enzyme regulating the conversion of estradiol into estrone, increases after SG-G100 administration.

Juvenile female rainbow trout have an extremely low plasma concentra-

*The vitellogenic gonadotropin is also referred to as the "carbohydrate-poor" or "Con AI" gonadotropin and was isolated from the pituitary polypeptides which are not adsorbed when an appropriate extract is passed through Concanavalin A–Sepharose (e.g., Campbell and Idler, 1976; Idler and Ng, 1979). The hormone isolated from the protein adsorbed on Cancanavalin A–Sepharose is referred to as the "Con AII" gonadotropin or the "carbohydrate-rich" gonadotropin or the "maturational" hormone.

tion of estrogen, and, as a consequence, the plasma vitellogenin level is negligible. The production of both estrogen and vitellogenin in these fish can be boosted by treatment with a salmon pituitary Con AII fraction (absorbed on Concanavalin A–Sepharose) which contains the maturational hormone. The Con AI fraction (unabsorbed on Concanavalin A–Sepharose (Idler *et al.*, 1975), immunologically purified by adsorption with an antiserum to the maturational hormone, manifests no such activity. Male trout treated with a salmon pituitary Con AII fraction do not respond by producing vitellogenin as in the female because of the inability of their gonads to produce estrogen (Campbell and Idler, 1979; Idler and Campbell, 1980). Upadhyay conducted ultrastructural investigations on the ovaries and livers of juvenile rainbow trout that had received injections of estrogen, salmon maturational hormone (Breton *et al.*, 1976), or the injection vehicle (Upadhyay *et al.*, 1978); he observed morphological changes in the protein synthetic machinery of the cell such as the endoplasmic reticulum and the Golgi apparatus. His data are suggestive of the induction of hepatic vitellogenin production following challenge with estrogen or with gonadotropin. Accumulation of lipid bodies in the oocytes in fish treated with maturational hormone indicates endogenous vitellogensis. However, no development of micropinocytotic processes occurs. This observation is consistent with the finding of Campbell (1978) that chum salmon pituitary Con AII fraction does not stimulate *in vitro* incorporation of vitellogenin into juvenile trout oocytes. However, in the hypophysectomized mature flounder maturational hormone stimulates the formation of oocytes with a prominent zona radiata (Ng *et al.*, 1980b) and also the incorporation of radiophosphate into ovarian yolk proteins (Idler and Ng, 1979; Ng and Idler, 1979) suggesting that maturational hormone stimulates vitellogenin incorporation. The disparity in the actions of maturational hormone on the juvenile and adult teleost may be attributed to the priming of the adult fish oocytes, by a hormone present in the pituitary of the adult which is absent in the juvenile, to facilitate response to maturational hormone by incorporating vitellogenin. A possible candidate is vitellogenic hormone. The vitellogenic activity of the gonadotropin preparations SG-G100 and carp GtH in the hypophysectomized teleost (Burzawa-Gérard, 1974; Sundararaj *et al.*, 1972) may therefore be attributable to either the combined actions of vitellogenic hormone and maturational hormone in the preparation (although the vitellogenic hormone content of the two gonadotropin preparations is unknown), or to a vitellogenic action of maturational hormone on oocytes which have been primed for responding to the hormone.

In a brief report, Nath and Sundararaj (1977) state that plasma vitellogenin level and ovarian weight in vitellogenic catfish decrease after treatment with an antiserum raised against a catfish gonadotropin fraction. Because of the method of gonadotropin preparation it is not possible to attribute the observed action to vitellogenic or maturational hormone.

Vitellogenic Atlantic salmon treated with an antiserum to maturational hormone have lower plasma levels of vitellogenin and estradiol than those treated with an antiserum to vitellogenic hormone or normal rabbit serum. A decreased incorporation of radioactive phosphate into gonadal yolk occurs in fish treated with either type of gonadotropin antiserum (Ng *et al.*, 1980a). An antiserum to vitellogenic hormone arrests vitellogenesis and induces follicular atresia in vitellogenic flounder; an antiserum to maturational hormone does not have any appreciable effect on the histological appearance of the ovary (Ng *et al.*, 1980b). When taken together these findings suggest that maturational hormone is responsible for initiating vitellogenesis, at least exogenous vitellogenesis by inducing ovarian estrogen secretion and therefore hepatic vitellogenin synthesis, but its role in promoting gonadal vitellogenin incorporation is relatively minor compared with that of vitellogenic hormone. The inhibitory effect of the maturational hormone antiserum on gonadal vitellogenin incorporation (Ng *et al.*, 1980a) may be a consequence of its influence on plasma estradiol and vitellogenin rather than a direct effect.

These roles of vitellogenic hormone and maturational hormone are supported by immunofluorescent localization of the hormones in the ovary. The hormones are detected, probably bound to their respective receptors, in specific regions of the ovarian section. Vitellogenic hormone is found in the ooplasm of both large immature and vitellogenic oocytes and in follicular envelopes of vitellogenic oocytes, implying that the hormone prepares or primes the large immature oocytes for the vitellogenic phase, and it stimulates the vitellogenic oocytes to incorporate vitellogenin. Maturational hormone is located in follicular envelopes in interstitial tissue and large immature oocytes suggesting its role in steroidogenesis and vitellogenesis through estrogen and vitellogenin production. However, it cannot be located in the ooplasm of vitellogenic oocytes (Ng *et al.*, 1980b).

J. Conclusion and Update

The process of vitellogenesis in teleosts has been shown to be similar to that operating in other oviparous vertebrates. The contribution of autosynthetic processes (endogenous vitellogenesis) to the yolk mass in the teleost ovary, relative to exogenous yolk acquired by incorporation of vitellogenin, has not been estimated. Exogenous vitellogenesis can be considered to consist of two phases. The first phase involves the induction of hepatic vitellogenin production under stimulation of ovarian estrogen. During the second phase vitellogenin is taken up from the blood stream and incorporated into ovarian yolk proteins.

An interpretation as to how gonadotropins function to control these pro-

cesses follows. In salmonids maturational gonadotropin occurs at high levels in plasma around spawning time but is near the lower limit of the radioimmunoassay during the phase of active incorporation of vitellogenin (Crim *et al.*, 1973, 1975). There appears to be a small increase in maturational gonadotropin coincident with an increase in estradiol in trout plasma early in vitellogenesis (Billard *et al.*, 1978), and antibody to maturational gonadotropin inhibits ovarian growth immediately prior to the rapid and massive increase in the ovarian weight (Wiegand and Idler, 1983). Therefore, there appears to be a low level of maturational hormone present when the fish resumes ovarian development after spawning, but it is sufficient to establish vitellogenin production by the liver. When flounder were treated during the stage of rapid ovarian growth (September) with antibody to carbohydrate-poor vitellogenic hormone the gonadosomatic index was greatly reduced and many vitellogenic oocytes underwent atresia. Immunofluoresence established that vitellogenic gonadotropin is bound to ooplasm of yolky oocytes and to the follicular envelope suggesting that the hormone acts on the yolky oocytes to stimulate incorporation of vitellogenin (Ng *et al.*, 1980b). This finding is consistent with the fact that the pituitary fraction containing the vitellogenic gonadotropin stimulated uptake of vitellogenin into trout oocytes *in vitro;* the maturational gonadotropin did not (Campbell, 1978).

Sundararaj *et al.* (1982) found that carp carbohydrate-rich gonadotropin induced some "vitellogenic oocytes" and at higher doses some yolky oocytes in postspawned hypophysectomized catfish and concluded that low levels maintained the sensitive system. It is not known what role the low levels of contaminating Con AI protein, known to be an impurity (Burzawa-Gérard, 1982a), play in the processes. In fact, it has only recently been reported that the Con AI (carbohydrate-poor) impurity in carp gonadotropin induced pinocytosis in a teleost, *Gobius niger* (Burzawa-Gérard, 1982b). Given the current state of knowledge, we suggest that low levels of maturational gonadotropin initiate vitellogenesis through its action on ovarian estrogen secretion which in turn augments hepatic vitellogenin synthesis although other actions on ovarian growth are not precluded. Ovarian yolk deposition and growth is then primed and maintained primarily by vitellogenic gonadotropin. One possible flaw in the preceeding observations is that knowledge gained from one species may not be directly applicable to another species. It is regretable that so much work was done and is still being reported on the maturational carbohydrate-rich gonadotropin from which the carbohydrate-poor vitellogenic hormone has not been totally removed. It has now been 7 years since we reported a simple way to achieve this goal using Concanavalin A–Sepharose (Idler *et al.*, 1975).

Teleost vitellogenin shares many of the characteristics of avian and amphibian vitellogenins. It is a glycolipophophoprotein inducible in the imma-

ture male and in the nonvitellogenic female. Teleost yolk proteins comprise lipovitellin and phosvitin which are similar in amino-acid composition to their avian and amphibian counterparts.

Radioimmunoassays have been developed to monitor the concentration of vitellogenin in plasma and that of yolk proteins in ovary. Generally, there is a correlation between the levels of plasma estrogen and plasma vitellogenin with vitellogenic growth of the ovary.

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9

AN INTRODUCTION TO GONADOTROPIN RECEPTOR STUDIES IN FISH

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I. INTRODUCTION

Hormones behave as chemical messengers in a complex system of cellular communication that coordinates and regulates metabolic processes. Although hormones are released into the general circulation, their actions are restricted to specific target cells. Primarily on the basis of mammalian studies, it has been established that hormone specificity is a consequence of molecules termed receptors which are located in those tissues that respond to hormone stimulation. Hormone receptors may be defined as cellular proteins which serve the dual functional properties of recognition and transmission of the hormone signal into a specific biological response. Functionally, recognition is manifested by the binding of the hormone to the

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receptor. After binding, the hormone–receptor complex undergoes a conformational change which results in the initiation of the chain of events leading to a specific response.

Although all hormone receptors share common functional properties, differences emerge with respect to hormone specificity, cellular localization, and the means by which the hormone signal is transmitted. Several excellent reviews discuss the means by which the binding of reproductive hormones to their receptors is coupled to a specific response (e.g., Khan, 1975; Catt and Dufau, 1976; Gorski and Gannon, 1976; Chan and O'Malley, 1978; Dufau and Catt, 1978; Hseuh, 1978; Insel, 1978; Means *et al.*, 1978; Baxter and Funder, 1979; Clark and Peck, 1979; Jensen *et al.*, 1982). Reproductive peptide hormones including luteinizing hormone (LH), human chorionic gonadotropin (HCG), follicle stimulating hormone (FSH), and luteinizing hormone releasing hormone (LHRH) bind to receptors located on the external surface of their respective target cell plasma membranes. For these hormones, receptor binding results in the activation of adenylate cyclase thereby increasing the intracellular concentration of cyclic 3′,5′-adenosine monophosphate (cAMP). Cyclic AMP then binds to a cAMP-dependant protein kinase causing activation of the enzyme and phosphorylation of intracellular regulatory protein substrates. Reproductive steroids (e.g., androgens, estrogens, and progestins) pass readily through the cell membrane and bind to soluble receptors located in the cytoplasm. Following binding the cytoplasmic hormone–receptor complex undergoes a conformational change termed activation or transformation to enable passage through the nuclear membrane. Following translocation to the nucleus, the activated steroid–receptor complex binds to specific acceptor regions of nuclear chromatin. This binding to nuclear chromatin regulates the levels of specific messenger RNAs and consequently the rates of synthesis of specific proteins.

This discussion is based on the premise that a working knowledge of receptor theory is essential to an understanding of reproductive endocrinology in fish. Because the literature dealing with the study of hormone–receptor interactions is so extensive, subsequent discussion is restricted to gonadotropin receptors. Although current knowledge pertaining to these receptors is largely a consequence of mammalian studies, no attempt is made to provide a detailed list of their properties; a more general approach has been adopted. The discussion focusses on the techniques used to identify gonadotropin receptors and the operational features that a hormone-binding site must possess to function as a receptor. Considerable emphasis is given to the technical aspects of receptor-binding studies performed by direct binding of hormone to gonadal tissue incubated *in vitro*. A final comment is made on the application of receptor-binding techniques to studies of fish gonadotropins. Because the investigation of gonadotropin receptors in fish is

currently in its infancy, it is hoped that this discussion will provide background for researchers entering this field. Technical aspects relevant to the identification of steroid-hormone receptors have been reviewed by Clark and Peck (1978, 1979) and Schrader *et al.* (1978).

II. GENERAL PRINCIPLES OF RECEPTOR BINDING

A. Experimental Approaches to Receptor Study

Gonadotropin (GtH) receptors, including those in nonmammalian vertebrates, have been studied by a variety of approaches which can be broadly classified as either indirect or direct (Licht, 1980a).

By the indirect approach, the characteristics of a receptor can be inferred from the measurement of a physiological response following hormone treatment. For example, by comparing the ability of a variety of hormones to elicit a response, a measure of receptor specificity can be obtained. Several *in vitro* assays have been useful for studies with fish gonadotropins. These are based on the activation of adenylate cyclase (Fontaine *et al.*, 1970, 1972; Idler *et al.*, 1975; Fontaine-Bertrand *et al.*, 1978), the induction of germinal vesicle breakdown (Jalabert *et al.*, 1974; Epler *et al.*, 1979; Hirose, 1980; Goetz, Chap. 3, Vol. 9B), and the activation of steroidogenesis (Yaron and Barton, 1980; Bona Gallo and Licht, 1981; Fostier *et al.*, Chap. 7, this volume; Yaron *et al.*, 1982; G. Van Der Kraak and E. M. Donaldson, unpublished). Although the initiation of these responses is specific to gonadotropins, individual species show wide variations in their specificity for gonadotropins of different origins. In the goby, steroid production follows the mammalian pattern by being highly specific for LH type gonadotropins, but cichlids in general lack gonadotropin specificity (Bona Gallo and Licht, 1981; Yaron *et al.*, 1982). In contrast, salmonids tend to be highly specific for homologous gonadotropins (Kagawa *et al.*, 1982; G. Van Der Kraak and E. M. Donaldson, unpublished). It remains to be determined whether these diverse patterns of hormone specificity in fish relate to changes in the native hormones or their receptors.

Although the expression of a biological response is the ultimate test of a hormone–receptor interaction, a different approach is required to investigate the molecular interaction of the hormone with the target cell. The development of techniques to produce "tagged" gonadotropins which retain their biological integrity has made it possible to conduct receptor studies by the direct approach and follow the actual binding of hormone to the target

tissue. Several variations of the direct approach have been used to localize hormone uptake to specific cell types. For example, Cook and Peter (1980) used autoradiography to study the uptake of ^{125}I-labeled carp gonadotropin by goldfish ovaries following injection *in vivo*. The ^{125}I-labeled carp gonadotropin was localized in the follicular epithelium of the largest follicles with no evidence of accumulation by first growth stage, by perinucleolar oocytes, or by atretic follicles. The greatest accumulation of label corresponded to the position described for "special thecal cells" (Hoar and Nagahama, 1978; Nagahama *et al.*, 1982) which are considered to be steroidogenic in this species. A more common route for autoradiographic analysis involves the incubation of tissue slices *in vitro* with either radiolabeled hormone alone or in competition with excess unlabeled hormone. When competition studies are conducted the specificity of hormone uptake can be examined. This approach has been successful for studies in all vertebrate groups, but it remains to be tested in fish. Alternatively, fluorescent hormone probes have been used following incorporation of ferritin (Luborsky and Behrman, 1979) or fluorescein groups (di Zerega and Hodgen, 1980) to mammalian gonadotropins. Developments in the field of immunohistochemistry have made it possible to conduct direct-binding studies thereby eliminating the use of modified gonadotropins. For example, Ng *et al.* (1980) used an indirect immunofluorescence procedure to localize the sites of gonadotropin accumulation in the ovary of the winter flounder. In this procedure, ovarian sections were incubated with gonadotropin followed by sequential treatment with anti-flounder-gonadotropin rabbit serum and a florescein isothiocyanate-labeled goat anti-rabbit-γ-globulin. The uptake of flounder gonadotropin was localized to ovarian interstitial tissue and the follicular envelope which is consistant with the role of this hormone in promoting steroidogenesis. By the same technique the uptake of a second pituitary fraction, termed vitellogenic gonadotropin, was restricted to the follicular envelope and ooplasm of yolky oocytes which is consistant with the actions of this fraction in promoting vitellogenin uptake. These results support the hypothesis that teleosts possess two gonadotropins with distinct sites of action (Ng *et al.*, 1980; Idler and Ng, see Chapt. 5, this volume).

Although the histological approach to receptor study is widely used, application to routine studies is hindered by the technique being qualitative rather than quantitative. By far the most powerful technique involves the direct binding of radiolabeled hormone to the target tissue with binding quantified through inhibition studies with unlabeled hormone. Cook and Peter (1980) using an *in vivo* approach were able to demonstrate gonadotropin binding sites in the ovary of goldfish undergoing gonadal recrudescence. Following intraarterial injection of ^{125}I-labeled carp gonadotropin, radiolabeled hormone accumulated in the ovary and its uptake was reduced

in a dose-dependent manner by preinjection with unlabeled hormone. Competitive uptake of ^{125}I-labeled carp gonadotropin by the ovary indicates the presence of a limited number of gonadotropin binding sites and corresponds to a major criterion for receptor identification.

Currently most receptor-binding studies are performed *in vitro* because a large number of tests can be performed using a single tissue preparation and the complicating effects of hormone turnover, common to *in vivo* studies, is minimized. The requirements for direct-binding studies performed *in vitro* include an isotopically labeled hormone, a suitable receptor preparation, and a means of separating the hormone–receptor complex from free hormone. The general principles involved in receptor studies are similar to those for other radioligand binding techniques such as radioimmunoassay (RIA). This involves establishing a competition between radiolabeled hormone and increasing amounts of unlabeled hormone for a limited number of hormone-specific binding sites. A receptor site can be provisionally identified provided that the tissue binds radiolabeled hormone and that this binding is reduced by unlabeled hormone. Several terms have been used to describe the competitive binding component including displaceable, saturable, and specific binding. Residual radioactive hormone bound to tissues in the presence of excess unlabeled hormone has been termed nonspecific binding. Although excess unlabeled hormone is sufficient to displace all the specifically bound radioactive hormone, nonspecifically bound radioactivity persists because of adsorbtion to the incubation tube and entrapment within the tissue preparation. The underlying assumption in this form of assay is that specific binding is caused by receptor binding. A frequent criticism of the *in vitro* approach is that binding is commonly studied under conditions in which the target tissue is no longer responsive to hormone stimulation. However, by careful preparation of the tissue for *in vitro* binding studies the binding of hormone can be quantified and also be directly related to the actions of the hormone by the simultaneous measurement of a biological response.

High-affinity FSH-binding sites have been identified by the binding of radiolabeled mammalian FSH preparations to gonadal tissues from mammals (Catt *et al.*, 1976; Darga and Reichert, 1978; Dufau and Catt, 1978), birds (Ishii and Farner, 1976; Licht and Midgley, 1976; Burke *et al.*, 1979b; Sakai and Ishii, 1980; Etches and Chen, 1981), reptiles (Licht and Midgley, 1976; Licht *et al.*, 1977a,b; Bona Gallo *et al.*, 1980), and amphibians (Adachi *et al.*, 1979; Adachi and Ishii, 1980). Similarly by using radiolabeled turkey FSH, specific FSH-binding sites have been identified in bird, mammal, and reptile gonads (Licht and Bona Gallo, 1978; Bona Gallo and Licht, 1979). Studies using radiolabeled sea turtle FSH have demonstrated the presence of FSH-binding sites in several reptilian species (Licht *et al.*, 1977a,b; Licht

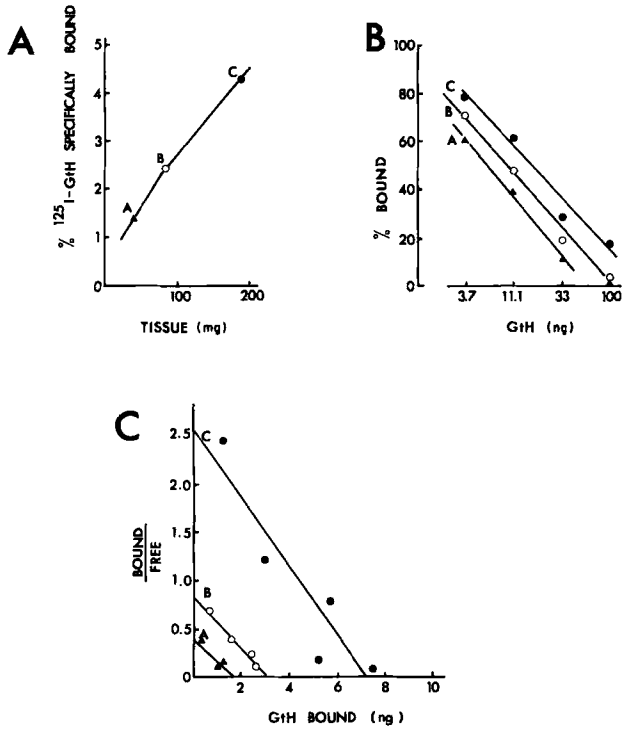

Fig. 1. Analysis of ^{125}I-labeled salmon gonadotropin binding to a 3000-*g* particulate fraction obtained by centrifugation of immature chum salmon ovarian homogenates. (A) Specific binding of ^{125}I-labeled salmon gonadotropin in relation to increasing amounts of ovarian tissue. For binding, 50 μl ^{125}I-labeled chinook salmon gonadotropin (SGA-2359, 3.5 × SG-G100 based on *in vitro* testosterone production) in 0.01 *M* phosphate buffer, 0.1 *M* NaCl, 2.5 m*M* $CaCl_2$, 0.1% bovine serum albumin, pH 7.5, was combined with 50 μl of buffer or unlabeled competitor and 200 μl of tissue suspension. Samples were incubated at 20° C for 20 hr with constant shaking. The reaction was stopped by the addition of 1 ml ice-cold buffer followed by centrifugation at 3000 *g* for 10 min. The supernatant was decanted and the centrifugation process repeated. Specific binding was calculated using the formula $(Tb - NSB)/Tc \times 100$, where *Tb* is the binding of ^{125}I-labeled salmon gonadotropin in the absence of competitor, *NSB* is the uptake of ^{125}I-labeled salmon gonadotropin in the presence of 10 μg SG-G100, and *Tc* is the total counts of radiolabeled hormone used in the incubation. Values are the mean of closely agreeing determinations made in triplicate. (B) Competition curves for ^{125}I-labeled salmon gonadotropin binding as a function of increasing amounts of unlabeled SGA-2359. Binding was expressed as the percentage of the total specific binding at each tissue concentration. (C) Scatchard plot for the competition data shown in (B) The bound/free hormone ratio and bound hormone were calculated as described in Section III after applying a correction for the maximum binding activity of the ^{125}I-labeled salmon gonadotropin preparation as described in Section IVB. Data are as follows: (A) tissue wt. 38 mg, k_a 1.5 × 10^9, capacity 1.7 ng, and capacity 44 pg/mg; (B) tissue wt. 83 mg, k_a 2.8 × 10^9, capacity 3.1 ng, and capacity 37 pg/mg; (C) tissue wt. 188 mg, k_a 3.5 × 10^9, capacity 7.1ng, and capacity 38 pg/mg.

and Bona Gallo, 1978). High-affinity LH-binding sites in mammalian gonad preparations have been identified using either radiolabeled mammalian LHs or HCG (Catt *et al.*, 1976; Saxena, 1976; Dufau and Catt, 1978). Radiolabeled turkey LH displays a high affinity for bird and lizard gonad preparations, but only a weak affinity for turtle and mammalian gonad preparations (Licht and Bona Gallo, 1978; Bona Gallo and Licht, 1979). Unlike FSH, mammalian LH and HCG are more species specific and cannot be used to identify high-affinity binding sites in nonmammalian species (Licht *et al.*, 1977b). Studies using radiolabeled sea turtle LH also did not reveal specific binding sites in sea turtle gonads (Licht, 1980b). These results indicate that receptor-binding techniques used for the identification of LH receptors in mammals may not be appropriate in all vertebrate classes (Licht *et al.*, 1977b; Licht, 1980a). Whether this represents a problem associated with methodology or a distinct form of hormone–receptor interaction occurring in some nonmammalian vertebrates remains to be determined.

Recent unpublished data from K. Aida and S. Ishii, B. Breton, and R. E. Peter and from Van Der Kraak and Donaldson, (1982) indicate that the *in vitro* approach may be appropriate for the measurement of gonadotropin-binding sites in fish gonads. The interaction of ^{125}I-labeled salmon gonadotropin with ovarian tissue from immature chum salmon is shown in Fig. 1. (Van Der Kraak and Donaldson, 1982). The specific binding of ^{125}I-labeled salmon gonadotropin as a function of increasing amounts of a 3000 *g* particulate ovarian fraction is shown in Fig. 1A. Specific binding increased in relation to tissue concentration such that 4.3% of the ^{125}I-labeled salmon gonadotropin bound specifically to 188 meq (original wet weight) of ovarian tissue. The specific binding of ^{125}I-labeled salmon gonadotropin was reduced in a dose-dependant manner by the addition of unlabeled salmon gonadotropin at all three tissue concentrations (Fig. 1B). The addition of 3.7 ng salmon gonadotropin markedly reduced specific binding, and 100 ng salmon gonadotropin reduced specific binding to near nonspecific binding levels. Analysis of the competition data using a Scatchard plot (Section II,B) revealed that specific binding was attributable to a single class of high-affinity binding sites present in low numbers (Fig. 1C). The high affinity of the salmon gonadotropin-binding sites (K_a 1.5–3.5 $\times$ 10^9 M^{-1}) was not unlike that described for gonadotropin receptors in other vertebrate groups (Catt *et al.*, 1976; Saxena, 1976; Bona Gallo and Licht, 1979). The close agreement between the binding capacities (37–44 pg gonadotropin/mg original wet tissue weight) suggests the same sites are measured at each tissue concentration. Additional data (not shown) indicates that ovine LH and FSH, which are essentially inactive when tested for their ability to stimulate ovarian steroid production in chinook and coho salmon, are virtually ineffec-

tive in reducing the specific binding of ^{125}I-labeled salmon gonadotropin to immature chum salmon ovaries. Additional research will be required to determine whether the actions of salmon gonadotropin are mediated by binding to these sites.

B. Mathematical Models of Receptor Binding

A number of mathematical models have been applied to data obtained from *in vitro* binding studies (Rodbard, 1973; Birnbaumer *et al.*, 1974; Catt *et al.*, 1976; Cuatrecasas and Hollenberg, 1976; Baxter and Funder, 1979; Clark and Peck, 1979). Hormone–receptor binding may be viewed as a simple bimolecular process with the hormone and the receptor as reactants and the hormone–receptor complex as the product. The binding reaction can be described by the equation

$$[H] + [R] \underset{k_2}{\overset{k_1}{\rightleftarrows}} [H{\cdot}R] \tag{1}$$

where [H] is the concentration of free hormone, [R] is the concentration of unoccupied receptor, and [H·R] is the concentration of the hormone–receptor complex.The rate of the forward reaction is dependant on [H] and [R] and a constant k_1 which describes the inherent rate at which they combine. Similarly the reverse reaction is dependent on [H·R] and a rate constant k_2. Therefore,

$$\text{Rate of forward reaction (association)} = k_1\,[H][R] \tag{2}$$

$$\text{Rate of reverse reaction (dissociation)} = k_2\,[H{\cdot}R] \tag{3}$$

At equilibrium, for each hormone and receptor which combine to form a hormone–receptor complex, a preformed hormone–receptor complex dissociates to give a free hormone and receptor. Therefore, Eq. (2) is equal to Eq. (3) and by rearrangement

$$K_a = \frac{k_1}{k_2} = \frac{[H{\cdot}R]}{[H][R]} \tag{4a}$$

or

$$K_d = \frac{k_2}{k_1} = \frac{[H][R]}{[H{\cdot}R]} \tag{4b}$$

where K_a is equilibrium association or affinity constant and K_d is the equilibrium dissociation constant. When 50% of the receptors contain hormone,

[H·R] and [R] are equal and may be cancelled in equation (4b) leaving K_d = [H]. Therefore, K_d represents the concentration of free hormone necessary to half-saturate the available receptor sites. This provides a working definition of affinity; the lower the K_d the greater the binding affinity.

Data suitable for the estimation of receptor characteristics can be obtained by incubation of a constant amount of receptor together with a constant amount of radioactive hormone and increasing amounts of unlabeled hormone. At equilibrium, radioactivity bound to the receptor preparation represents that bound in both a specific and a nonspecific manner (Section II,A). The nonspecific binding component must also be identified by parallel incubation with excess unlabeled hormone. Specifically bound radioactivity at each unlabeled hormone concentration can then be determined by subtraction of the nonspecifically bound radioactivity. From the total concentration of labeled and unlabeled hormone added and the proportion of total radioactivity specifically bound, the quantity of receptor-bound hormone can be determined. Similar data can also be obtained following incubation of the receptor preparation with increasing amounts of radioactive hormone.

Receptor-binding data are most frequently analyzed using the Scatchard plot (Scatchard, 1949), although the validity of this technique has been questioned (Klotz, 1982). For Scatchard analysis, the total receptor concentration is denoted by [N_{max}] such that:

$$[N_{max}] = [R] + [H \cdot R] \tag{5}$$

Solving for [R] in Eq. (4a) and by rearrangement the basis for the Scatchard plot is obtained:

$$\frac{[H \cdot R]}{[H]} = Ka\,([N_{max}] - [H \cdot R]) \tag{6a}$$

$$\text{or } \frac{\text{Specifically bound}}{\text{Free}} = Ka\,(N_{max} - \text{specifically bound}) \tag{6b}$$

When a single class of independent binding sites is present, a plot of specifically bound hormone-free hormone against specifically bound hormone results in a straight-line relationship with slope of $-K_a$. The X intercept of the resulting line equals [N_{max}]. By this method it has been possible to characterize the binding properties of gonadotropin receptors in the majority of studies. Nonlinear Scatchard plots can arise from the presence of a heterologous population of receptor sites as is frequently seen in steroid-receptor studies (Clark and Peck, 1979). In addition, they may result from a failure to meet one of the several assumptions necessary to perform this analysis (Khan, 1975; Catt *et al.*, 1976). These assumptions include:

1. Achievement of complete equilibrium.
2. Identical behavior of labeled and unlabeled hormone and their presence in a homogeneous form.
3. Absence of site-to-site interactions.
4. Univalence of hormone and receptor.
5. Behavior of receptor binding as a simple bimolecular reversible reaction.
6. Perfect separation of bound and free hormone without disturbing the equilibrium.
7. Absence of reactant degradation during incubation.

The equilibrium constant K_a can also be derived from the ratio of the association and dissociation rate constants (see Eq. 2 and 3). Determination of the association rate constant involves measuring receptor-bound hormone at various intervals following initiation of the binding reaction. The dissociation rate constant can be estimated by following the time course of dissociation of preformed hormone–receptor complexes. For a discussion of the theoretical and technical aspects of these approaches see Khan (1975), Catt *et al.* (1976), and Ryan and Lee (1976).

III. RECEPTOR CRITERIA

The demonstration that the target tissue binds radiolabeled hormone in a specific manner is not sufficient justification to state that a hormone–receptor interaction has been observed. Although receptor binding is characterized by specific binding, not all specific binding interactions necessarily reflect receptor binding. For example, specific binding could result from binding to a site responsible for hormone degradation and in certain instances from binding to the incubation tube or filters used in the separation of bound hormone (Cuatrecasas and Hollenberg, 1976). The identification of a hormone–receptor interaction depends on the ability to correlate the properties of a hormone-binding site with features which define the expected operational characteristics of a hormone receptor (Birnbaumer *et al.*, 1974; Khan, 1975; Cuatrecasas and Hollenberg, 1976; Munck, 1976; Ryan and Lee, 1976; Clark and Peck, 1978; Hecheter, 1978; Insel, 1978). These operational features include: (1) a finite number of binding sites, (2) a high binding affinity, (3) appropriate hormone specificity, (4) target organ specificity, (5) reversibility, (6) a functional relationship between binding and the initiation of an appropriate biological response. Each of these is considered further.

If the formation of a hormone–receptor complex is an obligatory first step in the initiation of a biological response, receptors must be present in limited

numbers so that they may be saturated at hormone concentrations which have physiological relevance. By possessing a limited number of receptor sites, the number occupied by hormone can be sensitive to small physiological changes in hormone concentration.

To function as a receptor, a hormone-binding site must have a binding affinity which is appropriate to the low concentrations of hormone found in body fluids (10^{-9}–10^{-11} *M*). Receptor affinity may be equated to the concentration of hormone required to half-saturate the receptor (Section II,B). If the affinity of a receptor was greater than the physiological concentrations of hormone, the receptor would be continually saturated. In this case, a tissue would be unable to respond to changes in hormone concentration. Should the receptor affinity be low, a receptor would be unable to recognize the hormone in the vast array of other molecules.

Hormone receptors are expected to display high affinity for a hormone or class of hormones. A given hormone–receptor binds one type of hormone and the binding of hormone to the receptor should be correlated with the biological activity of the hormone. This concept implies that there is no competition for receptor sites by substances unrelated to the hormone in question. More specifically, in the case of hormones which compete for a receptor-binding site, the relative potency of the competing hormones should be in direct proportion to their bioactivities. For an additional discussion of this aspect see Section V.

The establishment of an appropriate tissue distribution of receptor sites forms an important criterion essential to any receptor validation scheme. Because only certain tissues respond to a given hormone, the quantity of receptor present should parallel tissue responsiveness. The specific uptake of ^{125}I-labeled gonadotropin was restricted to the ovary from chum salmon and could not be demonstrated in the liver, kidney or muscle, tissues which are considered to be nonresponsive to gonadotropins (Fig. 2) (Van Der Kraak and Donaldson, 1982). Care must be taken in evaluation of this particularly when a tissue considered to be nonresponsive contains a binding site with properties synonymous with those of a hormone receptor. Such a case requires a close comparison of binding data with those obtained from a recognized target tissue.

Reversibility of binding is an important functional criterion for receptor identification because it is necessary to account for the reversal of physiological effects when hormone is removed from the incubation media. Further, receptor analysis (Section II) is based on the assumption that receptor binding is a reversible process. However, in practice, receptor binding is generally not completely reversible. Kinetic analysis of HCG receptor binding reveals an initial phase of binding which is readily reversible, followed by a second phase of slow and incomplete reversibility (Ketelslegers *et al.*, 1975;

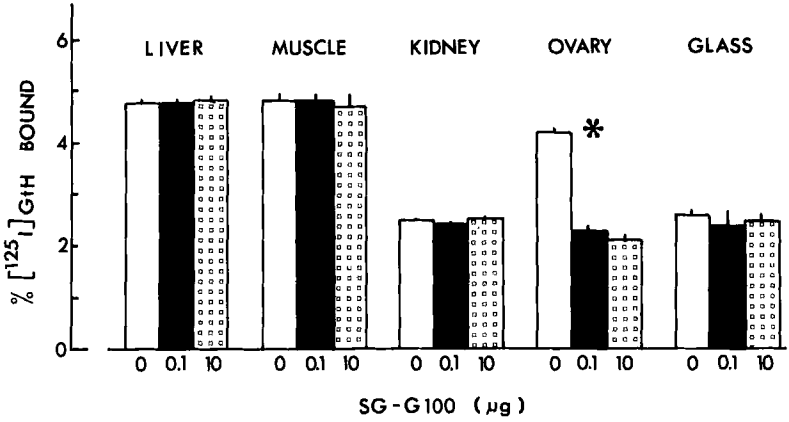

Fig. 2. The binding of ^{125}I-labeled salmon gonadotropin binding to 3000 *g* particulate fractions equivalent to 50 mg original-wet-weight of ovary, liver, kidney, and muscle from immature chum salmon. Results are expressed as the mean ± 2 SEM based on triplicate determinations of the total ^{125}I-labeled salmon gonadotropin uptake in the presence of buffer alone, 0.1 and 10 μg unlabeled SG-G100. In the case of binding to glass tubes, incubation buffer was used in replacement of the tissue.

Ryan and Lee, 1976). When the dissociation constant (K_d) is calculated from the association rate constant (k_1) and the initial rapid phase of the dissociation constant (k_2), there is excellent agreement with the value obtained by equilibrium experiments. Because of the formation of irreversible gonadotropin–receptor complexes there must be processes in addition to simple dissociation which operate to terminate receptor binding. Although these processes have not been completely elucidated, the formation of irreversible hormone–receptor complexes appears to be a more common feature of hormone–receptor interactions than traditionally believed (Catt *et al.*, 1979). Considerable evidence has accumulated that suggests gonadotropins, contrary to traditional views, enter their target cells following receptor binding (Chen *et al.*, 1977; Conn *et al.*, 1978; Anderson *et al.*, 1979; Catt *et al.*, 1979; Luborsky and Behrman, 1979; Suter *et al.*, 1980; Ahmed *et al.*, 1981). This process, termed *internalization,* involves entry of the hormone–receptor complex into the cell by way of small endocytotic vesicles. The internalized hormone subsequently undergoes degradation by the action of lysosomal enzymes. Internalization may represent a major route by which the cell terminates the stimulatory effects of receptor binding, because in the case of HCG binding to ovine corpora lutea, up to 84% of the membrane-bound HCG undergoes internalization within 24 hr (Ahmed *et al.*, 1981). Although the data are far from clear, evidence provided by Ahmed *et al.* (1981) and that obtained during periods of desensitization (see further discussion) sug-

gest that the functional life of the receptor may be restricted to a single binding of hormone.

Without question, the establishment of a functional relationship between binding and the initiation of a biological response is the most important criterion for identification of a hormone receptor. This is difficult to establish and is not often met. However, for studies with gonadotropins, three approaches have proven successful. These are as follows:

1. Correlation between binding and response at varied stages of the life cycle or physiological states.
2. Correlation between binding and response following an alteration in target organ sensitivity.
3. Simultaneous evaluation of binding and response at varied levels of added hormone.

If gonadotropin-binding sites are receptors, then their numbers should change in direct relation to tissue responsiveness. For example, the appearance of LH-binding sites in the fetal rabbit testis corresponds to the maturation of Leydig cells and the ability of these cells to produce testosterone in response to HCG (Catt *et al.*, 1975). The most frequent correlation has involved the relationship between the number of LH-binding sites and ovarian maturation. The number of LH-binding sites has been correlated with follicular size in pigs, sheep, and cows (Channing and Kammerman, 1973; Lee, 1976; Ryan and Lee, 1976; Gosling *et al.*, 1979) and with stage of the estrous or menstrual cycle in rats, sheep, and humans (Nimrod *et al.*, 1977; Diekman *et al.*, 1978; McNeilly *et al.*, 1980). For example, Lee (1976) reported that the binding of HCG to porcine ovarian follicles progressively increased in relation to follicle size and was accompanied by a concomitant change in cAMP production in response to HCG. Similarly, during the luteal phase, maximum binding of HCG to the corpus luteum coincides with the time of greatest progesterone secretion. No direct comparisons have been described for gonadotropin binding and tissue responsiveness in fish. However, Cook and Peter (1980) reported the increased total uptake of ^{125}I-labeled carp gonadotropin *in vivo* by the ovaries from maturing and mature goldfish compared to sexually regressed individuals. A necessary avenue for future research would be to complement these findings with the simultaneous evaluation of specific gonadotropin binding and steroidogenic activity.

The functional significance of LH-binding sites in the adult rat testis has been studied extensively following injection of LH and HCG *in vivo* (Conti *et al.*, 1976, 1977a,b; Dufau and Catt, 1978; Catt *et al.*, 1979, 1980; Sharpe and McNeilly, 1980). After an initial activation of cAMP production and steroid production the testis enters a refractory or desensitized state charac-

terized by a marked impairment of these responses when tested *in vitro*. Desensitized tissues display a reduced sensitivity to hormone stimulation when tested in dose-response experiments (i.e., response curve shifted to the right), or in extreme cases a reduction in the maximum response evoked by hormone stimulation. By simultaneous evaluation of the number of LH-binding sites, it has been possible to demonstrate a reduced number of binding sites during desensitization and a concurrent increase in the number of binding sites as hormone sensitivity returns. Studies of this type have proven most useful in the establishment of a functional role for gonadotropin binding sites in mammals and may have application in studies with fish.

In an analogous manner, it has been possible to establish a role for gonadotropin binding sites under conditions in which target tissue sensitivity is increased. Numerous mammalian examples can be found in the literature (see Catt *et al.*, 1979, 1980; Gosling *et al.*, 1979; Henderson, 1979; Richards, 1979; Ahren *et al.*, 1980). For example, in the ovary 17β-estradiol has a stimulatory effect on FSH receptor levels, whereas FSH and 17β-estradiol increase the amount of LH receptors.

One aspect of fish reproduction physiology that has received considerable attention relates to the use of a priming dose of gonadotropin prior to the induction of ovulation with a higher gonadotropin dose 3 days later (Donaldson and Hunter, Chap. 7, Vol 9B). Whether the effect of the priming dose of gonadotropin is to increase receptor numbers or to activate the synthesis of steroid precursers remains to be considered. Direct evidence for an effect of a fish gonadotropin on its own binding sites comes from the unpublished research of Breton and Peter. In their experiments, which involve the binding of ^{125}I-labeled carp gonadotropin to intact goldfish oocytes *in vitro*, no specific uptake of hormone could be distinguished during a 1-hr incubation. By exposing the oocytes to low doses of carp gonadotropin for 1 hr prior to addition of the labeled hormone, a competitive binding component could be recognized. Whether the action of carp gonadotropin is to induce the synthesis of new receptor or to effect a redistribution of receptors within the membrane remains to be established.

A third approach used to determine the significance of gonadotropin binding sites is based on the assumption that the relative degree of binding should be reflected by the intensity of the biological response. This approach necessitates the use of intact functional cells so that hormone binding and cellular response can be measured in the same preparation. The relationship between HCG binding, cAMP accumulation, and testosterone production has been extensively studied in the rat testis either by the use of interstitial cells prepared by collagenase dispersal or Leydig cells purified on Metrizamide gradients (Catt and Dufau, 1976, 1977; Dufau and Catt, 1978). In these cases, incubation of graded amounts of HCG was accompanied by

commensurate increases in hormone binding and cAMP production. Such findings provided evidence that binding sites can be regarded as functionally active receptors. However, when responses more distal to binding and cAMP production are considered, interpretation of dose-response data became more complicated. For example, the dose-response curve for testosterone production was shifted far to the left compared to that for hormone binding and cAMP production. Actually only about 1% of the available HCG binding sites need be occupied to elicit a full steroidogenic response. This marked discrepancy between hormone binding and steroid production in the testis and also in isolated luteal cells (Dufau *et al.*, 1977) casts doubt on the role of hormone-binding sites in controlling steroidogenesis. Subsequent analysis of intracellular cAMP pools including that bound to the regulatory subunit of protein kinase revealed good correlation between hormone binding, cAMP production, protein kinase activation, and steroidogenesis (Dufau *et al.*, 1977). Clearly, an examination of the significance of hormone-binding sites through dose-response relationships is a complex issue. These experiments led to the concept that gonadal tissues contain "spare" or excess receptors beyond those required for maximal steroid production. The existance of a receptor reserve need not imply that these excess binding sites are without physiological significance. For example, excess hormone-binding sites could serve to maintain tissue sensitivity in the presence of low hormone levels by enhancing the probability of a hormone–receptor interaction (Baxter and Funder, 1979; Catt *et al.*, 1980). A receptor reserve could also serve as a reservoir of sites to replace those utilized or processed during normal physiological control of steroid production (Catt *et al.*, 1980).

Whether gonadotropin receptors in excess of those needed for the activation of steroidogenesis are present in fish is not known. Such an arrangement would explain the low and often undetectable levels of gonadotropin in the plasma of salmonids prior to maturation (Crim *et al.*, 1975). Based on data regarding *in vitro* activation of adenylate cyclase and steroid production, it is possible to speculate that this may indeed be the case. Bioassays used to measure gonadotropin activity in fish based on steroid production are frequently greater than 100-fold more sensitive than those based on cAMP production (Fontaine *et al.*, 1972; Idler *et al.*, 1975; Nagahama *et al.*, 1982; Yaron *et al.*, 1982). Again further research is necessary to clarify this aspect.

IV. TECHNICAL CONSIDERATIONS

In this section, the experimental procedures for the investigation of gonadotropin receptors in fish is described. Discussion is based on the extensive literature available from other vertebrate groups. The general ap-

proach to gonadotropin-receptor studies involves the preparation of a radiolabeled derivative of a highly purified gonadotropin, characterization of the biological properties of the labeled derivative, and finally, direct-binding studies with the target tissue (Birnbaumer, 1978).

A. Preparation of Labeled Gonadotropins

The most critical step for receptor-binding studies is the preparation of a labeled derivative. It must be stressed that conditions routinely employed to prepare labeled gonadotropins for use in a RIA are generally not suitable for receptor-binding studies. Gonadotropins are susceptible to damage during radiolabeling and frequently show a marked reduction in biological activity while retaining the capacity to bind to specific antisera in a RIA. For example, using salmon gonadotropin iodinated to a level of about 150 μCi/μg, greater than 85% of the label was bound by excess antibody, but no specific binding could be demonstrated following incubation with gonadal tissue (G. Van Der Kraak and E. M. Donaldson, unpublished). Radiolabeled gonadotropins are commonly prepared using ^{125}I with iodination achieved by modification of the chloramine T (Hunter and Greenwood, 1962) or lactoperoxidase (Thorell and Johansson, 1972) methods. Several features identify ^{125}I as the isotope of choice, including its availability in a virtually carrier-free state, high isotope abundance, relatively long half-life, and high counting efficiency (Khan, 1975; Saxena, 1976; Birnbaumer, 1978). The obvious disadvantage of using ^{125}I is that iodide is not a normal constituent of gonadotropins. Incorporation of ^{125}I into gonadotropins involves an oxidation and in most methods a reduction step both of which may lead to a loss of activity. By careful selection of conditions used during iodination and maintenance of the level of incorporation to about 1 atom of ^{125}I per molecule, biological activity can be retained. As an alternative to iodination, labeled gonadotropin may be prepared with tritium which offers a longer half-life tracer and labeling accomplished by substitution rather than addition. The disadvantages of using tritium include a labeling protocol involving extensive chemical modifications which can result in biological inactivation coupled with the production of a very low specific activity tracer (Means and Vaitukaitas, 1972; Khan, 1975; Saxena, 1976).

The choice of chloramine T or lactoperoxidase methods for iodination depends to a large extent on the past experience and preference of the particular investigator. Although no difference has been reported for the binding properties of HCG iodinated by chloramine T or lactoperoxidase methods, mammalian LH and FSH preparations are generally more satisfactorily prepared by the lactoperoxidase method (Catt *et al.*, 1976). For studies with salmonids, no difference could be distinguished between the binding

properties of salmon gonadotropin iodinated to less than 1 mole ^{125}I/mole gonadotropin prepared by either method (G. Van Der Kraak and E. M. Donaldson, unpublished). Similarly B. Breton and R. E. Peter (personal communication) could distinguish no difference in the binding of carp gonadotropin prepared by either method when incubated with goldfish ovaries. For both methods, no single protocol has gained widespread acceptance; for specific protocols, refer to Miyachi *et al.* (1972), Catt *et al.* (1976), Reichert (1976), Saxena (1976), and Licht *et al.* (1977a). In general, the level of oxidizing agent, chloramine T or hydrogen peroxide in the case of the lactoperoxidase method, should be reduced to the lowest effective level. Other steps which have proven useful include performing the iodination for short periods at low temperature and when possible omitting the use of a reducing agent.

Apart from the chloramine T and lactoperoxidase methods which rely on the incorporation of ^{125}I to tyrosyl residues, alternate methods of labelling proteins with ^{125}I are available. Most common of these involves the conjugation of ^{125}I-labelled *N*-succinimidyl 3-(4-hydroxyphenyl) propionate to free amino groups of proteins (Bolton and Hunter, 1973). This method offers the potential advantage of reducing the problems associated with the exposure of proteins to oxidizing and reducing agents and warrants testing with fish gonadotropins.

Following iodination, labeled hormone is separated from free ^{125}I most frequently by chromatography on a small (1 × 10–20 cm) Sephadex G50 column. The protein peak may then be used directly for receptor-binding studies. A drawback of this procedure is that damaged hormone is not separated from intact hormone. To increase the proportion of labeled hormone capable of receptor binding, one of several chromatographic procedures may be employed. In each instance, a screening is done by direct-binding studies to select only that portion of the ^{125}I-labeled gonadotropin peak which displays the highest binding activity. Methods include gel filtration on a much larger column (up to 100 cm) of either Sephadex G100, Bio-Gel P60 or Sephacryl S200 which expand the protein peak (Catt *et al.*, 1976; Licht and Bona Gallo, 1978; Sairam, 1979). Damaged hormone may also be partially resolved from intact labeled hormone by chromatography on dry cellulose powder (Catt *et al.*, 1971, 1972; Tsuruhara *et al.*, 1972). In this procedure, damaged gonadotropin passes through the powder and intact gonadotropins are retained. The gonadotropin bound to the cellulose powder can then be eluted in buffers containing increasing amounts of bovine serum albumin or γ-globulin. A more frequently used technique involves group-specific affinity chromatography on Concanavalin A (Con A)–Sepharose (Dufau *et al.*, 1972a, 1973). In this procedure, intact gonadotropin binds to the gel and damaged hormone and nonglycoprotein contaminants pass through the gel when eluted with carbohydrate-free buffer. By adding competing carbohy-

drate in the form of either glucopyranoside or mannopyranoside the labeled gonadotropin is eluted from the column.

Two exeriments have been conducted to evaluate the effects of further chromatographic purification on the binding of ^{125}I-labeled salmon gonadotropin to chum salmon ovaries. By chromatography on Con A–Sepharose the specific binding of ^{125}I-labeled salmon gonadotropin was increased from 2.7% to 4.0%. Chromatography on a 1.3 × 35 cm Sephacryl S200 column increased the specific binding of the tracer from 1.7% to 2.5%. In both instances the increased binding was attributed to a reduction in the amount of nonspecific binding. Clearly further research is needed in this area.

The most effective means of increasing the proportion of labeled gonadotropin that binds to receptor sites is by receptor purification. This method is based on the premise that, in a mixture containing biologically intact and inactivated labeled gonadotropin, only the intact hormone will bind to the receptor site. Following incubation of labeled gonadotropin with the target tissue, the mixture is centrifuged to remove free gonadotropin. Labeled gonadotropin is then dissociated from the receptor by reduction of pH or elevated temperature and collected in the supernatant (Dufau *et al.*, 1972b; Ketelslegers and Catt, 1974; Catt *et al.*, 1976). Both of these procedures elute the receptor-bound gonadotropin with minimal release of nonspecifically bound radioactivity. Labeled gonadotropin recovered in this manner possesses an increased capacity to bind to receptor sites and a concomitant increase in biological activity when compared to the starting material. For example, ^{125}I-labeled human FSH eluted from testis receptors at pH 5 displayed 28% specific binding when incubated with fresh tissue compared to only 8.2% specific binding for the original tracer subjected to acid treatment (Ketelslegers and Catt, 1974). The major disadvantage of this technique is that the yield of labeled gonadotropin is low, representing about 5% of the starting radioactivity (Catt *et al.*, 1976). Receptor purification, although not a frequently used technique, can be particularly useful when the tracer preparation displays a low level of binding and may have application for studies with fish gonadotropins.

B. Characterization of Labeled Gonadotropins

Gonadotropin-receptor studies ultimately depend on the ability to characterize the labeled hormone. A primary consideration involves testing the hormone preparation for biological activity following radiolabeling. In addition, when kinetic parameters are to be measured it is necessary to accurately determine the quantity of hormone incubated in the receptor assay. A major complication arises in all gonadotropin-receptor-binding studies because purified gonadotropins are seldom of maximal attainable biological activity (Ketelslegers *et al.*, 1975; Dufau and Catt, 1978). Consequently,

even in the absence of damage during labeling, a portion of radioactivity taken to represent gonadotropin will be inactive. Although specific activity can be estimated from the incorporation of ^{125}I during iodination, a more meaningful value can be obtained in an assay which reflects the biological activity of the tracer preparation. Because it is generally assumed that only biologically active hormone is capable of receptor binding, this fraction must be identified and used to calculate specific activity. Failure to apply these corrections will lead to a false determination of both the affinity and capacity of gonadotropin receptor sites. These principles are detailed in Catt *et al.* (1976) and Dufau and Catt (1978). In an example provided by Catt and coworkers, 100,000 cpm of ^{125}I-labeled HCG corresponded to 1 ng of native hormone as determined by receptor assay. However, only 50% of this radioactivity was capable of receptor binding. Therefore, a more accurate estimate of the specific activity would be 50,000 cpm/ng because 50% of the radioactivity represents inert material. In the case where 20,000 cpm of ^{125}I-labeled HCG out of a total of 100,000 cpm binds specifically to a receptor preparation this corresponds to 0.4 ng of bound (B) HCG, 0.6 ng of free (F) HCG and a B/F ratio of 0.67. Failure to make these corrections leads to the erroneous conclusion that 0.2 ng of HCG was bound, and 0.8 ng HCG was free, with a B/F ratio of 0.25.

Conventional *in vivo* bioassays can be used to quantify the activity of labeled gonadotropins, but are of limited application because of their inherent imprecision which makes quantification of a loss of bioactivity difficult when this is less than 50% (Reichert, 1976). A convenient method for assessing the effect of iodination on gonadotropin activity involves testing serial dilutions of the iodination mixture by *in vitro* bioassay based on cAMP or steroid production. In addition, the activity of the starting material and hormone iodinated in the absence of iodine or oxidizing agent should be tested. If no damage is incurred during the iodination procedure, a series of identical curves should be obtained for all preparations (Birnbaumer, 1978; Abramowitz *et al.*, 1982). This approach was used to quantify the activity of salmon gonadotropin prepared by the lactoperoxidase method (Fig. 3) (Van Der Kraak and Donaldson, 1982). No difference could be distinguished between the capacity of intact hormone and iodinated hormones to augment testosterone production in minced testis pieces incubated *in vitro*. These results suggest the lack of effect of lactoperoxidase iodination on the biological activity of salmon gonadotropin.

Although *in vitro* bioassay can be used to determine the specific activity of labeled gonadotropin preparations, a convenient method involves "self-displacement" in a receptor assay. For this procedure, two displacement curves are required. The first is generated from the binding of a constant amount of labeled gonadotropin and competition resulting from the addition of increasing amounts of unlabeled hormone. A second displacement curve

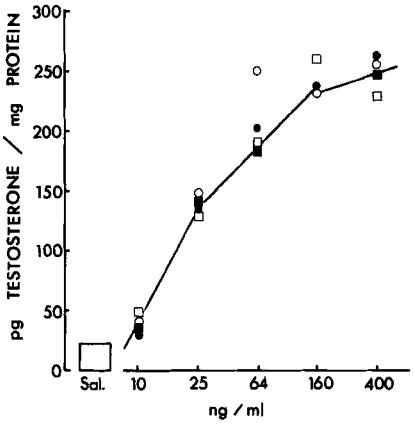

Fig. 3. The effect of lactoperoxidase iodination on the biological activity of salmon gonadotropin SGA-2359. Briefly, 10 μl of iodide (0.6 nmoles of KI supplemented with 100,000 cpm ^{125}I) was combined with 25 μl 0.5 *M* phosphate buffer followed by 50 μl (10 μg) gonadotropin and 10 μl lactoperoxidase (100 IU/ml Calbiochem). Iodination was achieved by two 10 μl H_2O_2 (30% H_2O_2 diluted 1:30,000) additions at 2-min intervals. The reaction mixture was then serially diluted in physiological fish saline for determination of biological activity by incubation with minced coho salmon testis pieces *in vitro* according to the methodology described by Bona Gallo and Licht (1981). The activity of this preparation was compared to that for intact hormone and SGA-2359 iodinated with either the KI or H_2O_2 omitted. Testosterone production was measured by radioimmunoassay. The λ for the assay was 0.19. The incorporation of ^{125}I to gonadotropin was determined by TCA precipitation described by Cook and Peter (1980). Symbols are as follows: ●, intact; ○ no iodine; ■, no H_2O_2; □ ^{125}I-labeled GtH (0.88 I per GTH).

is generated by incubating increasing amounts of labeled gonadotropin. By plotting the percentage bound as a function of unlabeled hormone concentration or cpm ^{125}I-labeled gonadotropin added, two parallel displacement curves can be obtained (Catt *et al.*, 1976; Diekman *et al.*, 1978; Barenton and Pelletier, 1980). Specific activity can be determined from the amount of labeled hormone (μCi) required to achieve a 50% reduction of specific binding divided by the quantity of unlabeled hormone (μg) to achieve the same inhibition. The self-displacement method can be performed in a RIA, although values may not be valid in terms of biological activity (Catt *et al.*, 1974). Specific activity estimated by receptor assay commonly provides a higher value than that determined by RIA (Catt *et al.*, 1974).

The proportion of radiolabeled hormone that binds to receptor preparation is routinely identified by incubation of labeled hormone with increasing amounts of tissue in a receptor assay. Generally, the specific binding of labeled hormone increases with increasing amounts of tissue and reaches a plateau corresponding to the proportion of hormone capable of receptor binding. When investigating this parameter, particular attention must be

directed to the effects of tissue concentration and temperature. For example, decreased binding may occur at high tissue concentrations and may relate to increased degradation of the hormone or the receptor (Catt *et al.*, 1976). Temperature affects both the rate of binding and the proportion of hormone bound to receptor preparations (Catt *et al.*, 1976; Licht and Midgley, 1976; Darga and Reichart, 1978; Adachi *et al.*, 1979; Kubokawa and Ishii, 1980). Some of the effects of temperature may relate to an influence on tracer or receptor degradation (Catt *et al.*, 1976). A second explanation for the effects of temperature may relate to the intrinsic nature of receptor molecules or the membrane–receptor complex for individual species (Kubokawa and Ishii, 1980). An additional discussion of these points is provided in Section IV,D.

The binding characteristics of labeled gonadotropins vary depending on the type of gonadotropin and the source of the receptor preparation. For example, up to 60% of labeled HCG will bind to excess mammalian gonad preparations compared to only 20–40% binding for mammalian LH preparations (Catt *et al.*, 1974, 1976). Turkey LH has a low binding activity when compared to these preparations because only about 5% of ^{125}I-labeled turkey LH binds specifically to avian gonad preparations, but 10% specific binding could be demonstrated in lizard gonad preparations (Bona Gallo and Licht, 1979). Labeled-FSH preparations commonly exhibit less than 15% specific binding to mammalian receptor preparations (Catt *et al.*, 1976; Nimrod *et al.*, 1976; Sairam, 1979) (Catt *et al.*, 1976; Nimrod *et al.*, 1976; Sairam, 1979) although values of up to 40% specific binding have been reported (Darga and Reichert, 1978; Chiauzzi *et al.*, 1982). Mammalian FSH preparations have a high binding activity (up to 40% specific binding) when tested in birds, reptiles, and amphibians (Licht and Midgley, 1976; Ishii and Farner, 1976; Adachi *et al.*, 1979; Bona Gallo and Licht, 1979). Nonmammalian FSH preparations have a low binding activity in that about 7–15% specific binding was obtained for turkey FSH and sea turtle FSH preparations (Licht *et al.*, 1977b; Bona Gallo and Licht, 1979). Radiolabeled fish gonadotropin preparations have only low levels of specific binding to fish gonad preparations. Katsumi Aida and S. Ishii (personal communication) observed a maximum of 2.5% specific binding of purified salmon gonadotropin when incubated with particulate fractions obtained from rainbow trout testes. Studies using a different purified salmon gonadotropin (G. Van Der Kraak and E. M. Donaldson, unpublished) demonstrated up to 6.0% specific binding to chum salmon ovaries. In studies of the binding of ^{125}I-labeled carp gonadotropin to goldfish ovaries B. Breton and R. E. Peter (personal communication) observed up to 7.5% specific binding. An explanation for the low levels of specific binding that characterizes the binding of radiolabeled nonmammalian gonadotropin preparations is not readily apparent. Although it may be suggested that nonmammalian gonadotropins contain a higher proportion

of biologically inactive molecules than mammalian gonadotropins, the available data dealing with the biological activity of these hormones generally does not support this hypothesis. Whether the inability to demonstrate high binding activity for fish gonadotropins is merely a technical problem will require additional research. The application of chromatographic or receptor purification schemes (Section IV,A) may be of considerable benefit for future studies with fish gonadotropins. Perhaps an alternate method of preparing labeled fish gonadotropin may be appropriate. For example, the incorporation of (*N*-succinimidyl 3-(4-hydroxy5-[^{125}I] iodophenyl propionate) (Bolton, 1977) to free amino groups rather than directly incorporating iodine to tyrosine residues as for chloramine T or lactoperoxidase methods may prove useful.

C. Receptor Preparations

The preparation of gonadal tissue as a source of receptors has taken several forms including tissue slices, dispersed cells, whole homogenates, crude particulate fractions obtained from homogenates, and membrane fractions at various stages of purification (Catt *et al.*, 1976; Ryan and Lee, 1976; Dufau and Catt, 1978). It is important to keep in mind that the gonads are composed of several cell types and that not all cell types contain gonadotropin receptors. Particularly when receptor concentration is low the inclusion of extraneous cell types can result in high levels of nonspecific binding which masks receptor binding. For example, in the mammalian testis, LH receptors are present in Leydig cells which form part of the interstitial cell compartment, but FSH receptors are restricted to Sertoli cells which are found in the seminiferous tubule compartment (Catt *et al.*, 1976). In the ovary, LH receptors are present in granulosa and thecal cells as well as the corpus luteum (Catt *et al.*, 1976; Richards, 1979). Ovarian FSH receptors are restricted to granulosa cells (Catt and Dufau, 1976). The use of specific cell types as the source of gonadotropin receptors has become a common practise in mammalian studies. Recently, Nagahama *et al.* (1982) reported that granulosa and thecal cells could be separated from fish oocytes. This technique may have application in the distinction of sites of gonadotropin binding in fish ovaries.

Gonadotropin-binding studies were initially performed using tissue slices (Lee and Ryan, 1971; Means and Vaitakaitis, 1972) which minimizes manipulation of the target tissue, but adds the complicating feature of a diffusion barrier. Currently most gonadotropin-binding studies utilize particulate fractions obtained by centrifugation of a crude gonadal homogenate. When particulate receptors are prepared, the centrifugal force required to sediment binding sites varies depending on the media used. For example,

80% of the LH-binding sites present in the rat testis are recovered by centrifugation at 1500 *g* when prepared in phosphate buffered saline (Catt *et al.*, 1974). For testis homogenates prepared in buffers containing 0.3 *M* sucrose, a broader distribution of binding sites is evident necessitating centrifugation at 10,000–15,000 *g* (Catt *et al.*, 1976; Dufau and Catt, 1978). In immature chum salmon, the greatest uptake of ^{125}I-labeled salmon gonadotropin was localized to a 3000 *g* particulate fraction of an ovarian homogenate prepared in phosphate-buffered saline (Fig. 4) (G. Van Der Kraak and E. M. Donaldson, unpublished). Crude particulate fractions offer the advantage of a rapid and reproducible technique for receptor preparations. Crude particulate fractions contain extraneous tissue in the form of nonreceptor cell types and intracellular organelles with associated degrading enzymes. Extensively purified membrane fractions may be advantageous because they lessen the risk of including degrading enzymes (Saxena, 1976). However, the use of purified membrane fractions poses additional problems. For example, the yield of membrane protein is frequently low, and the membrane preparation is often not representative of the target tissue because of preferential accumulation of membranes from a given cell type. Purification of plasma membranes is a lengthy process and comparative studies of tissues in different functional states could be complicated by different degrees of purification and recovery (Khan, 1976). For studies in which binding and biological response are to be correlated, intact viable cells are

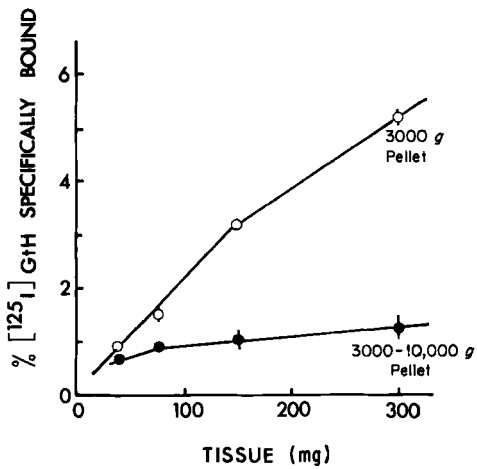

Fig. 4. Specific binding of ^{125}I-labeled salmon gonadotropin to increasing quantities of 3000 *g* and 3000–10,000 *g* particulate fractions of ovarian homogenates from immature chum salmon. Specific binding was calculated as described in Fig. 1. Values are the mean ± 2 SEM for duplicate determinations.

required. These may be obtained by enzyme or mechanical dispersal and by tissue culture. A potential problem associated with viable cells is that, along with the retention of metabolic activity, hormone degradation may be enhanced. Another problem that may arise because of enzyme dispersal is that enzyme treatment could affect the affinity or number of binding sites.

The ability to demonstrate receptor binding can be facilitated by use of animals in a particular stage of their life cycle. For example, rat testicular FSH receptors are most easily demonstrated in immature animals when receptors are most abundant on a wet tissue weight basis (Catt *et al.*, 1976). Similar results have been described for FSH-binding sites in the domestic chicken (Bona Gallo and Licht, 1979; Etches and Cheng, 1981) and in a number of reptilian species (P. Licht, personal communication). In the case of LH receptors in the ovary, receptor numbers increase markedly in the progression from small to medium to large follicles. Although LH receptors are present in low numbers in the immature rat ovary, pretreatment with pregnant mare serum gonadotropin to produce pseudopregnant ovaries provides an abundant source of receptors (Lee and Ryan, 1973; Catt *et al.*, 1976).

Although gonadotropin receptors are relatively insoluble in aqueous media, treatments with nonionic detergents such as Triton X-100, Lubrol PX, and Lubrol WX are effective in solubilization of the receptor (Dufau *et al.*, 1974; Catt *et al.*, 1976; Saxena, 1976; Dufau and Catt, 1978). The definition of a soluble receptor is somewhat arbitrary (Catt and Dufau, 1977). Soluble receptors are not sedimented by centrifugation at 360,000 *g* for 3 hr, and are retarded by gel filtration on 6% agarose. They can be identified by direct-binding studies, and various chromatographic techniques can be used to determine the physiochemical properties of the receptor. Technical problems associated with receptor solubilization tend to preclude the use of solubilized receptors as the initial protocol for receptor identification. Extraction with detergent usually results in the loss of the functional coupling between hormone binding and the activation of cellular response such as adenylate cyclase activity. Comparison of binding properties determined for solubilized and particulate receptors are often difficult to interpret. Solubilized receptors are generally more labile than particulate receptors. Quantitative studies of extraction efficiency indicate that not all gonadotropin receptors are extracted; performed hormone–receptor complexes are extracted with a greater efficiency than unoccupied receptors. A further complication arises from a change in receptor-binding affinity following solubilization. For example, LH-receptor affinity tends to decrease but FSH-receptor affinity increases following solubilization (Dufau and Catt, 1978). It should also be noted that the physiochemical properties of solubilized receptors differ depending on the detergent used.

D. Methods of Separation

Receptor studies require a simple and rapid means of separating free hormone from that bound to the receptor. For gonadotropin-receptor studies using intact cells or particulate fractions centrifugation or filtration methods are most common. The problems associated with these methods are discussed by Birnbaumer (1978).

For separation by centrifugation, the receptor preparation is diluted with 1–2 ml of incubation buffer and then centrifuged in the cold to compact the receptor preparation. Frequently, the receptor preparation is resuspended in incubation buffer and recentrifuged to reduce the quantity of radioactivity trapped on the compacted tissue. This procedure has been used in studies of gonadotropin binding in fish (Van Der Kraak and Donaldson, 1982; K. Aida and S. Ishii, personal communication). Particular care must be taken to ensure that the centrifugal or "*g*" force used is sufficient to compact the entire receptor preparation. Catt *et al.* (1974) suggest that the *g* force used for the separation of bound hormone should be the same as that used to prepare the original binding fraction. Failure to comply with this protocol can result in a proportion of the receptor-bound hormone being included in the free hormone fraction. Exceptions to this general rule exist, because centrifugation at 1500 *g* is sufficient to sediment all LH-binding particles in rat testis homogenates prepared by centrifugation at 20,000 *g*. This is because of aggregation of smaller binding particles during incubation (Dufau and Catt, 1978).

Separation of bound and free hormone can be effected by filtration through glass-fiber or millipore filters. By this method, receptor-bound hormone is retained on the filter but free hormone passes through the filter. Particular attention must be given to the nonspecific trapping (adsorption) of free hormone to the filter. Nonspecific binding can in some cases be minimized by selection of an appropriate type of filter and by presoaking the filter with a high concentration of competing protein, usually albumin (Khan, 1975; Birnbaumer, 1978). Following the initial binding of receptor to the filter, nonspecific binding can be reduced by washing with buffers containing albumin. Problems may arise as a result of clogging of filters by large amounts of membrane or cells, but the filtration technique can be of particular advantage when the mass of receptor protein is low (Catt *et al.*, 1974).

Alternate methods of separation are required for soluble receptors (Dufau *et al.*, 1974). Free and receptor-bound hormone can be separated by gel filtration or sucrose density centrifugation. Because these techniques are cumbersome, a more rapid means of separation involving precipitation of receptor-bound hormone by polyethylene glycol has been used (Dufau *et al.*, 1973; Catt *et al.*, 1976; Saxena, 1976).

E. Additional Considerations

A necessary requirement for gonadotropin-receptor analysis involves determination of the extent of labeled hormone and receptor degradation occurring during incubation. Damage during incubation has been associated with several variables including the nature and amount of the tissue preparation, time, and temperature (Lee and Ryan, 1973; Ketelslegers *et al.*, 1975; Catt *et al.*, 1976; Ryan and Lee, 1976). The process of degradation or inactivation is not clearly understood. For example, LH inactivation which occurs following incubation with particulate rat ovarian receptors has not been accompanied by fragmentation of the molecule or a loss in immunoreactivity. Incubation damage tends to be greatest in whole homogenates and crude membrane fractions. In some cases it has been possible to reduce incubation damage by further purification of the tissue preparation and by the use of proteolytic enzyme inhibitors such as Trasylol or phenylmethyl sulfonylfluoride (Saxena, 1976; Morris and Saxena, 1980). Temperature appears to be a major factor contributing to incubation damage. In mammalian studies, incubation damage tends to be minimal at 4° C with the degree of damage increasing at elevated temperatures (Ketelslegers *et al.*, 1975; Diekman *et al.*, 1978; Cameron and Stouffer, 1982). The rate of incubation damage at 37° C was greater than that observed at 24° C for HCG binding to rat testicular homogenates. The extent of hormone damage can vary depending on the gonadotropin, with HCG being more stable than LH (Lee and Ryan, 1973; Cameron and Stouffer, 1982).

To determine the extent of hormone inactivation during incubation, labeled gonadotropin is first preincubated with the receptor preparation and the supernatant or filtrate remaining after separation of bound hormone is assessed for its ability to bind to a fresh receptor preparation. The degree of inactivation can be determined from the loss of binding activity when compared to fresh tracer or labeled gonadotropin preincubated in buffer alone. For this calculation it is necessary to account for inactive hormone initially present in the labeled hormone preparation and the amount bound to the receptor during the first incubation (Catt *et al.*, 1976). Receptor degradation can be monitored in a similar fashion. This involves preincubation of the receptor preparation in the absence of hormone for various times followed by an examination of the capacity of the preincubated tissue to bind radiolabeled hormone. Comparisons can be made with the binding activity of ice-stored or freshly prepared receptor preparations (Ketelslegers *et al.*, 1975; Ryan and Lee, 1976) or with portions of the receptor preparation not subjected to a period of preincubation (Diekman *et al.*, 1978).

Inactivation of the labeled hormone and receptor during incubation will

obviously have a marked effect on the calculation of binding parameters. Catt *et al.* (1976) have suggested that inactivation will affect only free hormone and free receptor owing to the observation that receptor-bound hormone retains its biological activity. When equilibrium-binding studies are performed, damaged hormone will affect the calculation of free hormone and consequently the calculation of K_d, but not the number of binding sites. Receptor damage affects the bound fraction and, therefore, the calculation of both K_d and the number of sites. Although corrections can be applied to account for incubation damage (Ketelslegers *et al.*, 1975; Ryan and Lee, 1976), the obvious solution is to select incubation conditions under which damage is minimal.

The potential that an inhibitor of receptor binding may be present or formed during incubation must be recognized. Aqueous extracts from pseudopregnant or pregnant ovaries from several mammalian species have been demonstrated to inhibit the specific binding of LH and HCG to ovarian receptors *in vitro* (Yang *et al.*, 1976, 1978, 1979; Sakai *et al.*, 1977). Similarly, inhibitors of FSH binding to both testicular and ovarian receptors have been reported (Reichert and Abou-Issa, 1977; Reichert, 1978; Darga and Reichert, 1978; Sato *et al.*, 1980). Both LH- and FSH-binding inhibitors are low-molecular-weight compounds; therefore, inhibition of binding can not be explained by the presence of gonadotropins in the tissue extracts. Several approaches can be used to determine the presence of binding inhibitors, for example, by testing receptor binding in crude homogenates versus extensively washed or purified tissue particulate fractions.

V. THE APPLICATION OF RECEPTOR-BINDING STUDIES

This section reviews the application of studies involving gonadotropin receptors and discusses the future application for studies with fish gonadotropins.

Gonadotropin receptors have been used extensively to measure gonadotropins in a manner analogous to the use of antibodies in a RIA. When a radioreceptor assay (RRA) is used, the quantity of hormone in a sample will be reflected by the extent to which the receptor binding of radiolabeled hormone is inhibited. In theory, RRA more closely resembles a bioassay than does RIA because the detection of hormone by RRA depends on the specific molecular conformation for receptor binding. This property differs from RIA which uses antibodies that are more likely to be directed to other portions of the molecule (Catt and Dufau, 1976). It must be stressed that

RRA analysis will not ncessarily provide the same information as bioassay and as such should not be viewed as a replacement. Results from a series of experiments by Licht and co-workers indicate a marked difference in gonadotropin activity may occur when determined by bioassay and RRA (Licht *et al.*, 1977a,b, 1979; Bona Gallo *et al.*, 1978; Bona Gallo and Licht, 1979; Licht, 1980a). An example of this phenomena comes from studies of LH- and FSH-binding sites in turkey gonads using homologous radioligands. Turkey LH-binding sites are highly specific for turkey LH in that turkey FSH has less than 1% LH activity. In sharp contrast, turkey FSH-binding sites were less specific because turkey LH had 13–48% FSH activity in binding studies although immunological studies indicate less than 0.2% contamination by FSH in the LH preparation. Similarly Licht *et al.* (1977a) demonstrated an unexpected high potency of LH for FSH-binding sites in the sea turtle when either human FSH or sea turtle FSH was used for radiolabeling. Additional studies indicate a marked discrepancy between the activity of equine LH when tested in several hetrologous FSH RRA and bioassay methods (Licht *et al.*, 1979). In summary, these results indicate that although a gonadotropin shows a high activity when tested by RRA it does not necessarily imply a high potency in physiological action (Licht, 1980a). These results also suggest that the properties of a hormone that are related to receptor binding must be considered distinct from those that enable it to stimulate a physiological response. For example, Channing *et al.* (1978) demonstrated that the removal of carbohydrate groups from HCG had little effect on receptor binding when compared to its ability to augment cAMP production. One possible explanation for these discrepancies between binding data and bioassay data may be that a hormone or a derivative may behave as an antagonist and bind to the receptor but fail to elicit a response. Such a situation occurs for asialogonadotropins. Asialo-HCG has a lower activity than does HCG when tested by *in vivo* or *in vitro* bioassay, but has a higher binding affinity for HCG receptors than does HCG (Tsuruhara *et al.*, 1972; Catt *et al.*, 1976; Saxena, 1976; Brand *et al.*, 1981).

Although the previous discussion suggests that RRA may not always be appropriate for the prediction of biological activity of gonadotropins, there are instances when RRA may be the appropriate analysis. For example, RRA results in a higher potency estimate than does conventional *in vivo* bioassay when a gonadotropin is characterized by a short plasma half-life. This has been documented for comparisons of ovine LH and human menopausal gonadotropin which have short and long plasma half-lives, respectively (Dufau and Catt, 1978). In such cases, an *in vitro* bioassay or RRA provides a more accurate measure of the intrinsic biological activity.

Despite these limitations, the RRA technique has widespread application

(Catt *et al.*, 1974, 1976; Reichert, 1976; Dufau and Catt, 1978). The most common use of the RRA technique has been for the measurement of gonadotropin activity in purified hormone preparations. The high sensitivity afforded by the RRA makes it a valuable tool when used to follow gonadotropin activity in individual column fractions during purification (Reichert, 1976; Sakai and Ishii, 1980). The RRA technique offers a rapid method for quantifying gonadotropin activity following chemical modification and in studies of subunit dissociation and association (Catt *et al.*, 1974, 1976; Burke *et al.*, 1979a; Sairam and Schiller, 1979). Application to studies with fish gonadotropin(s) would be most valuable. Through the use of techniques to quantify receptor binding it may be possible to distinguish whether fish species contain one or two gonadotropins and also evaluate their specificity.

An additional application, although not widely used, is the measurement of plasma gonadotropins by RRA (Catt *et al.*, 1976; Saxena, 1976; Dufau and Catt, 1978). The work of Tan and Dodd (1978) and Bye *et al.* (1980) suggests that the measurement of plasma gonadotropin in a wide array of fish species will require the purification of gonadotropins from at least each family and probably genus. Such an undertaking would be an enormous task as would be the search for a heterologous RIA system. A number of studies indicate that gonadotropin from one species can be used to quantify gonadotropin receptors in gonadal preparations from other vertebrate classes (Licht *et al.*, 1977b; Bona Gallo *et al.*, 1978; Adachi *et al.*, 1979; Burke *et al.*, 1979b; Kubokawa and Ishii, 1980). Notable in this regard has been the use of radiolabeled human FSH to define FSH-binding sites in birds, reptiles, and amphibians (Section II,A). However, studies using human FSH as a means of detecting gonadotropin receptors in fish gonad preparations have been unsuccessful (Adachi *et al.*, 1979; Adachi and Ishii, 1980). Whether other gonadotropins of nonpiscine origin or some fish gonadotropin preparations display a lack of species specificity toward fish gonadotropins warrants additional research.

Apart from the foregoing applications, gonadotropin-receptor studies have provided new insight into the mechanism of hormone action and the endocrine control of gonadal development in mammals. Several of these features have been discussed in Section III as a means of defining a functional role for gonadotropin-binding sites. In mammals, three distinct patterns of hormone regulation of receptor numbers have been defined. These include: (1) autoregulation where hormones affect the concentration of their own receptor, (2) coordinate regulation in which a steroid and protein hormone interact and affect the receptor concentration for the same or a different protein hormone, and (3) heteroregulation in which one hormone regulates the concentration of a different hormone (Richards *et al.*, 1976). Studies

of this type have resulted in major advances toward the understanding of ovarian development and the necessary coordinate hormone interactions required for oocyte maturation (Henderson, 1979; Richards, 1979; Ahren *et al.*, 1980).

It would be of considerable theoretical interest to define gonadotropin-receptor numbers throughout the life cycle of fish with different reproductive strategies. Studies pertaining to the control of receptor number and gonadal responsiveness will provide new insight to controlled reproduction studies. Induction of oocyte maturation, ovulation, and spermiation by the classical hypophysation technique, for example, assumes that gonadotropin receptors are present in suitable number to respond to hormone stimulation. In the future, techniques may be available to assess if this is indeed the case. With increased understanding of the factors controlling receptor numbers it may be possible to develop humoral schemes to alter receptor numbers and subsequent gonadal responsiveness.

VI. CONCLUDING REMARKS

Recent developments in the bioassay of fish gonadotropins and promising preliminary results on the identification of binding sites for these hormones open this field as a major area for research pertaining to fish reproductive physiology.

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Addendum: In a recent paper Schlaghecke (*Gen. Comp. Endocrinol.* **49,** 261–269, 1983) demonstrated the presence of high-affinity limited-capacity binding sites for ^{125}I-labeled HCG in testicular extracts from adult rainbow trout. These binding sites were restricted to testicular tissue and could not be demonstrated in brain, heart, kidney, or muscle tissue. Crude rainbow trout pituitary extracts and partially purified pituitary fractions, which were retained on Con A-Sepharose, inhibited the binding of ^{125}I-labeled HCG. In contrast, pituitary fractions, which were not retained on Con A-Sepharose, failed to compete for these binding sites. Although the ^{125}I-labeled HCG binding sites in rainbow trout testis share several features associated with a hormone–receptor interaction, their physiological significance must be questioned in view of the high temperature (37°C) used to measure binding in this study.

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SYSTEMATIC INDEX

Note: Names listed are those used by the authors of the various chapters. No attempt has been made to provide the current nomenclature where taxonomic changes have occurred. Boldface letters refer to Parts A and B of Volume 9.

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Note: Boldface **A** refers to entries in Volume IXA; **B** refers to entries in Volume IXB.

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