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Oxidative Stress and Hormesis in Evolutionary Ecology and Physiology

A Marriage Between Mechanistic and Evolutionary Approaches

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To my mother

Preface

Research programmes on oxidative stress and hormesis are wide ranging. Biomedical and toxicological sciences have traditionally centralised such research, but in the last years we have seen the merger of laboratory-based biochemical and physiological approaches to the study of oxidative stress and hormetic mechanisms with ethological, ecological and evolutionary principles. Although research in this area is still in its infancy, many new fields of research are emerging from the marriage between these mechanistic and functional perspectives. What is critically lacking yet is a synthesis geared towards evolutionary ecologists, ecophysiologists and behavioural ecologists that seeks to consolidate a vast body of literature whilst, at the same time, remains sensitive to readers who may not have the depth of knowledge often necessary to navigate new fields of scientific enquiry. This book seeks to fill in this gap. Its goal is to synthesise and integrate research on oxidative stress and hormesis, but as seen from the perspective of an evolutionary ecologist or physiologist. Work in this area can open new frontiers for research to further our understanding on how and why biological diversity has evolved. This book does not have, however, the presumption to claim that oxidative stress or hormesis are vital, nor does it wish to fully describe biochemical mechanisms. Rather it wishes to emphasise that oxidative stress and hormesis are missing pieces of a complex biological puzzle. Focussing on these missing pieces and integrating them with other biological features can provide fascinating insights into how organisms work, how they evolve to sustain physiological function under a vast array of environmental conditions and why they work the way they do.

The book is organised into ten chapters. Chapter 1 provides general historical information, definitions and background of research on oxidative stress physiology, hormesis and life history; Chaps. 2–10 analyse how oxidative stress and hormesis have influenced many aspects of life, from the incipient stages of development to the strategies of reproduction and the ways organisms age. While every chapter deals with a specific topic, it should not be forgotten that they are all related to each other. For example, when interpreting the effect of reproductive effort on oxidative damage, it is also important to consider that such effect will be possibly dependent on other aspects, like early life experiences, predation risk, or food quality and quantity. Moreover, the physiological machinery in turn may influence the way a specific factor impacts on the individual. For example, over evolutionary time, diet has been likely adjusted to the physiological needs of an

individual or species, while at the same time the physiological strategies have been adjusted to make use of what is present in the diet itself. We clearly need to integrate all this information in order to further our understanding of how variation in physiological systems and life history strategies comes out. In this regard, the term adaptation has been used in a general way throughout the book, including both real adaptations and exaptations. For a character to be regarded as an adaptation, it must be a derived character that evolved in response to a specific selective agent. The emergence of an antioxidant mechanism could have been maintained, for example, by natural selection because it confers current selective advantage, but this is not evidence of why it has been evolved. This means that protection against oxidative stress may not be the primary reason for why all antioxidant mechanisms have been evolved, but evolution of a certain antioxidant mechanism could have provided secondarily a selective advantage.

Opportunities for studying truly natural processes are diminishing because pristine or near-pristine environments are rapidly shrinking and becoming more difficult to reach. This should remind us that we need to know more about how the changing and emerging environments are influencing natural animal populations. The many examples reported throughout the book will actually show how oxidative stress and hormesis can represent two important links between environmental changes and evolutionary fitness, hence they can represent two relevant tools of investigation for conservation biologists, as well.

The book is not exhaustive, hence many relevant studies have not been included. The book has also never taken into consideration discussion of methodological approaches to quantify reactive species production, antioxidants or damage levels. Obviously, methods for the quantification of components of the redox machinery have to satisfy specific technical criteria. We have to recognise, however, that each method has limits; hence using a combination of more oxidative status parameters is essential. It is also crucial that the biochemistry of what is being measured is taken into consideration when interpreting experimental results. We have not to lose sight of the fact that there are also specific restrictions inherent to many ecological research programmes, such as the availability of only small amounts of blood and the requirement of non-terminal sampling.

The biological meaning of many mechanisms or molecules that are currently measured to quantify damage or antioxidant defences is often hard to gauge, because most in-depth information about the physiological functions of many of them come from studies on a limited number of taxa. We also do not know in many cases what differences in oxidative stress mean; this is because we still do not know whether the impact on evolutionary fitness differs among oxidative damage parameters. Consequently, any generalisations and extrapolations about function should be made with considerable caution. Focusing too much on the limited array of model organisms generally used, and on highly conserved pathways, narrows our understanding of what works under what circumstances. We need to appreciate that evolution has likely modified or changed the basic defence toolkit and that understanding how different organisms have solved the challenges posed by their environments and lifestyles is interesting and important.

The complexity and diversity of the natural world is a powerful experimental resource that is often forgotten by many scientific disciplines. Appreciating such a complexity will be instructive and will offer us new opportunities for exploring new frontiers of biological research.

David Costantini

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Contents

Chapter 1
Historical and Contemporary Issues of Oxidative Stress, Hormesis and Life-History Evolution

Abstract The transition from a reducing to an oxidising chemistry in the atmosphere and oceans paved the way for the diversification of life. Oxygen expanded metabolic and biochemical capacities of organisms. Over the incipient stages of evolution of oxidative metabolism, organisms also needed to develop mechanisms to mitigate the toxic effects of oxygen derivatives, such as free radicals and non-radical reactive species. This chapter provides a general historical background, with definitions and information of free radicals, antioxidants and oxidative stress. This chapter also examines how mild doses of stress can have stimulatory effects on organismal performance through hormetic mechanisms and that this may significantly relate to evolutionary fitness and to the ecology of species. Finally, the chapter explains the concept of life-history trade-offs and highlights how the need to manage oxidative stress in an optimal way may be an important mechanism driving the outcome of many of these trade-offs.

1.1 The Great Oxidation Event: From a Reducing to an Oxidising World

The planet Earth is approximately 4.5 billion years old. The atmosphere of the primeval Earth was quite different from what we observe nowadays. It was mildly reducing, with large proportions of methane, ammonia and hydrogen and a low concentration of oxygen (Schopf and Klein 1992; Sessions et al. 2009). Around 2.45 billion years ago, atmospheric oxygen rose suddenly in what is now termed the Great Oxidation Event (Sessions et al. 2009). A second significant increase in atmospheric oxygen occurred at around 600–800 million years ago and was accompanied by the oxygenation of the deep oceans and emergence of multicellular animals (Sessions et al. 2009). The increase in oxygen concentration in the atmosphere and oceans paved the way for the diversification of life (Fig. 1.1).

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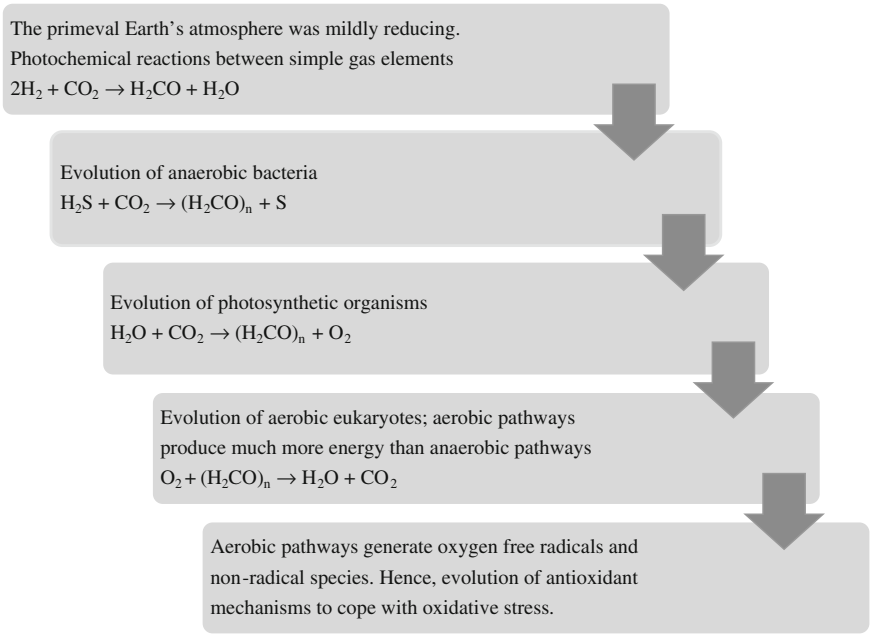

Fig. 1.1 Sequence of main transitions in energetic metabolism induced by changes in atmosphere and ocean chemistry (see Falkowski 2006)

The transition from a reducing to an oxidising atmosphere was characterised by the evolution of metabolic networks of increasing complexity (Raymond and Segrè 2006). Adaptation to molecular oxygen has also likely taken place independently in species from diverse lineages, even if it is unclear whether it contributed to shaping taxonomical diversity (Raymond and Segrè 2006). Certainly, oxygen expanded metabolic and biochemical capacities of organisms. The stimulatory effect of oxygen on the evolution of metabolic networks was not cost-free. Beyond diversification of mechanisms using oxygen to produce energy, organisms also needed to evolve mechanisms to mitigate the toxic effects of oxygen derivatives, such as free radicals and non-radical reactive species.

1.2 Reactive Species, Antioxidants and Oxidative Stress

1.2.1 On the Nature of Free Radicals and Other Reactive Species

The discovery of organic free radicals dates back to over a century ago, when the scientist Gomberg (1900) at the University of Michigan identified the triphenylmethyl

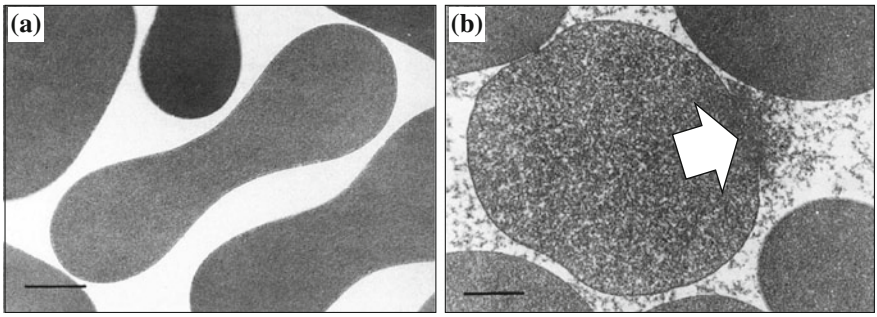

Fig. 1.2 Transmission electron micrographs of (**a**) intact rabbit erythrocytes and (**b**) oxidised rabbit erythrocytes undergoing haemolysis induced by free radicals generated from 2,2′-azobis(2-amidinopropane) dihydrochloride. Reprinted from Niki (2014) with permission from Elsevier

radical. For this discovery, Gomberg has been recognised as the founder of radical chemistry. A free radical was later defined as *any transient* (*chemically unstable*) *species* (*atom, molecule or ion*) (Bernthsen 1942; Herzberg 1971). Recently, Halliwell and Gutteridge (2007; p. 19) proposed to define a free radical as *any species capable of independent existence that contains one or more unpaired electrons*, attributes that make free radicals unstable and prone to react with other chemical species, hence able to cause oxidative damage to macromolecules and tissues (Fig. 1.2). It is, however, now well established that the generation of oxidative damage is not only a consequence of free radical activity, but also of other chemicals that lack radical properties (e.g., hydrogen peroxide, hypochlorous acid, peroxynitrite and singlet oxygen). To refer to this high variety of pro-oxidants, the term Reactive Species has been proposed because it would be inclusive of chemicals of both radical and non-radical nature, as well as chemicals that are derivatives of oxygen (reactive oxygen species) or other elements (e.g., reactive nitrogen species; Halliwell and Gutteridge 2007).

Free radicals can originate through various mechanisms (Halliwell and Gutteridge 2007). For example, they can arise from the loss of a single electron from a non-radical chemical

$$A - e^- \rightarrow A^{\cdot +} \text{ (radical cation)}$$

or from the gain of a single electron

$$A + e^- \rightarrow A^{\cdot -} \text{ (radical anion)}$$

A free radical can also originate from homolytic fission of a molecule (a covalent bond is broken, and one electron from the bonding electron pair remains on each atom)

$$A : B \rightarrow A^{\cdot} + B^{\cdot}$$

On the other hand, heterolytic fission occurs when both electrons remain on one of the two atoms

$$A : B \rightarrow A^{-} + B^{+}$$

Reactive species largely differ in chemical properties and damaging potential. For example, while hydroxyl ($^{\cdot}OH$) is one of the most reactive free radicals described so far, it can only cause oxidative damage locally because, for its high reactivity, it has a very short life, so it cannot migrate across cells. In contrast, hydrogen peroxide (H_2O_2), which is much less reactive than hydroxyl, can work as a Trojan horse diffusing away from sites of production to other cellular locations, where it can propagate the oxidative cascade through the generation of other reactive species.

Reactive carbonyl species are another interesting case: they originate from the oxidation of carbohydrates and lipids (predominantly polyunsaturated fatty acids) and have a much longer half-life (from minutes to hours) than that of reactive oxygen or nitrogen species (microseconds) (Pamplona 2008; Pamplona and Costantini 2011). The non-charged structure allows reactive carbonyl species to migrate with relative ease throughout hydrophobic membranes and hydrophilic cytosolic media, hence facilitating the spread of oxidative damage from the site of production to other body compartments.

A certain pro-oxidant typology may also generate a new reactive species with a pro-oxidant potential different from that of the originating molecule. For example, the reaction of the superoxide anion with nitric oxide generates the peroxinitrite anion, which, thanks to its higher diffusion rate across cell membranes (Marla et al. 1997; Denicola et al. 1998), might be responsible for many of the effects ascribed to the superoxide anion (Halliwell and Gutteridge 2007). The superoxide anion can also give rise to the more histolesive hydroxyl through its reaction with hydrogen peroxide in the so-called Haber–Weiss reaction:

$$O_2^{\cdot -} + H_2O_2 \rightarrow O_2 + {}^{-}OH + {}^{\cdot}OH$$

This large variation in chemical characteristics and pro-oxidant potential among reactive species might be ecologically relevant where different activities (e.g., reproduction, foraging) generate different kinds of reactive species. However, we do not know the exact biological meaning of reactive species yet, which would help us to establish whether such variation in production of reactive species is functionally and ecologically relevant and may be a target of natural selection.

Although the discovery of organic free radicals raised a lot of interest, it was not until 50–60 years later that the existence of free radicals in living organisms was demonstrated and their responsibility for cell senescence suggested (Commoner et al. 1954; Gershman et al. 1954; Harman 1956; McCord and Fridovich 1968, 1969). Since then, research on chemicals with pro-oxidant activity increased dramatically and today the study of oxidative stress physiology and redox status regulation has gained a very important role in medicine, biochemistry, physiology, pharmacology, ecotoxicology and, more recently, in evolutionary ecology (Fridovich 1978; Halliwell and Gutteridge 2007; Costantini 2008; McGraw et al. 2010).

It is important to point out that reactive species are not simply toxic products of metabolism, but are also essential molecules for cell signalling and regulation (Thannickal and Fanburg 2000; Dröge 2002). Reactive species work as redox messengers in regulatory processes in which the signal is delivered through the redox chemistry (redox signalling; Thannickal and Fanburg 2000; Dröge 2002). The organism response to a social or non-social environmental stimulus depends on a cascade of processes, starting from the perception of the stimulus to its translation into hormonal secretions, which in turn regulate the response itself. Research in the area of behavioural endocrinology has contributed to the identification of several mechanisms that regulate the extent and rate at which organisms respond to environmental influences (Nelson 2005). Because of these signalling properties in cell-cell communication, reactive species might also be important regulators of the way organisms respond to their environment. At low concentrations, reactive species act as secondary messengers responsible for a signal transduction from extracellular signalling molecules and their membrane receptors to the intracellular regulatory systems that control gene expression (Turpaev 2002). Addressing the regulatory role of reactive species is certainly methodologically complex and not easily applicable to field studies. The rapid technological advance might, however, make this possible in the years to come.

1.2.2 Antioxidant Mechanisms

In the nineteenth century, engineers discovered that certain substances could prevent metals from corroding by shutting off the oxidation processes. Although the reasons behind this were poorly understood, scientists began adding compounds with antioxidant properties to foods high in unsaturated fatty acids and found that this prevented the onset of rancidity (Halliwell and Gutteridge 2007). However, the strongest support to the importance of antioxidants and, indirectly, to free radicals in living systems came later with the discovery of superoxide dismutase, an enzyme responsible for scavenging the superoxide radical (McCord and Fridovich 1968, 1969). Interest in antioxidants rocketed. We now know that living organisms have evolved many differing ways of coping with the pro-oxidant activity of reactive species, including a large variety of molecules and metabolic pathways (Pamplona and Costantini 2011). Although many of these antioxidant mechanisms are highly conserved across species, some are peculiar to certain taxa and, as we will see in the following chapters, specific antioxidant mechanisms appear to be more important in certain species than in others.

As with free radicals, several definitions of antioxidant have been proposed so far, but it has been proven surprisingly difficult to define clearly. Halliwell and Gutteridge (2007; p. 81) defined an antioxidant as *any substance that delays, prevents or removes oxidative damage to a target molecule*. Cornelli (2009) provided a more clinical (and operational) definition of antioxidant in order to take into account the differences among the many biomarkers of oxidative stress

available: *an antioxidant is a product that inhibits oxidation* in vitro *and reduces oxidative stress* in vivo*, irrespective of how oxidative stress is measured*. The distinction between in vitro and in vivo is important because there are compounds that have antioxidant activity in vitro, but do not reduce oxidative damage in vivo. Recently, Pamplona and Costantini (2011) provided a broader view of the definition, proposing that an antioxidant is *any mechanism, structure and/or substance that prevents, delays, removes or protects against oxidative non-enzymatic chemical modification* (*damage*) *to a target molecule*. In this definition of antioxidant, Pamplona and Costantini (2011) proposed that organism structures (e.g., cell membrane composition) might be considered as antioxidant mechanisms because they influence the cell resistance to oxidative stress. Moreover, they made the point that the oxidative modification of a molecule should be of non-enzymatic nature, in order to distinguish it from oxidative modifications typical of many normal redox reactions that occur in the body.

It is, therefore, clear that antioxidant mechanisms are numerous and work in quite different ways. In the following paragraphs, I review some typologies of antioxidant mechanisms.

1.2.2.1 Enzymatic and Non-enzymatic Molecular Antioxidants

Organisms have evolved various molecules to defend themselves from the prooxidant action of reactive species. Antioxidant enzymes play an important role in the removal of reactive species or in their transformation into less reactive compounds (Halliwell and Gutteridge 2007). They are also important in the removal of intermediate derivatives of oxidative damage (hydroperoxides) from the body. The organism also relies on non-enzymatic antioxidants, which may work as cofactors of the activity of antioxidant enzymes, sequester metal ions or quench reactive species through their passive oxidation (Halliwell and Gutteridge 2007). Major classes of molecular antioxidants include the following groups:

- Manganese superoxide dismutase (it is located in the mitochondrion of animal cells) and copper–zinc superoxide dismutase (it is located in the cytosol and in the intermembrane space of the mitochondrion in animal cells) catalyse the dismutation of the superoxide anion into oxygen and hydrogen peroxide (Halliwell and Gutteridge 2007):

$$2O_2^{\cdot -} + 2H^+ \xrightarrow{SOD} O_2 + H_2O_2$$

There is also an extracellular form of copper–zinc superoxide dismutase (SOD3) that occurs in extracellular fluids such as plasma, lymph and synovial fluid. Various similar enzymes containing, for example, iron or nickel were described in bacteria, yeast, algae and plants (Halliwell and Gutteridge 2007).

- The enzymes catalase and glutathione peroxidase work coordinately to eliminate hydrogen peroxide from the body. Catalase reduces hydrogen peroxide to

water and oxygen at high rates, but its low affinity for hydrogen peroxide might make it more important during peaks of hydrogen peroxide production or accumulation:

$$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$

Glutathione peroxidases, present in selenium- and non-selenium-dependent forms, use the reduced form of glutathione to reduce peroxides and hydroperoxides to water and alcohols, respectively. However, given their higher affinity for hydrogen peroxide than catalase, glutathione peroxidases are important to remove the small amounts of peroxides continuously produced by cells (Halliwell and Gutteridge 2007). Various selenium-containing glutathione peroxidases isozymes have been identified so far (Behne and Kyriakopoulos 2001). A phylogenetic analysis of the glutathione peroxide family, including plant, fungi, bacteria, invertebrate and vertebrate taxa, showed complex relationships within this molecular family, suggesting that basal classes of glutathione peroxidases have originated from independent evolutionary events, such as gene duplication, gene losses and lateral gene transfer (Margis et al. 2008). It is further recognised that there is an evolutionary pattern in animals leading to the origin of most isozymes and a second pattern leading to the glutathione peroxide 4 isozyme, which occurs in animals, plants and fungi. This second pattern would suggest evolutionary convergence mediated by functional pressures, but it is unclear why this pattern was not also observed for other isozymes (Margis et al. 2008).

- Glutathione is a tripeptide that occurs at concentrations as high as 10 mM in its reduced protective form in most tissues (Halliwell and Gutteridge 2007). In animal cells, it is synthesised in the cytoplasm through the action of the enzyme glutathione synthetase and the consumption of energy. Glutathione is very important for the maintenance of redox balance. Measurements of both reduced and oxidised forms of glutathione are used to detect any perturbations of the cell redox state. The antioxidant activity of glutathione is due to the reduced thiol (i.e. sulfhydryl, SH) group of its cysteine residue. Once oxidised, the cysteine residue can be recycled back to its reduced form through the action of the enzyme glutathione reductase. Glutathione is also involved in metabolism of ascorbate, communication between cells through gap junctions, and protection of thiol proteins against oxidation and cross-linking (Halliwell and Gutteridge 2007). Another thiol-related redox-active molecule is the protein thioredoxin (Mitsui et al. 2002). Thioredoxin can regulate transcription factors (e.g., nuclear factor-kappaB, activated protein-1) and scavenges reactive species in cooperation with the peroxiredoxin/thioredoxin-dependent peroxidase (Mitsui et al. 2002). Finally, recent studies highlighted a potential protective role of haemoglobin against reactive species (Reischl et al. 2007). For example, Hausladen et al. (1998) found that haemoglobin protects *Escherichia coli* against nitrosative stress; similarly Nishi et al. (2008) found that overexpression of haemoglobin reduced production of hydrogen peroxide and enhanced cell viability

against exposure to hydrogen peroxide in rats. Haemoglobins contain thiol groups, whose number largely varies among species. Erythrocytes face high partial pressures of oxygen, high oxygen flow and high concentrations of iron. Hence, selective pressures may have favoured the evolution of thiol-rich haemoglobins in order to make red blood cells buffered against oxidation. An opposite view has also been proposed: the propensity of haemoglobin to oxidation may fuel a state of oxidative stress and, possibly, of loss in red blood cell function (Tappel 1955; Katsu et al. 2010). Taken together, it may be hypothesised that natural selection has operated in order to optimise a molecular compromise among costs and benefits of having or not having haemoglobin.

- The so-called dietary antioxidants (vitamin E, carotenoids, polyphenols) eliminate free radicals through a synergic action (ascorbate may also be involved): when a given antioxidant is oxidised through the reaction with a free radical, another antioxidant can recycle it back to its reduced form (see Chap. 4). For example, the carotenoid radical can be converted back to its reduced form by tocopherols:

$$\mathrm{Car}^{\cdot+} + \alpha\mathrm{TOH} \longrightarrow \mathrm{Car} + \alpha\mathrm{TO}^{\cdot} + \mathrm{H}^{+}$$

Therefore, in order to achieve a successful protection against oxidative damage, a balance in the concentrations of molecules involved in a certain reaction is needed. Of the various dietary antioxidants, vitamin E plays an important role in the protection of fatty acids working as a chain-breaking antioxidant (Niki et al. 1988; Niki 2014). The term vitamin E refers to a class of lipophilic compounds that includes tocopherols (d-α, d-β, d-γ and d-δ tocopherol) and their corresponding tocotrienols (Wang and Quinn 1999; Surai 2002). Tocopherols reduce lipid peroxyl groups to hydroperoxides, thus inhibiting the propagation of lipid peroxidation (Esterbauer et al. 1991). Moreover, tocopherols promote repair of oxidative damage in cell membranes of myoblasts (Howard et al. 2011). Carotenoids are also lipophilic antioxidants in animal cells and protect against ultraviolet light in plants. Hundreds of different carotenoids have been described so far, although only some of them, such as α- and β-carotene, lutein, lycopene, zeaxanthin or cryptoxanthin, are present at relevant concentrations in animal tissues and plasma (Krinski 1993; Surai 2002). Carotenoids are synthesised by photosynthetic organisms, hence animals are generally assumed to obtain them from their diet. However, recent evidence of laterally transferred genes for carotenoid biosynthesis in some arthropods (Moran and Jarvik 2010; Altincicek et al. 2011) challenged this view. Despite their in vitro antioxidant properties, empirical evidence for carotenoids being important antioxidants in vivo is weak (Costantini and Møller 2008; Simons et al. 2012). Moreover, they may also have toxic effects on the organism, supplementation trials of carotenoids and of other dietary antioxidants (vitamins C and E), administered singly or together in humans (e.g., Lee et al. 1999; The Age-Related Eye Disease Research Group 2001; Muntwyler et al. 2002) failed to stop

disease and ageing, and increased the mortality among participants of the trial. Results on non-human animals are also not supportive enough for an important antioxidant role of dietary antioxidants (see Chap. 4). Another large class of dietary antioxidants are polyphenols. Numbering several thousands of distinct species, polyphenols represent a group of secondary metabolites mostly derivatives and/or isomers of flavones, isoflavones, flavonols, catechins and phenolic acids (Halliwell and Gutteridge 2007; Han et al. 2007). They have diverse biological properties, such as antioxidant, anti-apoptosis, anti-ageing or anti-inflammation protection; improvement in the endothelial function; inhibition of angiogenesis and cell proliferation activity (Halliwell and Gutteridge 2007; Han et al. 2007). Like other dietary antioxidants, however, polyphenols can also have pro-oxidant effects (Rietjens et al. 2002).

- Ascorbic acid (vitamin C) is endogenously synthesised, but there are many taxa (e.g., various primate species including humans, various bats, many rodent and passerine species, teleost fish) that have lost the capacity to synthesise it. These species lack L-gulonolactone oxidase, which is the enzyme responsible for its synthesis, hence they must obtain ascorbic acid from the diet (e.g., Jenness et al. 1980; Martínez Del Rio 1997; Cui et al. 2011). Once oxidised, the ascorbic acid can be returned back to its reduced form by reduced nicotinamide adenine dinucleotide phosphate-dependent (Rose and Bode 1993) or glutathione-dependent (Maellaro et al. 1994; Wells and Xu 1994) dehydroascorbate reductases or by reduced nicotinamide adenine dinucleotide phosphate-dependent plasma membrane ascorbate free radical reductase (Navas et al. 1994).
- Nitrogen metabolism gives rise to compounds, like uric acid, that can have antioxidant properties in the organism (Iqbal et al. 1999; Klandorf et al. 1999). However, the interpretation of the protective role of uric acid against oxidative damage is complex as changes in uric acid concentration are possibly influenced by both its excretory pathway and its links to protein turnover (proteins are substrates of oxidation) and by antioxidant demands. Multiple selective pressures may have therefore operated to generate among species variation in concentration and dynamics of uric acid (Monaghan and Costantini 2014).
- Luciferins are luminescent substrates of the luminous reactions that occur in many marine bioluminescent species; they also have antioxidant properties, as they are highly reactive with molecules, such as the superoxide anion or peroxides. Rees et al. (1998) suggested that the primary function of luciferins was originally the detoxification of oxygen derivatives. The functional shift from antioxidant to light-emitting function might have occurred when the strength of selection for antioxidant defence mechanisms decreased. Rees et al. (1998) suggested that this might have been made possible when marine organisms began colonising deeper layers of the oceans, where exposure to oxidative stress is considerably reduced because of reduced light irradiance and lower oxygen levels.

- Free metal ions promote the pro-oxidant activity of hydrogen peroxide through its one-electron reduction to the more reactive hydroxyl radical through the Fenton reaction:

$$M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + {}^{\cdot}OH + {}^{-}OH$$

(M^{n+} and $M^{(n+1)+}$ are transition metal ions; M^{n+} stands for ions, such as Cu^{+} and Fe^{2+}; $M^{(n+1)+}$ stands for ions, such as Cu^{2+} and Fe^{3+})

Free metal ions can also cleave hydroperoxides into highly reactive free radicals, such as the alkoxyl ($R-O^{\cdot}$) and alkylperoxyl ($R-OO^{\cdot}$) radicals, through the Fenton reaction. However, living organisms have evolved specific proteins (e.g., ferritin, ceruloplasmin) specialised in the sequestration of free metal ions, hence making them innocuous (Halliwell and Gutteridge 2007).

1.2.2.2 Cellular Structural Components

Macromolecules show differing degrees of susceptibility to oxidative damage, a property intrinsically related to the chemical characteristics of the molecules themselves. Hence, it has been proposed that the macromolecular composition of a cell might be another antioxidant mechanism natural selection has shaped to make cells more resistant to reactive species (Hulbert et al. 2007; Pamplona and Barja 2007; Pamplona and Costantini 2011). This hypothesis is based on the following evidence (see Chap. 10):

- Polyunsaturated fatty acids are the macromolecules most susceptible to oxidative damage. They are the least abundant fatty acids in cell membranes and are less abundant in species that live longer (Hulbert et al. 2007; Pamplona 2008).
- Guanine is the least abundant nucleotide in mitochondrial DNA (Samuels 2005) and, of the four nucleobases, guanine is generally most easily oxidised (Kovacic and Wakelin 2001; Bjelland and Seeberg 2003).
- Cell proteins have a low content of the amino acid methionine (Aledo et al. 2011; Portero-Otín et al. 2004; Pamplona et al. 2005; Ruiz et al. 2005), which is one of the amino acids most susceptible to peroxidation (Stadtman et al. 2003).
- Evolution has possibly favoured glucose as the most important carrier of energy because it is the more stable glycolytic intermediate, hence more resistant to oxidation (Bunn and Higgins 1981; Monnier et al. 1991). This might explain why the high basal blood glucose concentrations found in birds do not compromise their longevity.

1.2.2.3 Regulators of Reactive Species Production

In animal cells, the major sites of physiological reactive species generation are the complexes I and III of the mitochondrial electron transport chain, which contain several redox centres (flavins, iron–sulphur clusters and ubisemiquinone) capable

of transferring one electron to oxygen and so generating the superoxide anion (Brand et al. 2004; Hulbert et al. 2007; Pamplona and Barja 2007). Several mechanisms regulate the activity of these complexes, thus indirectly influencing the production of reactive species:

- Mechanisms that influence the concentration of respiratory complexes. For example, birds have a lower content of complex I than mammals, as well as a lower degree of reactive species production (St-Pierre et al. 2002; Lambert et al. 2007; Pamplona and Barja 2007). Therefore, regulation of the expression of the mitochondrial complexes (and especially complex I) could be part of an adaptive mechanism to control reactive species production (Pamplona and Costantini 2011).
- Mechanisms that influence the degree of reduction of respiratory complexes. For example, an increase in the ratio between the reduced and oxidised forms of nicotinamide adenine nucleotide is accompanied by an increase in the degree of reduction of complex I (Kushnareva et al. 2002; Barja 2007). This is important because respiratory complexes promote production of reactive species when their degree of reduction is high (Pamplona and Barja 2006, 2007).
- Regulation of uncoupling proteins. Cells produce energy through the oxidation of substrates like glucose. To do so, the respiratory complexes reduce oxygen to water, and use energy released by electrons to pump protons from the matrix of the mitochondrion into its intermembrane space, creating a proton motive force. Protons move back to the matrix following an electrochemical gradient, and adenosine-5′-triphosphate (ATP) synthases use such an energy to produce ATP. Under these conditions, there is coupling between electron transport and oxidative phosphorylation. However, there may be formation of the superoxide anion because electrons can also react with oxygen. The reaction of superoxide with membrane phospholipids forms the hydroxynonenal, an end product of lipid peroxidation, which is capable of activating uncoupling proteins. Uncoupling proteins provide a path for protons to diffuse from the intermembrane space to the matrix without ATP synthesis. Therefore, energy is released as heat. In this way, electron transport is uncoupled from oxidative phosphorylation. This mild uncoupling lowers the proton motive force and stimulates the electron transport, causing the oxidation of respiratory complexes and lowering the local concentration of oxygen and so of superoxide production. Thus, the induction of proton leak by hydroxynonenal limits mitochondrial reactive species production and works as a feedback response to overproduction of superoxide by the respiratory chain (Echtay et al. 2003; Brand et al. 2004).
- Chemical modifications of a protein after its translation (e.g., acetylation, S-nitrosation, glutathionylation; Pamplona and Costantini 2011). For example, glutathionylation of complex I increases superoxide production by the complex itself, and when the mixed disulphides are reduced, superoxide production returns to basal levels (Han et al. 2003; Taylor et al. 2003).
- Regulation of the mitochondrial partial pressure of oxygen. Animal cells are normally exposed to low oxygen concentrations; this is interpreted as an

antioxidant mechanism (Halliwell and Gutteridge 2007). When the organism enters a prolonged state of hypoxia (i.e. dramatic drop in tissue oxygen concentration), hypoxia inducible transcription factors (e.g., Hif-1α or Hif-2α) promote the expression of genes encoding proteins that help cells to cope with a hypoxic or anoxic status (Fandrey and Gassmann 2009). There exists, however, high variation in how species can tolerate hypoxia (see Chaps. 3 and 4).

- Regulation of the cardiolipin content in mitochondria. Cardiolipin is a phospholipid located almost exclusively within the inner mitochondrial membrane, where it contributes to the regulation of mitochondrial bioenergetics (Hoch 1992; Portero-Otin et al. 2001). Cardiolipin is, however, a potential target of reactive species attack because its chemical structure contains unsaturated fatty acids. This is very important because peroxidation of cardiolipin influences the activity of mitochondrial complexes I, III and IV (Paradies et al. 2000, 2001, 2002). However, cardiolipin has a low unsaturation degree, which makes it more resistant to peroxidation than other phospholipids. This might be one reason why cardiolipin has been positively selected as a regulator of mitochondrial bioenergetics (Pamplona 2008; Pamplona and Costantini 2011).

1.2.2.4 Repair and Detoxifying Systems

Oxidative damage is an unavoidable consequence of oxidative metabolism, and the primary antioxidant mechanisms are unable to avoid it completely. Therefore, some oxidative damage always occurs, hence it is important to repair damaged molecules or to remove them from the organism because they may have detrimental effects on organism functionality. Some examples of these antioxidant mechanisms are reported in the following paragraphs:

- Although most protein oxidative modifications are irreversible and, therefore, need to be catabolised, certain oxidative modifications, such as methionine sulfoxide and disulphides, can be repaired by the methionine sulfoxide reductase enzyme in a thioredoxin-dependent reaction (Moskovitz et al. 2001).
- Degradation of oxidised proteins is needed to avoid aggregation and the build-up of modified proteins. This is done through the activity of various systems: lysosomal proteases, calcium-dependent proteases, proteasomal system and caspases (Pamplona and Costantini 2011).
- Phospholipase A2 is an enzyme that removes acyl chains from cell membrane phospholipids, while acyltransferase and transacylase enzymes are responsible for reacylation of phospholipids (Farooqui et al. 2000).

1.2.2.5 Signalling Mechanisms

Redox state is regulated through the action of reactive species, which act as second messengers in signal transduction networks (Giles 2009). In doing so, they activate various antioxidant mechanisms:

- The heat shock response, through activation of heat shock transcription factors and the elevated expression of heat shock proteins and molecular chaperones, protects the cell against the accumulation of non-native proteins (e.g., Parsell and Lindquist 1993; Morimoto 1998; Feder and Hofmann 1999; Sun and MacRae 2005). Activation of heat shock factor-1 during this process subsequently induces the expression of a variety of heat shock proteins (HSPs). HSPs are evolutionarily conserved and show many homologies within and among all prokaryote and eukaryote organisms, indicating strong stabilising selection (Kristensen et al. 2003; Sørensen 2010).
- Reactive species can affect the expression of many redox-sensitive genes (e.g., glutathione peroxidase, quinone reductase, catalase, superoxide dismutase, haeme oxygenase-1, γ-glutamylcysteine synthase and metalloproteinases) and cellular functions, including cell–cell communication, metabolism, structure, motility, proliferation, differentiation and apoptosis (Giles 2009; Pamplona and Costantini 2011): (1) oxidant administration to differing cell types regulates the expression of 1–5 % of the genome; (2) antioxidant levels modulate the effect of reactive species and also act independently to regulate signalling; (3) changes in the cellular redox balance result in both the up and downregulation of subsets of genes; (4) the expression of individual genes is dependent on the chemical properties of the regulatory oxidant or antioxidant; and (5) the extent to which redox regulation occurs is governed by both the magnitude and duration of the applied stimulus. The activation of an antioxidant response by a chemically diverse range of reactive species indicates that common sensing mechanisms act as generic stress sensors. This signalling cascade culminates in the nuclear translocation of and transactivation by the nuclear factor (erythroid-derived 2)-like 2 called Nrf2 (Copple et al. 2008; Kaspar et al. 2009). As a consequence, there may be initiation of metabolic phase I and II enzymes, which are, for example, responsible for oxidising xenobiotics and conjugating them to glucuronyl, sulphate and glycyl groups in order to facilitate their cellular export or upregulation of redox-sensitive genes and metabolic enzymes (Copple et al. 2008; Pamplona and Costantini 2011).

1.2.3 Oxidative Stress

Cells live in a dynamic redox environment, whose status is determined by a complex balance between pro-oxidant and antioxidant molecules. It is this balance that determines the oxidative stress level the cell is exposed to (Fig. 1.3). Definition of oxidative stress is complicated by the fact that (1) many molecules and biochemical/genetic pathways are involved and (2) oxidative stress is basically a latent variable because we cannot directly measure it, so we have to rely on proxy variables to quantify the oxidative stress level. Definitions of oxidative stress proposed so far highlighted the importance of oxidative damage production in general or of oxidation of thiol groups in particular as the main determinants of

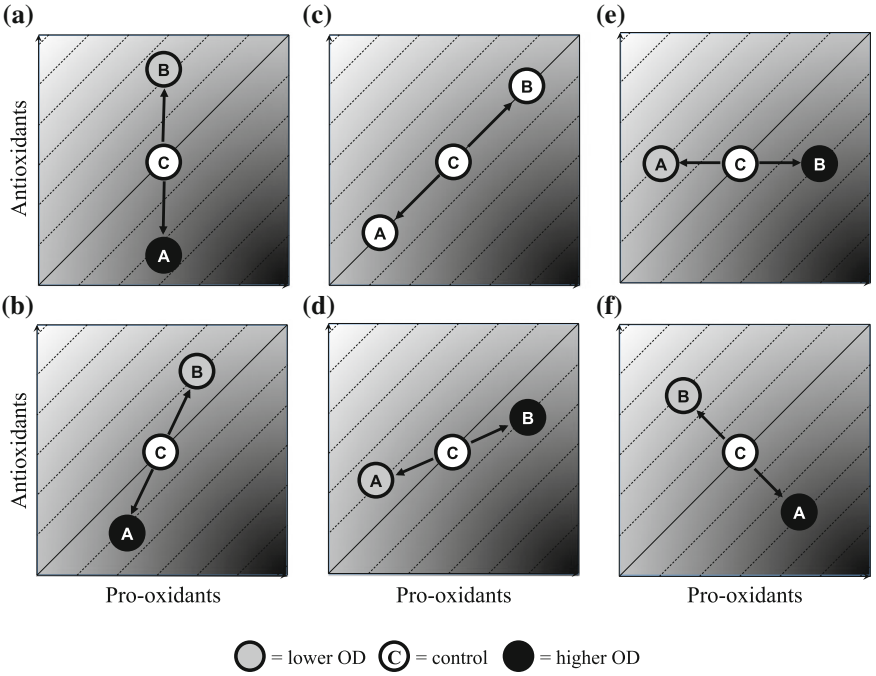

Fig. 1.3 Relationships among pro-oxidants, antioxidants and oxidative stress. Oxidative damage (and so oxidative stress) increases when antioxidant levels decrease for a given level of pro-oxidants. Therefore, oxidative stress increases from *top left* corner to *bottom right* corner as indicated by background shading (*darker* indicates higher oxidative stress level). *Dashed lines* are "iso-oxidative stress" lines, in that all combinations of anti- and pro-oxidant levels on the line yield the same level of oxidative stress. The *solid line* represents combinations of anti- and pro-oxidants with an equal level of oxidative stress as the control group. In **a**–**f** different hypothetical situations are compared with respect to possible ways that two (experimental) groups *A* and *B* could differ from a control (*C*) group. In scenario (**a**), *A* and *B* do not differ in pro-oxidant production, but *B* has more antioxidants per unit of pro-oxidants and, consequently, attains a lower level of oxidative stress than *A* (e.g., Gaál et al. 2006). In scenario (**b**), *A* has lower levels of pro-oxidants but also of antioxidants than *B*, hence *A* suffers more oxidative stress than *B* (e.g., Lin et al. 2004; Gaál et al. 2006). In scenario (**c**), antioxidant levels of both *A* and *B* are matched to the different pro-oxidant levels, and consequently, there is no difference in oxidative damage between the two groups (e.g., López-Torres et al. 1993; Pérez-Campo et al. 1993). In scenario (**d**), *B* is exposed to higher oxidative damage than *A* because the antioxidant response of *B* to a pro-oxidant challenge, while still operating, is not sufficient to maintain oxidative damage at the same level of *A* (e.g., Gianni et al. 2004; Lin et al. 2008). In scenario (**e**), *A* and *B* do not differ in antioxidant levels, however, *B* suffers higher oxidative damage because its production of free radicals is higher than *A* (e.g., Costantini et al. 2008). Finally, in scenario (**f**), *B* has lower pro-oxidant production and higher antioxidant levels than *A*, hence suffering lower oxidative damage and so lower oxidative stress than *A* (e.g., Costantini and Dell'Omo 2006). *OD* = oxidative damage. Reprinted from Costantini and Verhulst (2009) with permission from John Wiley and Sons

cell deterioration and senescence. Sies (1985, 1991) was the first to provide a definition of oxidative stress. He proposed that oxidative stress is caused by a *disturbance in the pro-oxidant–antioxidant balance in favour of the former, leading to potential damage*. In 2007, Halliwell and Gutteridge (p. 187) proposed a revised version of Sies' definition: *oxidative stress is a disturbance in the pro-oxidant–antioxidant balance in favour of the former, leading to potential biomolecular damage caused by attack of reactive species upon the constituents of living organisms*. In both definitions, it is implicit that oxidative stress may or may not occur depending on the balance; however, the generation of oxidative damage is continuous and it is its rate of generation that varies. Accordingly, Costantini and Verhulst (2009) defined oxidative stress as *the rate at which oxidative damage is generated*. Implicit in this definition is that oxidative stress is a continuous variable that is unlikely to ever be exactly zero since pro-oxidants are continually produced and some oxidative damage is always generated. Basal levels of oxidative damage may change consequently to exposure to environmental stressors or across time, reflecting shifts of oxidative balance towards a more oxidised or reduced state (Dröge 2002).

Other authors proposed definitions of oxidative stress highlighting that oxidation of thiol groups rather than generation of generic oxidative damage is critical because thiols are the main regulators of a cell's redox environment. Jones (2006) defined oxidative stress as a *disruption of redox signalling and control*, which regulate the redox balance and the antioxidant response to oxidative insults. According to this new definition of oxidative stress, Sohal and Orr (2012) proposed the redox stress hypothesis of ageing, which states that ageing is caused by a pro-oxidising shift in the redox state of the cells, which leads to the overoxidation of redox-sensitive protein thiols and the consequent disruption of the redox-regulated signalling mechanisms. Hence, oxidative damage to thiol proteins would be biologically more relevant than damage to other molecules. Specific tests of biological effects of different kinds of damages are, however, lacking in wild animals.

Although the term oxidative stress is used rather universally, encompassing all kinds of oxidative damage, Dotan et al. (2004) suggested that there could be different subcategories of oxidative stress, depending on which molecules are damaged. Molecules differ in their susceptibility to peroxidation and in the molecular pathways that repair them (Bielski et al. 1983; Porter et al. 1995; O'Connor et al. 2002; Pamplona and Barja 2007; Pamplona and Costantini 2011). Given that an oxidative insult can damage, for example, proteins but not lipids or nucleic acids, the level of damage to a certain molecule might not be a good measure of organismal oxidative stress (Dotan et al. 2004). It is not known yet whether differences in peroxidation by molecular type are biologically relevant; while it may be that measuring, for example, a biomarker of lipid peroxidation provides a general picture regarding resource-based trade-offs, it is also plausible that different fitness-related traits might be differentially influenced by oxidative damage of different molecules or tissues (Costantini et al. 2010b). Also, it is not always clear whether the damage level is functionally relevant. For example,

oxidative damage may increase to a level that is still under a threshold of tolerance. Moreover, it can be damage to those molecules that do not significantly compromise body functions, such as in the case whereby reactive species damage DNA (which is commonly measured), but such damage is limited to the non-coding sequences, the so-called junk DNA. Much of the genome is actually non-coding and oxidative damage in these regions might be expected to have little biological effect, an inference supported by observations that there is preferential repair of coding sequences (Evans and Cooke 2004).

1.2.4 Biochemical Integration and Modularity of Redox Systems

The traditional approach in ecological and physiological studies is to focus on one or a few aspects of physiology at a time. Because many physiological mechanisms impact on more than one process and interact with each other, the merger of multiple physiological variables and other endpoints (e.g., genetics, toxicology) in a multivariate approach may open up exciting new perspectives of research (Pamplona and Costantini 2011; Cohen et al. 2012). A univariate approach may, for example, miss to detect a change in a single physiological variable whether any changes in other related variables modify the structure of relations among variables, without altering average values.

Generally speaking, all biological systems exhibit an integration of morphological, biochemical and genetic components, such that these are intercorrelated through functional, structural, developmental or evolutionary interdependency (Ravasz et al. 2002; Giuliani et al. 2004; Papin et al. 2004; Schlosser and Wagner 2004; Klingenberg 2008; Mitteroecker and Bookstein 2007; Costantini et al. 2011b, 2013; Pamplona and Costantini 2011; Cohen et al. 2012). In particular, biochemical integration refers to the functional interdependency among biomolecules through a direct or indirect reaction or regulation: for example, the concentration of molecule A depends on the concentration of molecule B and vice versa; molecule A influences molecule C through molecule B; and reaction A influences reaction B (Papin et al. 2004; Pamplona and Costantini 2011). In order to make rigorous inferences on integration, it is, therefore, important that a basic knowledge of the biochemistry of molecules under study is available.

Implicit in the biological property of integration is that the effectiveness of the defence machinery depends not only on how good the antioxidants are at preventing oxidative damage, but also on the synergistic and competitive interactions among different antioxidants, as well as how changes in levels of oxidative damage are accompanied by parallel changes in antioxidants. This information may provide clues about the organism's response to environmental stressors that a univariate approach would not allow to detect.

The dynamics of correlation and variance of multiple components in biological systems provides information on the organismal stress response and consequences of exposure to a stressor (Gorban et al. 2010). The analysis of correlation or covariance matrices of oxidative status measures can be used to quantify the integration of a certain biological system (Pavlicev et al. 2009; Haber 2011; Costantini et al. 2013). The integration of redox state of a tissue may be estimated using the relative standard deviation of eigenvalues of the correlation matrix (Pavlicev et al. 2009; Haber 2011):

$$\mathrm{SDrel}(\lambda) = \frac{\sqrt{\mathrm{Var}(\lambda)}}{\sqrt{N-1}}$$

It is calculated as the ratio of the square root of the variance of eigenvalues (λ) to the square root of the number of variables (N) minus 1, and varies between zero (corresponding to no relationships among the variables) and one (perfect correlation among all variables). Eigenvalues are calculated by running a Principal Component Analysis on the correlation matrix for each experimental group, separately. Graphical representation of the integration can be done, for example, using networks (Fig. 1.4), where a node represents a certain biomolecule and a link between nodes indicates that those two molecules are significantly correlated or, depending on its thickness, can simply reflect how strong the correlation between the two nodes is, regardless of whether it is significant or not. In the specific case of integration of redox systems, the analysis of correlation or covariance matrices may help to identify, for example, which molecules can constrain the antioxidant response. Some antioxidant mechanisms rely on a delicate equilibrium among molecules involved. Hence, a failure of one of them could compromise the effectiveness of the antioxidant response. For example, deficiency in vitamin E may compromise the use of vitamin C (Lebold et al. 2013). However, it cannot be entirely excluded that maintaining a variety of components in the antioxidant machinery could provide a "fail-safe" to avoid the case that the failure of one antioxidant compromises the whole system. To answer these questions would mean identifying any biochemical constraints present in the network and how these can limit the plasticity of the stress response. This could be done, for example, by experimental removal or downregulation of an arbitrary or most-connected node (i.e. hub) of the network.

Increases in the concentration of a given antioxidant may also result in no net reduction in oxidative damage because any potential beneficial effects are offset by compensatory reductions in other antioxidants. For example, long-term vitamin C supplementation to laboratory mice reduced expression of several genes linked to free radical scavenging, with no net effect on various oxidative damage measures (Selman et al. 2006).

There is evidence that integration of a metabolic or genetic system may change with exposure to environmental stressors (Fig. 1.4), possibly depending on the degree of prioritization of processes that promote self-maintenance and survival or reproduction (e.g., Giuliani et al. 2004; Csermely and Söti 2006; Tun et al. 2006;

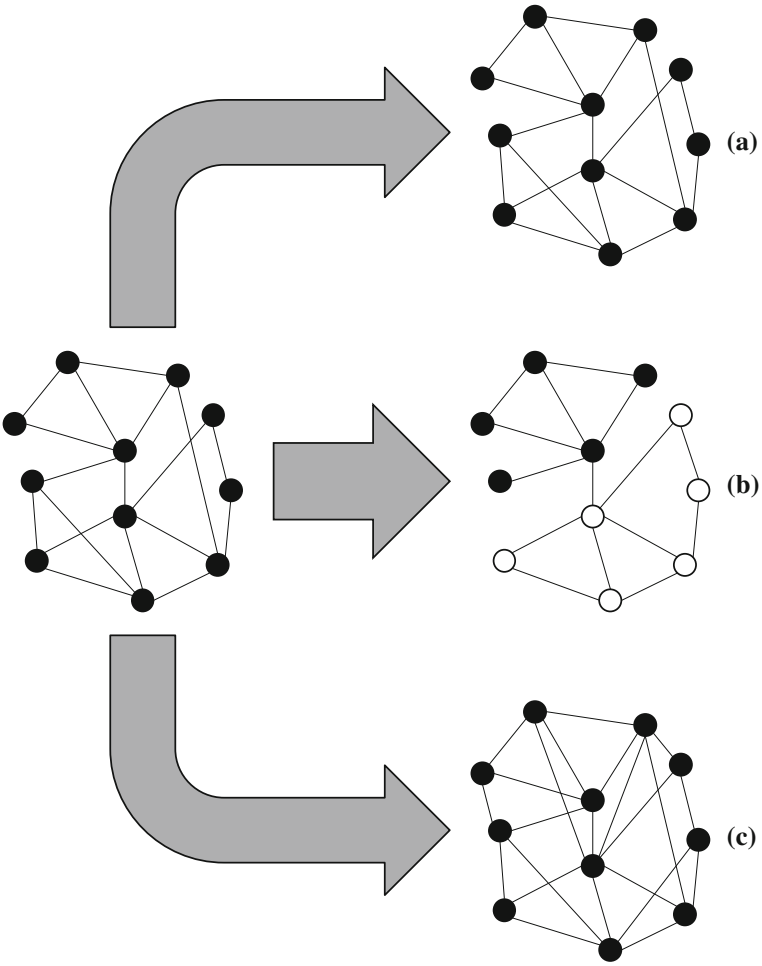

Fig. 1.4 Schematic representation of three hypothetical scenarios of temporal variation in degree of redox network integration and modularity in a tissue. In scenario (**a**), the network integration remains stable over time; in (**b**), the network loses integration and two modules (*white and black circles*, respectively) can be recognised by the fact that nodes within each module are highly connected, while the two modules are connected to each other by one link only; in (**c**) the network becomes more integrated compared with the original one. The three scenarios can also be directly comparable with each other without consideration of the original network whether nodes refer to the magnitude of the change over time (e.g., within-individual change values over a certain experimental period) rather than to the concentration of a certain molecule. In this case, we would, therefore, compare the degree of integration of changes in variables (i.e. how a change in concentration of molecule A is correlated to that of molecule B) rather than the integration among baseline measures before and after a certain time or experimental treatment, respectively

Parter et al. 2007; Southworth et al. 2009). Gene and metabolic networks can, for example, become less integrated as the individual ages, hence becoming noisier and less stable because of a decrease in the effectiveness of communication among functional units (Csermely and Söti 2006; Xue et al. 2007; Southworth et al. 2009; Soltow et al. 2010).

A decrease in integration might be expected whether a disruption of homoeostatic mechanisms (e.g., decrease in molecular communication, deregulation of redox balance control by thiols) makes the cells less capable of efficiently controlling the activity of free radicals (Dröge 2002; Jones 2006). For example, an increase in production of free radicals might not be tackled by the antioxidant response, leading to an increase in oxidative damage. Moreover, oxidation of molecules involved in redox regulation and control might alter their functionality, hence reducing their capacity of interacting with other molecules. The strength of correlations (and hence integration) among parameters of oxidative status may change, depending on whether they reflect measurements made under baseline or stressful conditions (Dotan et al. 2004; Costantini et al. 2011b, 2013). An important question, therefore, is whether the level of integration changes linearly with the increase of a stressor intensity or shows a biphasic response (showing greater levels of integration after mild exposure to the stressor), indicating a link with hormesis. For example, it has been found that the integration of blood oxidative status decreased linearly with a short-term increase in physical activity (Costantini et al. 2013).

It may also be informative to look at integration in a comparative framework because differences in correlations among antioxidant levels between species might exist (e.g., Cohen and McGraw 2009; Sepp et al. 2012), hence the degree of integration might be subject to different selective pressures. Finally, it is important to look at how increases or decreases in biochemical integration of the oxidative stress response impinge on evolutionary fitness.

From analyses of integration, it is also possible to describe the hierarchical organisation of modularity of the system under study (Ravasz et al. 2002; Pamplona and Costantini 2011). A module is a discrete unit of x number of elements interacting in a tightly integrated way that performs a specific task, separable from the functions of other modules (Hartwell et al. 1999; Hasty et al. 2001). Only relatively few or weak interactions will then connect different modules, possibly depending on their functions and environmental fluctuations (Fig. 1.4). The identification and study of single modules may also facilitate the examination of biochemical processes and impact on organismal physiological state by deconstructing complex networks into components that are easier to describe. Ravasz et al. (2002) described, for example, a hierarchical organisation of modularity in the metabolic network of *E. coli*. The authors found that there are many highly integrated small modules (submodules), which group into a few larger modules. The authors also suggested that the accumulation of changes in the submodules could slowly impact the properties of the larger ones, hence affecting the evolution of the whole system. Further modular organisations have been recently characterised in metabolic networks and stress response pathways of

bacteria. Parter et al. (2007) found that metabolic networks of bacteria living in variable environments are more modular than networks of bacteria living in more stable conditions. The authors suggested that unstable environmental conditions promote modular organisation because each module deals with a specific task. Given that in an unstable environment an organism can be faced with several environmental pressures, it could be more functionally efficient to separate the processing of the stimulus and the response among different modules according to the nature of the stimulus itself (Parter et al. 2007). Accordingly, Singh et al. (2008) reported that stress response modules vary in both number and degree of cohesiveness in bacteria. Moreover, they showed that most of the modular variation is in environmental sensing and signal transduction genes at the onset and completion of the response, while structural proteins show the least variation. These findings also suggested that the modular architecture might reflect a real functional organisation of the metabolic system in relation to environmental pressures. Modularity of bacteria (117 species) also appeared to be more correlated to the ecology of the species than to phylogenetic relatedness (Parter et al. 2007). The lowest modularity was found for obligate species, intermediate modularity levels occurred in specialised and aquatic species and the highest modularity was found for facultative, multiple and terrestrial species.

1.3 Hormesis

1.3.1 Historical Scenario: Birth, Death and Resurgence of Hormesis

That some substances may be stimulatory or even beneficial at low doses, but toxic at high doses, is something we are all familiar with. However, for compounds that are considered always stressful or toxic, it is more surprising that, at low doses, they can actually stimulate physiological adaptive responses of the organism, possibly resulting in beneficial effects (Calabrese 2010b; Costantini et al. 2010a; Mattson and Calabrese 2010). That chemicals with toxic effects at high doses may have beneficial effects when administered at low doses was already recognised around five centuries ago by Paracelsus (Mattson and Calabrese 2010). Then, in the mid 1880s, the pharmacologist Hugo Schulz (1888) observed experimentally that a broad range of chemical disinfectants stimulated the metabolism of yeast at low doses, while inhibited it at high doses (Mattson and Calabrese 2010). Schulz believed that the biphasic responses he observed in his experiments could explain the adaptive responses observed in homoeopathy that permit patients to resist an infection and to recover (Mattson and Calabrese 2010). This kind of biphasic responses was later named hormesis by Southam and Ehrlich (1943), who observed a stimulatory effect of solutions of red-cedar extract on the growth rate of various fungal species. Unfortunately, scientific investigation of hormetic responses lost credibility in the 1920s and 1930s because they were erroneously

linked to homoeopathy, a practise shunned by traditional medicine (Mattson and Calabrese 2010). It is since the 1980s that there has been a resurgence of interest in hormesis, especially in the fields of toxicology and pharmacology. Many studies showed that hormetic responses occur across a wide range of organisms (from plants to bacteria and vertebrates) exposed to a diverse range of at least 1,000 chemical and environmental stressors (Calabrese and Baldwin 2003; Calabrese and Blain 2005, 2009; Costantini et al. 2010a; Mattson and Calabrese 2010). Moreover, it has been realised that many scientists have reported observations supportive of hormesis using other terms (e.g., U-shaped, J-shaped, bimodal, inverted U-shaped, Arndt-Schulz law, bell-shaped curve, Hueppe's rule, heat or cold hardening), which has contributed to underestimate the occurrence of hormesis in the literature (Calabrese 2010b).

1.3.2 Types of Hormesis

The term hormesis is currently used in two different, albeit-related, ways. The first relates to its original use in describing a biphasic response to an environmental stressor, with a mild dose stimulation and a high dose inhibition of the response of a trait (e.g., antioxidant activity, longevity) compared with a control group. Let's take the relationship between contaminants and oxidative damage as an example. We might expect the negative effects of exposure to contaminants to become more severe (and oxidative damage to therefore increase) as exposure increases (Fig. 1.5a). However, there may be no noticeable effect until the level of contaminant the organism is exposed to reaches a threshold value (termed the *No Observed Adverse Effect Level* in toxicology), above which oxidative damage increases linearly or non-linearly with dose (Fig. 1.5b). In another case, the change in oxidative damage level in relation to contaminant exposure is biphasic (Fig. 1.5c): a mild contaminant dose would elicit the organism stress response (possibly beneficial), hence reducing oxidative damage level; on the other hand, a high contaminant dose would cause inhibition of stress response pathways or toxicity, hence inducing an increase in oxidative damage level (Costantini et al. 2010a; Costantini 2013; Mattson and Calabrese 2010). This last kind of dose-response relationship is termed hormesis. In this definition, we have to distinguish between stimulatory and beneficial. Stimulatory physiological responses might not be beneficial in terms of reproduction or longevity in case, for example, trade-offs occur among fitness traits. Moreover, it is suggested that the stimulatory response can be direct without initial disruption in homoeostasis (e.g., that operating within the normal maintenance function; a chemical has two actions with opposing effects on a biological endpoint) or can be induced by a disruption in homoeostasis, followed by an overcompensatory response (Calabrese and Baldwin 2002; Calabrese 2010a, b). The biphasic nature of the response might also arise from prioritisation of self-maintenance mechanisms at mild doses of stress exposure because this is not so demanding to deal with if compared with exposure to high doses.

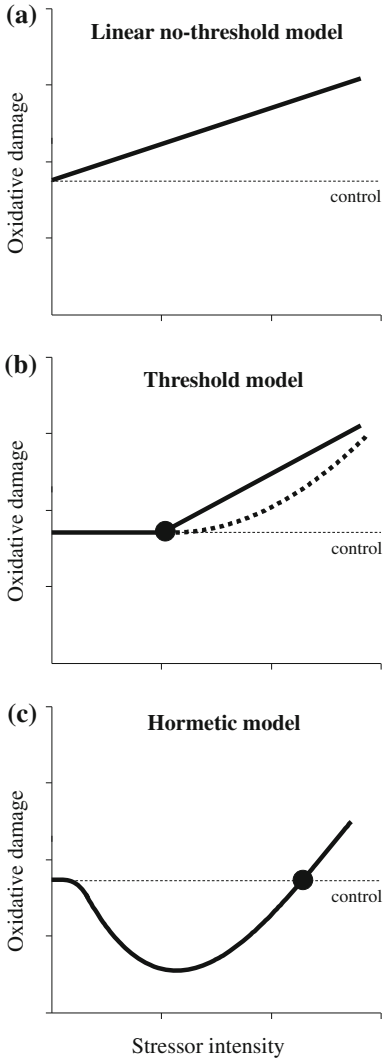

Fig. 1.5 Examples of common dose–response models. In these examples, oxidative damage is used as an endpoint. **a** In the linear no-threshold model, oxidative damage increases linearly with the stressor intensity, with no threshold of activation of the physiological response. **b** In the threshold model, the stressor has no biological effect until a threshold (indicated by the *black circle*) is reached, above which oxidative damage can increase linearly or non-linearly. (**c**) In the hormetic model, the response to the environmental stressor is biphasic, with oxidative damage decreasing and increasing at mild and high doses of the stressor, respectively. The zero equivalent point (*black circle*) refers to the area of the hormetic curve where oxidative damage equals that of the control (i.e. zero exposure). Reprinted from Costantini (2013) with kind permission from Springer Science + Business Media B.V

In most experimental investigations of this process, various treatment groups are exposed to differing levels of a stressor and the average response of each group is then measured and compared with a control group. The timescale over which the response is measured can vary from the immediate (as is typical with biochemical measures such as concentration of oxidative damage molecules) to the long term (as in the case of longevity; Costantini et al. 2010a). For example, the viability of *Saccharomyces cerevisiae* increases with the concentration of hydrogen peroxide they are exposed to (peak of stimulation around 0.45 mM); after a certain threshold (0.8 mM), the viability goes below the control level, showing that

hydrogen peroxide becomes toxic after a certain concentration (Davies et al. 1995).

A second use of the term hormesis is in relation to the conditioning or priming effect, whereby exposure to a mild level of a stressor results in the organism being better able to cope with exposure to higher levels of the stressor when encountered on subsequent occasions (Costantini et al. 2010a). This type of hormetic response is called conditioning hormesis according to the terminology proposed by Calabrese et al. (2007). Experimental investigations of this process require some individuals to be not exposed or exposed to mild and high levels of the stressor initially. At some later point, half of the individuals from each group are allocated to one of two new groups: control or exposed to high levels of the stressor. The extent to which individuals can then cope with the high level exposure (i.e. the extent to which they show detrimental effects on evolutionary fitness, or some proxy for fitness, such as tissue damage) is compared among the groups (Costantini et al. 2010a). It would be expected that individuals previously exposed to the mild level of the stressor would withstand the subsequent exposure to the high level of the stressor better than individuals that did not experience previous exposure or were previously exposed to a high level. For example, conditioning of PC12 cells (a rat cell line derived from pheochromocytoma cells) with low concentrations of hydrogen peroxide made them more resistant to subsequent exposure to high levels of hydrogen peroxide compared with cells that were not previously exposed to or were exposed to high concentrations of hydrogen peroxide (Tang et al. 2005). Specifically, cells were conditioned with 0, 5, 10, 20 or 30 μmol L^{-1} hydrogen peroxide for 90 min, followed by 24 h recovery, and subsequent exposure to 50 or 100 μmol L^{-1} hydrogen peroxide for 24 h. Apoptosis was significantly lower in cells that were conditioned with 10 or 20 μmol L^{-1} hydrogen peroxide in both experiments (Tang et al. 2005). Moreover, conditioning with 10 μmol L^{-1} hydrogen peroxide had a protective effect on hydrogen peroxide-induced dissipation of the mitochondrial membrane potential, inhibited hydrogen peroxide-induced intracellular accumulation of reactive species and induced overexpression of the antiapoptotic protein Bcl-2 (Tang et al. 2005).

The timing of the first (mild level) exposure may be important to determine the strength of the hormetic conditioning, for example, if there is a critical time window or developmental stage in which the priming effect can occur (Costantini 2013). Studies on rats showed that exposure of pups to a mild stressor can dampen the stress response mediated by glucocorticoids when exposed to a stressor later in life (Laviola and Macrì 2013). Such a dampening in the secretion of glucocorticoids might be beneficial. In contrast, exposure to high stress in early life might cause subsequent hypersensitivity to stressors, manifested in an oversecretion of glucocorticoids. Such hypersensitivity has been shown to have detrimental effects on health (Levine et al. 1967; Liu et al. 1997; Romero 2004; pp. 692–694 in Nelson 2005) and can lead to excessive energy mobilisation (Wingfield et al. 1998), immunosuppression (Webster Marketon and Glaser 2008), oxidative stress (Costantini et al. 2011a) and abandonment of reproductive activity (Wingfield and Hunt 2002). Clearly, environmental conditions (e.g., food availability, predation

risk) would then determine the extent to which high secretion of glucocorticoids may still fall within an adaptive range regardless of any costs associated. There may or may not be a cost associated with the first exposure; the existence of such a cost would explain why natural selection has not favoured a system design that does not require a priming effect in order to work effectively (Costantini et al. 2010a). The negative fitness consequences of the priming process would only be evident if there were no subsequent exposure to a higher level of the stressor, as in the case of environmental mismatching (Costantini et al. 2010a, 2014). The probability of encountering high levels of the stressor in the environment will likely influence the evolution of the response, as will the extent to which the first exposure carries costs. As illustrated by the rat example above, exposure to differing intensities of a stressor in early life can have differing effects. Recent work in rodents suggested that exposure to relatively high stress levels (e.g., 24 h of maternal deprivation, a model for maternal neglect; e.g., Cirulli et al. 2003) can, in addition to having negative effects, actually have beneficial effects on some aspects of brain function (hippocampal synaptic plasticity, emotional learning). This is especially apparent in the hippocampus, and these beneficial effects are particularly evident under conditions of high stress in adulthood (Oomen et al. 2010). However, we know little about optimal exposure levels, i.e. those that give the maximum overall benefit at the minimum cost, and how this varies with different stressor types (Costantini et al. 2010a).

1.3.3 Quantitative Features of Hormesis and Problems with Its Detection

The maximum stimulation commonly observed in a hormetic response appears to be 30–60 % greater than control group (Calabrese and Baldwin 1998), and it is consistent across many species, endpoints, agents tested and both kinds of hormetic responses. The range of the stimulatory response looks generally modest (Calabrese 2008). However, in about 2 % of the cases analysed so far, the range of the stimulatory zone was very wide (Calabrese 2010a). The above characteristics of a hormetic response are very important to bear in mind because, given the modest magnitude and width of the stimulation, a rigorous study design along with considerable statistical power might be needed in order to detect a hormetic response (Calabrese 2010a). This is even more important when background levels of, for example, oxidative damage are very low and so the stimulatory effects of the upregulation of antioxidant mechanisms (e.g., repair systems) consequently to a hormetic response can be difficult to detect analytically (Hoffmann 2009).

Another aspect that may make the hormetic response difficult to detect is the interaction among differing chemicals or environmental stressors (Calabrese 2010b). This interaction might influence the dose at which the stimulation occurs or the maximum response. For example, Flood et al. (1983, 1985) analysed the

effects of various drugs on mouse memory. Although drugs individually enhanced memory by around 50 %, the maximum response was not influenced when administered in combination. However, the dose at which the maximum response occurred was reduced for each drug. In another study on the water flea *Daphnia carinata*, it was found that low concentrations of glyphosate improved the number of offspring per female and the time to the first brood when the experiment was conducted in sea salt medium, but not in a medium designed for daphnids (Zalizniak and Nugegoda 2006). It was suggested that the hormetic effect induced by glyphosate in the sea salt medium occurred because glyphosate enhanced the uptake of elements that were in limited supply in that medium (Zalizniak and Nugegoda 2006). In another experiment, Zalizniak and Nugegoda (2006) also showed that the glyphosate concentration at which they previously found a hormetic effect had an effect when cadmium was absent or was 5–10 % of its 48 h LC_{50} (dose required to kill half the individuals of a population under study after a certain test duration). Similarly, Zhang et al. (2013) found that mixtures of ionic liquids induced time-dependent hormetic effects on the growth of *Vibrio* bacteria, but the maximum stimulation took more time to be achieved than when *Vibrio* cultures were exposed to individual ionic liquids. Overall, these findings clearly show that complex interactions among chemicals make difficult to predict hormetic responses. These results might have important implications when, for example, we evaluate whether the intensity of an environmental perturbation (e.g., ambient temperature, pH) is within the stimulatory or toxicity range. The interaction of one environmental stressor with another may drastically change the overall effect on the organism, hence affecting our interpretation of the effects of that stressor on individual life-history adjustments. For example, if an increase of 5 °C in ambient temperature is needed to induce a stimulation of antioxidant response, it might only need an increase of 2 °C to induce a biological effect if it occurs simultaneously to, for example, an increase in humidity. Moreover, the stimulatory area and so the peaks of stimulation might differ among traits because differing mechanisms may be activated or the individual may prioritise investment in one trait at the expense of another one.

Another feature that may make the assessment of hormetic responses problematic is the variability within a population. For example, in epidemiological evaluations, the occurrence of intergroup dose-response variability within a population may produce a blended dose-response relationship that could mask the dose-response dynamic that occurs within each subgroup (Calabrese 2010b). For example, a study on two crustacean species (*Gammarus aequicauda* and *G. insensibilis*) showed a differential genotypic resistance to thermal stress, which resulted in differing mortalities (Patarnello and Battaglia 1992). Moreover, the relationship between temperature and activity of the enzyme glucose phosphate isomerase (positively correlated with survival rate in both species) was linear or hormetic-like biphasic, depending on genotype and species (Patarnello and Battaglia 1992).

Finally, it cannot be assumed that a hormetic response we describe in a species is comparable with that of another one because the hormetic zone (the stimulatory area under the curve) can differ among species, possibly reflecting specific adaptations to differing habitats (Holmes et al. 1980; Parsons 2001; see Chap. 9).

1.3.4 Hormesis and Evolutionary Fitness

Stimulation of a trait through hormesis would be evolutionarily relevant whether this increased the ability of an individual to have its genes represented in future generations and so to spread them throughout the population. In this case, evolutionary changes would occur through changes in gene frequencies. The approach of hormesis from an evolutionary perspective has created a debate on whether hormesis is an evolutionary expectation. Forbes (2000) suggested several scenarios to argue that hormesis of evolutionary fitness cannot be expected: (1) because of trade-offs among life-history traits, it is not possible for all traits to exhibit hormetic responses simultaneously, and therefore, fitness is unlikely to be enhanced at mild levels of exposure to stressors; (2) stimulation of certain phenotypic traits might be selectively neutral; selection of non-adaptive or neutral genes coding for hormesis might occur as a consequence of linkage, i.e. non-independent assortment of genes having their loci on the same chromosome, which would make them more likely to be inherited together; (3) hormesis of a trait might buffer fitness against environmental changes, slowing down its decrease as the level of exposure to a stressor increases or maintaining fitness at a constant level; therefore, stimulation of certain organism traits might be selectively favoured.

Forbes' argumentations are supported by studies carried out on *Drosophila* fruit flies showing opposite trends in longevity and reproduction in response to heat stress (Maynard Smith 1958; Le Bourg et al. 2001; Le Bourg 2005; Sørensen et al. 2007, 2008) or on western fence lizards (*Sceloporus occidentalis*) showing that 2,4,6-trinitrotoluene-induced hormesis for growth occurs under high but not low food availability (McFarland et al. 2012). However, hormesis of growth was observed under suboptimal diet conditions in house crickets *Acheta domesticus* (Luckey 1968). It is quite hard to generalise from these results given that they are based on a very narrow range of laboratory model species. It should also be considered that the production and consumption of energy might be altered as part of the hormetic response, so making hormesis less energetically demanding than expected. In other words, energy use could be optimised in terms of energetic trade-offs between self-maintenance and other activities, such as reproduction (Costantini et al. 2010a). Hormesis might also tend to evolve in habitats where a certain species may achieve its maximum metabolic efficiency (Parsons 2001, 2007), which would make energy less constraining for individual decisions. Moreover, there is a large body of literature showing that mild exposure to

stressors can simultaneously increase both longevity and reproductive output (Parkhurst et al. 1981; Giesy et al. 2000; Cardoso et al. 2002; Calabrese and Baldwin 2003; Calabrese and Blain 2005; Cutler et al. 2009). Such discrepancies might be, for example, explained by the extent to which the environmental conditions (e.g., food availability, ambient temperature, stage of life cycle) under which hormesis of fitness is tested are challenging for the individual and can so strengthen or weaken constraints to individual decisions. Finally, a recent study found that natural variation in hormetic effects on lifespan for heat shock response may be genetically determined (Rodriguez et al. 2012), suggesting that hormesis could be a target of natural selection (see Chap. 9).

1.4 Life-History Evolution

The study of life-history evolution seeks to describe the major features (e.g., growth trajectories, age at maturity, longevity, reproduction) of the life cycle of an individual or a species and to determine what causes differences in evolutionary fitness among life-history variants within and between species (Stearns 1992). This is based on the idea that a phenotype consists of a series of traits connected to each other by constraining relationships called trade-offs, and it is the interaction among these traits that determine evolutionary fitness (Stearns 1992).

Classic life-history research showed that species strongly differ in life-history strategies. There is also large variation in life-history styles within the same species, reflecting differentiation among phenotypes and genotypes. Moreover, in sexually reproducing species, sexes have different life histories, and so, they may differ in the behavioural and physiological strategies they adopt in response to the same environmental stimuli.

Over space and evolutionary time, individuals or species have been faced with the need to solve an important problem: the use of available resources (e.g., food, cover, time). This is because resources occur in limited supply in the environment, and nearly all life-history traits and activities require these resources. For these reasons, the current theoretical framework used in evolutionary ecology to describe and understand life-history diversity is based on the concept of trade-offs in the allocation of resources to life-history traits (Roff 1992; Stearns 1992). The theoretical underpinnings of life-history theory can be summarised as follows. Given that resources are finite, investment of a resource to one trait, such as reproduction, means that less can be invested to other traits, such as body maintenance. As a consequence, investment of a resource in a certain trait, activity or process has negative consequences for other traits or activities that require that same resource. Moreover, it has become clear that (1) it is also the performance of one activity (e.g., immune response) that may generate negative consequences for other traits (e.g., oxidative damage to cells), (2) trade-offs can also arise from

switches in molecular pathways rather than from resource allocation (Barnes and Partridge 2003), and (3) there are limits to organism functions imposed by basic physics and chemistry (Bialek 2012). The existence of these trade-offs gives rise to the central concept in evolutionary ecology that all fitness-related traits cannot be simultaneously maximised because of constraints on trait expression and costs originating from life-history strategies. A constraint implies a limitation to evolutionary change that inhibits the ability of the phenotype to evolve or canalises its evolution along certain paths. Hence, constraints would serve as regulators of evolution (Schwenk 2002). Natural selection is expected to operate in order to optimise a compromise among the costs and benefits of different investment strategies. The resulting evolutionary fitness outcomes (i.e. lifetime reproductive success of individuals with different investment strategies, hence the degree to which their genetic architecture is represented in the next generation), will drive evolution of life histories, provided that fitness differences are heritable.

The relationship of the organism to its environment is mediated by a physiological basis that constrains micro-evolutionary optimisation. It is recognised that elucidating the physiological mechanisms that may work as constraints is essential to our understanding of the diversification of life histories (Zera and Harshman 2001; Ricklefs and Wikelski 2002). It should not be forgotten, however, that the evolutionary benefits of a particular investment strategy depend on many factors. For example, extrinsic mortality factors, such as disease or predation, produce important selection pressures that drive the evolution of reproductive strategies, senescence rates and lifespan (Reznick et al. 1990; Stearns 1992). Therefore, it would be a counter-selective strategy where resources are invested in a long-lasting body where the probability of, for example, being predated is very high. Under these conditions, a better evolutionary strategy would be to invest more resources into reproduction rather than self-maintenance (Fig. 1.6a). High investment of parents in reproduction can, in turn, increase the growth rate of their offspring, making them reach sexual maturity at an early age. This may have costs for offspring in terms of oxidative stress and a resulting high rate of senescence (Fig. 1.6b). Environmental conditions under which we investigate trade-offs are, therefore, very important to take into account because they can exacerbate or mitigate costs associated with a certain strategy, hence they may shape ecological trade-offs (Jessup and Bohannan 2008).

There are several physiological mechanisms that may drive trade-offs. We, therefore, need to quantify these mechanisms. The benefit is that there are laboratory techniques that allow measurement of, for example, molecular end products to be used as markers of a potentially relevant biological signal. Evolutionary ecologists and physiologists have relied on various aspects of physiological mechanisms, such as energy production and expenditure, immune response or hormones. In the last few years, some authors have suggested that oxidative stress might be a prime physiological mechanism mediating short- and long-term life-

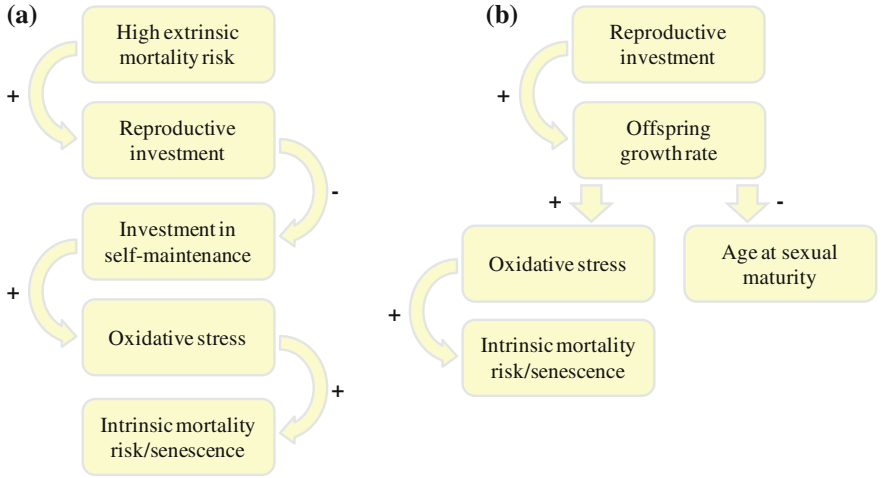

Fig. 1.6 Hypothetical relationships among life-history traits, senescence and oxidative stress. In (**a**) individuals exposed to high extrinsic mortality risk (e.g., predation risk) invest lots of resources in reproduction, which comes at a cost for self-maintenance, especially under conditions of limiting resources. A main consequence is an increase in oxidative stress level and so in the intrinsic mortality/senescence rate. In (**b**) a higher investment in reproduction results in offspring growing faster and attaining sexual maturity earlier than offspring of parents that invested less in reproduction. Growing at a faster rate is paid in terms of high oxidative stress and, consequently, a higher intrinsic mortality/senescence rate. + indicates an increase, while − indicates a decrease of a certain variable

history trade-offs because the resultant tissue degradation might influence reproductive performance, growth patterns, senescence and survival (Costantini 2008, 2010; Dowling and Simmons 2009; Monaghan et al. 2009; Costantini et al. 2010b; McGraw et al. 2010; Metcalfe and Alonso-Alvarez 2010; Isakkson et al. 2011; Selman et al. 2012). More recently, it has been emphasised that we should look at oxidative stress (and other stressful agents) under a hormetic framework because the degree and direction of effects will depend on its intensity and will not necessarily be detrimental (Costantini et al. 2010a). Therefore, measures of oxidative damage or antioxidant molecules would offer a unique currency to quantify the result of trade-offs and predict consequences for evolutionary fitness. The utility of this approach extends beyond understanding life-history variation among individuals or species. It can also be used to understand the factors that have shaped the evolution of mechanisms that regulate oxidative balance and how such regulation relates to other physiological pathways and varies across differing social and non-social environmental contexts. Oxidative stress may have represented an important internal selective pressure that contributed to the production of evolutionary innovations.

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Chapter 2
Early-Life Hormesis and Oxidative Experiences Fine-Tune the Adult Phenotype

Abstract The early environment in which an individual grows up and develops has profound, long-lasting and often irreversible consequences throughout the individual lifetime. Growing individuals are faced with different physiological and life history demands; hence, they may adopt differing strategies for allocating resources between growth and self-maintenance. Costs and benefits vary across contexts and are likely to be species-specific; hence, their magnitude does not appear to be peculiar to a certain strategy of growth and development. Oxidative stress and hormesis have been suggested to be particularly important in programming the phenotype. This chapter discusses the central role of maternal sexual or stress hormones in fine-tuning the redox physiology of progeny and provides evidence in favour of a role of oxidative stress as a universal proximate mechanism underlying the trade-off between growth strategy and self-maintenance. This chapter also examines how hormesis in early life shapes a plastic phenotype adapted to variable conditions in its adult environment and how such long-lasting effects may be inherited. Understanding the physiological mechanisms mediating trade-offs during development is critical for our understanding of how the diversity in growth and developmental strategies has been evolved and why a strategy may be successful under certain environmental circumstances but not others.

2.1 Early Environment and Phenotypic Development

The adult phenotype is the outcome of complex interactions among the individual genetic background and the pre- and post-natal environmental conditions the individual experiences from conception through its growth and development until sexual maturity. It is in fact now well established that molecular regulators of development do not all reside only within the embryo or the egg, but also do work after birth. Post-natal environmental factors, such as temperature, photoperiod, diet, competition, parents, or the presence of predators, are a continuous source of external stimuli that carry information about the environment. The organism filters this information and decodes it in order to coordinate appropriate development

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physiological and life history responses. Post-natal environmental factors are therefore very important not simply because they select for genetic variation, but also because they contribute to build up phenotypic variation (Gilbert 2001; Metcalfe and Monaghan 2001a, b; West-Eberhard 2003; Monaghan 2008; Gilbert and Epel 2009).

Developmental systems are also guided by processes other than genetic control or natural selection, such as self-organisation properties of components of a developing organism, neutral evolution, epigenetic modifications of the genome or compensatory rearrangements of anatomical traits and metabolic networks (Szyf et al. 2005; Gerhart and Kirschner 2007; Badyaev 2011; Curley and Branchi 2013).

The complex interplay among the differing processes quoted above has fuelled the evolution of a multitude of growth and developmental strategies (e.g., metamorphosis, altricial–precocial axis). There are taxa that continually grow across their lifetime (indeterminate growth) or that stop at adulthood (determinate growth). There are also taxa whose growth is dramatically influenced by environmental conditions (e.g., food, temperature) more than that of others. For example, the growth of reptiles is indeterminate, slows down after sexual maturity and is strongly influenced by fluctuations in environmental conditions (Andrews 1982). Plant seeds can delay development and germination for years until environmental conditions become good enough to sustain the growth of the plant. This diversity in growth/development patterns is not random, but tends to be canalised along a few main routes. For example, the slow-to-fast pace of life continuum is typical for a large number of species. Moreover, within a same species, it is also possible to observe large among population or individual variation in the rate of growth and development, which may, for example, result in notable variation in body size at adulthood. Animal body size has a significant impact on evolutionary fitness in many animal species because early attainment of a large body size may confer selective advantages, such as in competition among males to attain a dominant position in the group, in laying large quantities of eggs or in the reduction in predation risk (Metcalfe and Monaghan 2001a, b). It could therefore be expected that in many species individuals grow as fast as possible in order to achieve adult body size as soon as possible to increase their reproductive success. However, growth rates are usually slower than the maximum achievable. Life history theory suggests that this pattern arises because of constraints in the allocation of limited resources (e.g., energy, nutrients) among competing functions and of physiological costs (e.g., tissue damage, accelerated rate of senescence) associated with a certain style of growing. Attaining sexual maturation earlier has certainly benefits because individuals may have higher fitness than those that mature later. Evolution of this strategy is, however, dependent on a variety of costs, from those that influence the chances of the developing individual to become sexually mature to those that can carry over through the start of reproductive activity. There is, for example, no point in developing fast if then cell oxidative damages compromise future fecundity. Delaying maturity would therefore pay more in evolutionary fitness terms. The sex is also important because a same developmental trajectory may have different fitness consequences for males and

females. For example, in breeding systems where males access to females after a strenuous competition, males might invest more in attaining a large body size at the expense of, for example, oxidative stress resistance, while females might do just the opposite. Consequently, individuals are faced with different physiological and life history demands; hence, they adopt differing strategies for allocating resources between growth and self-maintenance (Ricklefs 1984, 2003; Case 1978; Stearns 1992; Starck and Ricklefs 1998). Moreover, developmental (e.g., errors that lead to defects of the embryo) and ecological (e.g., need of a high foraging activity) factors also contribute to shape growth strategies (Gotthard 2001). Growth rate is so optimised with respect to selective environmental pressures, which change with maturation, and to physical and physiological constraints imposed by the organism design. It is well documented in the ecological literature that early-life experiences influence survival and reproduction in many invertebrate and vertebrate species (Lindström 1999; Metcalfe and Monaghan 2001a, b). Being born in a drought period to an inexperienced mother, for example, had adverse consequences for longevity, adult size and reproductive potential in African elephants *Loxodonta africana* (Lee et al. 2013b). It is also recognised that environmentally induced variation among individuals may impact on population dynamics and evolution of life histories (Lindström 1999). Hence, a particular state of development may turn to be a point of vulnerability or a window of opportunity (Andersen 2003), depending on how much adaptive the induced phenotype will be. Understanding the proximate physiological mechanisms mediating development trade-offs is therefore critical to further our understanding of how the diversity in growth and developmental strategies has been evolved and why a strategy may be successful under certain environmental circumstances but not others (Lindström 1999; Zera and Harshman 2001; Ricklefs and Wikelski 2002; Monaghan 2008).

In this chapter, I discuss the role of maternal hormones in fine-tuning the redox physiology of progeny (see Chaps. 4 and 8 for an examination of maternal nutrition, antioxidants and antibodies), how oxidative stress may act as a proximate mechanism underlying the trade-off between growth strategy and self-maintenance, and the way the oxidative phenotype can be shaped by early-life experiences. I also examine how hormetic processes contribute to explain how the early environment can help shape a phenotype adapted to the conditions the organism is most likely to experience in its adult environment.

2.2 Pre-natal Maternal Effects: How Mothers Use Hormones to Shape Their Offspring

Females pass to their embryo through the placenta or deposit in their eggs a large variety of molecules (e.g., hormones, nutrients, antioxidants) that will contribute to shape the anatomy, physiology and behaviour of the offspring. It is thought that the way mothers do so is shaped by natural selection because the quality and quantity

of the investment will influence both the mother and offspring fitness. Most studies testing the effects of these maternal effects, however, tend to be of short-term. In order to fully evaluate the adaptive value of a certain maternal effect, we need to track free-living individuals over long periods of time and to consider the environmental context and mechanisms inducing the effect within a life history framework (Sheriff and Love 2013).

2.2.1 *Effects of Maternal Androgens: Examples from Birds*

Mothers may influence phenotypic development of their offspring through the deposition into the egg of sexual hormones (Schwabl 1993; Groothuis et al. 2005; Carere and Balthazart 2007; Groothuis and Schwabl 2008). The importance of maternal hormones was first highlighted in the 1990s, when Schwabl (1993, 1996) demonstrated that female canaries (*Serinus canaria*) deposited testosterone in their eggs and did so not randomly across the laying sequence. Although yolk testosterone may have a beneficial effect on offspring (Schwabl 1993), it can also carry costs for the offspring, such as suppression of immune activity, oxidative stress and mortality (Sockman and Schwabl 2000).

Long-term effects of yolk androgens on oxidative balance may come through various routes, such as organisational effects of cell metabolism, differential growth rates or influences on social status and behavioural profile. There has not been so far any attempt to tease apart the various ways through which androgens may influence adult oxidative balance. In the last few years, however, it has been attempted to quantify the effects of androgens on oxidative state by injecting testosterone into the yolk. Results of these studies have been quite conflicting. Tobler and Sandell (2009) found that injecting eggs with 0.5 ng of testosterone on day 3 of incubation reduced plasma non-enzymatic antioxidant capacity in zebra finch (*Taeniopygia guttata*) males, but not females, at 10 days of age, while no effects emerged at 34 days of age. In another study using a same injection dose on day 3 of incubation, Tobler et al. (2013) found that males from testosterone-injected eggs appeared to have higher antioxidant levels than control males over a period lasting from fledging to the age of 7 months, whereas females had antioxidant levels that were intermediate compared with the two male groups. Galván and Alonso-Alvarez (2010) did not find any effects of injecting eggs of a same clutch with 26 ng of testosterone on red blood cell glutathione or plasma non-enzymatic antioxidant capacity in nestling great tits (*Parus major*). In contrast, Noguera et al. (2011) found that injecting third-laid eggs with 261 ng of testosterone on the day of laying increased transiently the plasma non-enzymatic antioxidant capacity and reduced the plasma concentration of malondialdehyde (an end product of lipid peroxidation) in yellow-legged gull (*Larus michahellis*) nestlings. Treidel et al. (2013) found that injecting eggs with 5 ng of testosterone prior to incubation did not influence the oxidative status of 17–18-day-old chickens (*Gallus gallus*), but reduced their DNA damage repair efficiency in

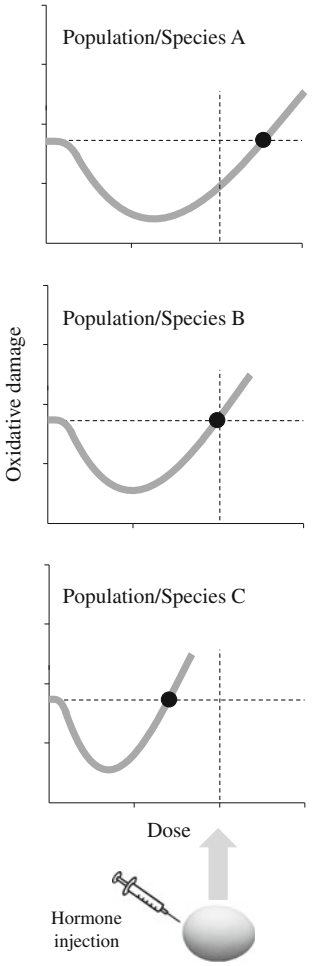

Fig. 2.1 The range of hormonal doses (or stressor intensity) over which the stimulation of organism stress response occurs can vary among populations or species, possibly reflecting evolutionary adaptations. These differences indicate the importance of the hormetic zone (the area under the *curve* and above the zero equivalent point) when predicting the responses of a population or species to an environmental perturbation. The zero equivalent point (*black circle*) is the point of the hormetic *curve* where oxidative damage equals that of the control (i.e. zero exposure), which is represented by a *horizontal dashed line*. Reprinted from Costantini (2013) with kind permission from Springer Science+Business Media B.V

comparison with control individuals. There might be several reasons that explain the discrepancies among these studies (Costantini 2013): (1) the dose of testosterone could have been, for example, in the stimulatory range of the dose–response curve for the gull, but in the inhibitory range for the zebra finch or the chicken (Fig. 2.1); however, zebra finches might have been conditioned to cope with low antioxidant levels during the nestling stage, which then stimulated them to more easily maintain high antioxidant levels during adulthood (Tobler et al. 2013); (2) the temporal window of manipulation of yolk androgens, which has a critical role for the organism response (e.g., hyporesponsive and hyperresponsive periods), might have differed among studies; (3) zebra finches and chickens have a higher pace of life than gulls; hence, they might have invested less in antioxidant protection against the pro-oxidant effects of testosterone, but this does not explain why blood antioxidants were unaffected in great tits, that have a high pace of life

too, and why the effect of a same dose of testosterone varies across studies on the zebra finches themselves; (4) the age at which the birds' response to treatment was tested differed among studies—thus changes in blood oxidative status across development could have caused the discrepancies among studies; (5) the distribution of testosterone across the yolk layers could have differed among studies, therefore exposing birds to different levels of androgens during differing phases of embryonic development (e.g., Lipar et al. 1999); (6) zebra finches, great tits, gulls and chickens may have differentially metabolised the injected testosterone, which is a precursor of the androgen dihydrotestosterone and the oestrogen estradiol (aromatisation: biosynthesis of oestrogens from testosterone catalysed by the enzyme aromatase)—the latter can have pro-oxidant effects in birds (Casagrande et al. 2012a, b); (7) species differ in the developmental strategies (altricial vs. precocial); hence, comparison is difficult because any effects might become evident at different time windows.

2.2.2 *Stress Hormones and the Developmental Programming Hypothesis*

The *Developmental Programming Hypothesis* states that adverse conditions during development may lead to adulthood disease. This hypothesis was proposed to explain why in mammals (including humans) exposure to environmental stressors during pregnancy, the pattern of foetal growth/development and later pathophysiology are connected to each other (Barker et al. 1993; Seckl 1998). It has been proposed that foetal malnutrition and foetal overexposure to glucocorticoids (also known as stress hormones) are two potential mechanisms underlying this early programming (Barker et al. 1993; Edwards et al. 1993; Seckl 1998). In particular, it has been suggested that overexposure of foetus to maternal glucocorticoids provides offspring with adaptive cues that would prepare their phenotype to a future stressful environment through a matching of the developmental and adult environmental conditions (Edwards et al. 1993; Seckl 1998). Vertebrates translate environmentally stressful stimuli into secretions of hormones that through a cascade mechanism activates the physiological stress response (Sapolsky et al. 2000; Romero 2004). Glucocorticoids are the end products of this hormonal cascade (Sapolsky et al. 2000; Romero 2004). Glucocorticoids induce a set of physiological and behavioural responses, which characterise the so-called emergency life history stage (Wingfield et al. 1998). It was therefore hypothesised that glucocorticoids would influence the plasticity of offspring stress response and prime the individual to the future stressful experiences. This would then confer to the offspring an adaptive advantage, as long as there is some matching between the early and adult environments (Monaghan 2008). Studies of wild European starlings (*Sturnus vulgaris*) showed that induction of pre-natal stress through *in ovo* injection of corticosterone decreased stress responsiveness of nestlings at fledging

(Love and Williams 2008a). The authors suggested that such plasticity in hypothalamic–pituitary–adrenal axis activity may be the result of a predictive adaptive response, that is, maternal stress hormones might provide offspring with a predictive signal of how their future environment sensu lato will be (Love and Williams 2008a, b). This form of maternal-induced developmental plasticity might be adaptive later in life because a hyperresponsive stress system could be too costly to sustain under harsh conditions (Love and Williams 2008a). Moreover, the transfer of stress hormones to eggs would also match the quality of a mother to offspring demands, hence increasing maternal fitness (Love and Williams 2008b). For example, when a mother is unable of providing enough food to her offspring because it is not available or she and her mate perform poorly in foraging for various reasons, it is more advantageous to produce a thrifty offspring because it would then require not too much food (Hayward and Wingfield 2004). The growing individual may therefore use stress hormones as maternal cues to predict its future environment and pave the way for a developmental strategy that might better prepare it for the expected future environment. It is the predictive nature of the environmental cue that might determine whether the developmental strategy will be adaptive or maladaptive (Monaghan 2008; Laviola and Macrì 2013). This paradigm has been criticised because the adult environmental conditions may not be predictable (e.g., in environments with marked seasonality), which would make the organism phenotype mismatched to its adult environment and likely incapable of surviving and having fitness (Bateson et al. 2004). The Developmental Programming Hypothesis, therefore, might prove unreliable when trying to predict the effects of early life on adult condition. However, it has been suggested that it is the intensity of early stress experienced that is important because this will programme the degree of stress responsiveness in relation to environmental conditions. In this context, hormesis could provide a useful conceptual and mechanistic model to fill in the gaps of the Developmental Programming Hypothesis because it highlights the importance of stressor intensity so that its consequences for individual growth, health and survival can be considered. For example, stimulation of growth through hormesis may occur when being exposed to low levels of stress early in life (in house crickets *Acheta domesticus*, Luckey 1968; in tobacco budworms *Heliothis virescens*, Stipanovic et al. 1986; in cotton bollworms *Helicoverpa armigera*, Celorio-Mancera et al. 2011; in western fence lizards *Sceloporus occidentalis*, McFarland et al. 2012). Hormesis might also increase the stress tolerance and response effectiveness of an organism (homeodynamics sensu Yates 1994; Lloyd et al. 2001; Stebbing 2009). In ecological terms, conditioning hormesis early in life might translate to an increase in phenotypic plasticity, hence the ability of an organism to respond to environmental stimuli. In general, the phenotypic response to the current environment may depend on both its similarity to previous environments (mismatches leading to ecological limits on plasticity; Auld et al. 2010), and on the nature of previous responses, since trait expression early in ontogeny may affect later trait expression (so-called plasticity–history limits; Auld et al. 2010). However, some evidence suggests that hormetic responses can be generalised across stressors, so that conditions experienced in early life allow an

individual to withstand other kinds of stressful conditions later in life (Le Bourg and Minois 1997; Bartling et al. 2003; Honma et al. 2003). Hormesis thus has the potential to increase phenotypic plasticity, so having the opposite effects of both ecological and plasticity–history limits (Costantini et al. 2010). Consequently, hormetic mechanisms could provide a fail-safe to buffer maladaptive effects of maternal investment when the maternal or early growth environment is not predictive of the adult environment (Costantini 2013).

2.2.2.1 Birds

Following pioneering studies of organisational effects of stress hormones on phenotypic development in mammals, several studies on birds reported the presence of stress hormones (corticosterone) in the egg (e.g., Downing and Bryden 2002; Eriksen et al. 2003; Hayward and Wingfield 2004; Love et al. 2005; Rubolini et al. 2005; Saino et al. 2005; Love and Williams 2008a, b; Marasco et al. 2012; Boogert et al. 2013), which led to the hypothesis that stress hormones might have a role in phenotype programming in birds, as well. Henriksen and collaborators (2011) recently reviewed studies on prenatal corticosterone-mediated effects in birds (see also Spencer et al. 2009; Schoech et al. 2011 for a review on post-natal corticosterone-mediated effects) and concluded that findings are inconsistent. Discrepancies among results might have been because the effects of yolk corticosterone depend on the interaction among pre- and post-natal environmental conditions, sex, age, development mode and type of treatment; alternatively, maternal corticosterone could have affected offspring behaviour and physiology through the alteration of other maternal factors (e.g., androgens, progesterone).

Two recent studies provided evidence that the mother might influence her offspring's oxidative balance and ageing phenotype by passing corticosterone to her eggs (Haussmann et al. 2012), but also that the nature of the effect depends on the interaction between the pre- and post-natal environments (Marasco et al. 2013). Haussmann et al. (2012) injected chicken eggs with a low (5 ng g^{-1} yolk) or high (10 ng g^{-1} yolk) dose of corticosterone, which elevated corticosterone levels within the physiological levels of chicken's yolk (Haussmann et al. 2012). While plasma baseline levels of corticosterone were not elevated compared with controls in chicks when 25 days of age, high-dose chicks had the strongest response to a restraint stress protocol. Chicks from corticosterone-injected eggs had baseline levels of oxidative damage higher than controls; moreover, chicks from the high-dose group had also an over-representation of short telomeres (indicating higher cell senescence) compared with control and low-dose chicks (Haussmann et al. 2012).

The effect of pre-natal stress may, however, depend on the early post-natal experiences. To answer this question, Marasco et al. (2013) used an experimental setting including four groups: pre-natal and post-natal untreated birds; pre-natal corticosterone-treated and post-natal untreated birds; pre-natal untreated and post-natal corticosterone-treated birds; pre- and post-natal corticosterone-treated birds.

For pre-natal stress treatment, eggs of Japanese quail (*Coturnix japonica*) were injected with 8.5 ng of corticosterone dissolved in peanut oil; for the post-natal stress treatment, chicks were given one mealworm (*Tenebrio molitor*) per day injected with 45 μg (between 5 and 15 days of age) or 90 μg (between 16 and 19 days of age) of corticosterone dissolved in peanut oil. Both pre- and post-natal treatments with corticosterone were chosen in order to increase corticosterone within the physiological range of quail. The effects of experiment were then tested on four parameters of oxidative status, analysed in red blood cells collected at 64 days of age and in the brain (cerebellum and midbrain) at 69–73 days of age (Marasco et al. 2013). In red blood cells, there was no effect on superoxide dismutase or protein carbonyls (biomarker of oxidative protein damage); the glutathione peroxidase was higher in all the corticosterone-treated birds than in controls, but there was an additive effect in birds that experienced both the pre- and post-natal treatment; a measure of non-enzymatic antioxidant capacity was lower in corticosterone-treated birds than in controls. All the parameters of oxidative status were not affected in the midbrain, while in the cerebellum the glutathione peroxidase was marginally higher in the three corticosterone-treated groups and the non-enzymatic antioxidant capacity was lower in the birds that experienced both the pre- and post-natal treatment than those that experienced only one of the two treatments (Fig. 2.2). Overall, increases in corticosterone concentration in yolk influenced the adult oxidative phenotype, possibly through direct effects on cell metabolism or gene expression. It might also reflect early-life costs that carried over until adulthood. Importantly, the nature of effects depended on the interaction between pre- and post-natal environments, suggesting a certain degree of plasticity in the regulation of oxidative balance. The strategy of depositing glucocorticoids into the eggs may be still adaptive whether any physiological costs for the chicks are lower than the benefits. This may be especially true for chickens and quail, as well as for other precocial species. Compared with altricial chicks, precocial chicks leave the nest soon after hatching and rely less on maternal care. Therefore, they have to be programmed to survive almost on their own very soon in life. In this regard, glucocorticoids may be very important because they enhance fear and vigilance behaviours, so allowing precocial chicks to avoid predators or to stay close to their siblings (Hayward and Wingfield 2004; Janczak et al. 2006). Moreover, chickens and quail are short-lived species; therefore, they might have been programmed to prioritise investment in growth and reproduction at the expense of investment in protection against oxidative stress.

The interaction effect between pre- and post-natal environments on the oxidative balance was also shown using unpredictable food supply as a source of pre- and/or post-natal stress. Wild grey partridges (*Perdix perdix*) had higher blood antioxidant capacity when they experienced no stress in both the pre- and post-natal stages; in contrast, antioxidants were significantly lower in grey partridges that experienced food stress only after hatching (Homberger et al. 2013). However, the production of free radicals in blood was not influenced by the stress regime. Hence, some of the reduction in antioxidants might have been because of the reduced access to food.

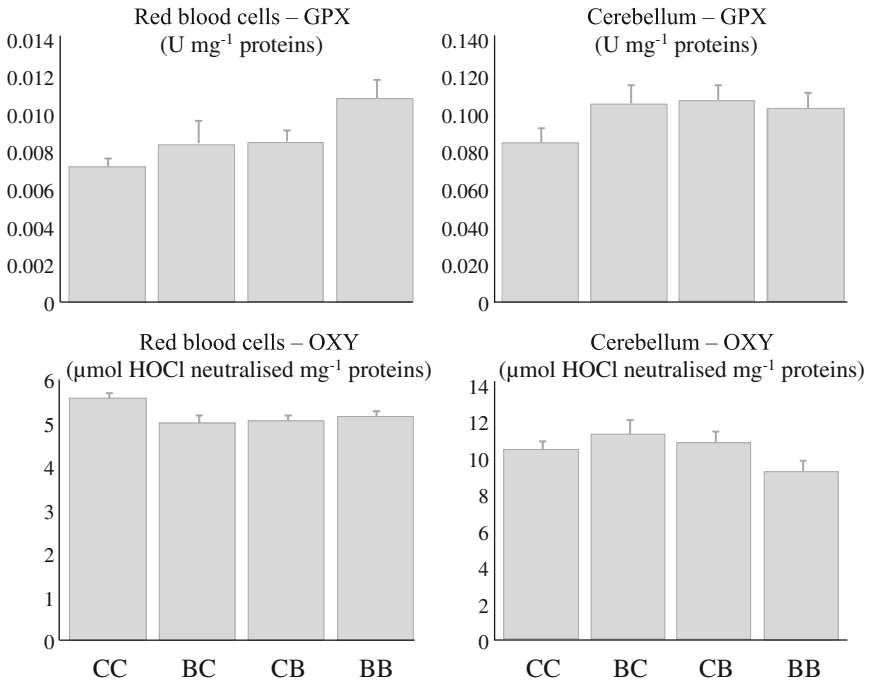

Fig. 2.2 Effects of physiological exposure to corticosterone (*B*) during the pre- and/or post-natal development on glutathione peroxidase (*GPX*) and non-enzymatic antioxidant capacity (*OXY*) in red blood cells and cerebellum of adult Japanese quails (Marasco et al. 2013). CC = controls; BC = pre-natal B-treated and post-natal untreated birds; CB = pre-natal untreated and post-natal B-treated birds; BB = pre-natal B-treated and post-natal B-treated birds. Data are shown as untransformed means + standard error. Reprinted with minimal alterations from Marasco and collaborators (2013) with permission from Elsevier

2.2.2.2 Mammals

It is well established in mammals that stress hormones can pass from the mother to her foetus, where they induce a cascade of downstream effects on embryo development and its future phenotype (e.g., Ward 1972; Dahlöf et al. 1977; Barker et al. 1993; Seckl 1998; Hauser 2013). Various experimental reports showed that early-life exposure to glucocorticoids can also have short- or long-lasting impacts on oxidative balance and resistance to oxidative stress (e.g., Mendez-Armenta et al. 2003; Atanasova et al. 2009). They also showed that the pregnancy window during which the embryo becomes exposed to stress hormones plays a significant role in fine-tuning the adult oxidative balance. Atanasova et al. (2009) treated orally for one week with dexamethasone (a synthetic glucocorticoid) two groups of common marmosets (*Callithrix jacchus*), a primate species endemic to South America. The treatment was performed during days 42–48 (early) or 90–96 (late) of gestation, which lasts around 144 days in this particular species. They found

that two-year-old offspring of mothers treated during late pregnancy had higher aortic mRNA expression for glutathione peroxidase 1, superoxide dismutase 2, glutamate–cysteine ligase and glutathione peroxidase 4 than control marmosets. On the other hand, early treated marmosets had only higher expression of glutathione reductase than controls. Levels of oxidative damage, as measured by concentration of F2-isoprostanes in urine, were significantly higher in late-treated marmosets than in other groups (Atanasova et al. 2009). Finally, neither body mass at birth nor that at two years of age was affected by treatment.

2.3 Epigenetic and Transgenerational Hormetic Effects

Epigenetics refers to the non-Mendelian transgenerational transfer of phenotypic characters without modification of gene sequences (Ho and Burggren 2010). It is becoming widely recognised that epigenetic alterations early in life may have long-lasting effects on the individual through changes in gene expression regulation (Jaenisch and Bird 2003; Delcuve et al. 2009; Crews et al. 2012). These modifications comprise chromatin remodelling (DNA methylation and histone modification) or micro-RNA-regulated transcriptional silencing. Although the strongest epigenetic effects emerge when occurring early in life, they are not confined to this temporal window, but can also occur in adulthood (Vaiserman 2011).

There is growing evidence that epigenetic variation may occur in natural animal populations and may be inherited by future generations (Bossdorf et al. 2008). However, it is not clear yet if and, if so, to what degree heritable epigenetic variation translates into among individuals variation in evolutionary fitness. Demonstrating this would be a first important step needed to assess whether an environmentally induced epigenetic trait is genetically assimilated.

It is increasingly recognised that at least some of the epigenetic reorganisation of phenotype is possibly explained by hormetic mechanisms. Many hormetic responses induced by a variable range of agents, including irradiation, heat or cold stress, dietary restriction, reactive species or dietary antioxidants have been characterised by large-scale changes in gene expression (Vaiserman 2008, 2010, 2011). In 1998, Rutherford and Lindquist proposed that the heat shock response could be a molecular mechanism underlying the emergence of epigenetic effects in natural animal populations. They found that when the *Drosophila* Hsp90 gene functions are experimentally altered, phenotypic variation affecting nearly any adult structure is produced, with specific variants depending on the genetic background and occurring both in laboratory strains and in wild populations (Rutherford and Lindquist 1998). They also found that multiple, previously silent, genetic determinants produced these phenotypic variants, suggesting that widespread variation affecting morphogenetic pathways exists in nature, but is usually silent (Rutherford and Lindquist 1998). Results of this study provided evidence for a molecular mechanism that assists the process of evolutionary change in response

to the environment. Importantly, this molecular mechanism (i.e. heat shock proteins) is involved in protection against oxidative stress. In a later study, further evidence in favour of this mechanism was provided in plants (Sangster et al. 2008), suggesting that the stress response mediated by heat shock proteins might be an epigenetic mechanism highly conserved across taxa.

It has been further suggested that the hormetic changes in gene expression in response to stressors are induced by a long-lasting epigenetic memory (Scott et al. 2009) and that these effects may carry over through generations (Zalizniak and Nugegoda 2006; Drake and Liu 2010). Variations in the quality of rat uterine environment induced by maternal nutrition can, for example, induce changes in genome methylation state of offspring (Lillycrop et al. 2005). DNA methylation of offspring induced by maternal nutrition has been found to have beneficial effects on longevity and disease resistance in mice (Wolff et al. 1998; Dolinoy et al. 2006). Epigenetic effects in rats may also come through behavioural maternal care. For example, maternal behaviours like grooming or pup-licking may increase the expression of glucocorticoid receptor expression and so influence the hypothalamic–pituitary–adrenal axis stress reactivity in the offspring (Champagne and Curley 2009; Weaver 2009). These effects can be, for example, associated with an increase in histone acetylation or DNA demethylation (Champagne and Curley 2009; Weaver 2009). Epigenetic modifications may also occur over the lifetime of an individual and induce measurable effects on longevity. This is particularly evident in studies of human twin pairs, which showed that the epigenetic modifications, while similar at a young age, differed in several tissues when the twins were elder (Fraga et al. 2005; Poulsen et al. 2007). These studies further supported the importance of environmental experiences as modulators of epigenetic modifications.

Although inheritance of hormetic epigenetic modifications and their impact on micro-evolutionary patters are still under intense debate, there is evidence indicating that hormesis may have transgenerational effects. Studies on the plant species *Arabidopsis thaliana* showed that mild heat stress induced transgenerational hormetic effects on fitness (seed production per individual plant) lasting at least until generation F_3 (Whittle et al. 2009). In contrast, a study on the water flea *Daphnia carinata* showed that exposure to the insecticide chlorpyrifos induced hormetic effects on survival, fecundity or the time to the first brood in two generations, but hormetic effects disappeared in the third generation (Zalizniak and Nugegoda 2006). Positive transgenerational hormetic effects on reproduction were also found in green peach aphids *Myzus persicae*, with aphids exposed to intermediate concentrations of insecticide producing offspring with the highest reproductive rate (Cutler et al. 2009). It has also been found that transgenerational hormetic effects improve the individual ability to colonise new territories, although this appeared to be dependent on how much stressful the new environment was (Piiroinen et al. 2013). Stress responses, whose plasticity is enhanced by hormesis, might generally enable invasive populations to persist under unfavourable conditions (Sexton et al. 2002) and, whether stress responses have a genetic basis, hormesis may further adaptation to stressful environments through genetic assimilation (Parsons 1991; Badyaev 2005).

A weak point of all these studies is that they were carried out mostly with artificially selected lines of laboratory animals; hence, any links among epigenetic modifications, hormesis and life history traits might have arisen as a simple product of artificial selection. There are, however, studies carried out on wild animals, suggesting that epigenetic modifications may drive adaptation to the prevailing environmental conditions. For example, offspring of Southern grass tussock skink (*Pseudemoia pagenstecheri*) females exposed to the scent of predator snakes were larger and more sensitive to predator odours (Shine and Downes 1999). Therefore, in an environment where the predation risk is high, females might equip their offspring with anti-predatory tools through epigenetic effects. However, we need studies that demonstrate the epigenetic molecular mechanisms for the above observations.

2.4 Post-natal Hormetic Priming of Organism to Withstand Stress Later in Life

That early life highly stressful conditions prior to sexual maturity have long-term negative effects on evolutionary fitness is well established (Monaghan 2008). But the extent to which early stress exposure is detrimental may depend on its severity, with mild stress exposure actually having a priming stimulatory effect (hormesis) that makes the individual more resistant to future stressful challenges. Understanding such hormetic processes may provide new insight to our understanding of how the early environment shapes a phenotype adapted to the conditions it is most likely to experience in its adult environment. There are many examples in the literature that provided evidence in favour of a long-lasting effect of early-life conditioning hormesis with or without subsequent exposure to stress. These studies also showed that the effects emerge across a broad variety of organisms in response to different combinations of early and adult stressors.

2.4.1 Plants

Exposure of seeds of aubergine (*Solanum melongena*) to high concentrations of cadmium caused inhibitory effects on growth of plants (Siddhu et al. 2008). In contrast, at lower concentrations, cadmium stimulated growth and increased biomass. Similarly, the growth of maize (*Zea mays*) plants was stimulated when exposed at mild concentrations of the antibiotic tetracycline (Migliore et al. 2010).

2.4.2 Invertebrates

Early-life exposure of nematodes (*Caenorhabditis elegans*) to heat stress (35 °C for 2 h), hyperbaric oxygen or juglone (a chemical that generates reactive species) significantly increased subsequent resistance to the same challenge, resulting in a

longer lifespan (Cypser and Johnson 2002). Moreover, it has been observed cross-tolerance between hyperbaric oxygen and juglone (Cypser and Johnson 2002). Similarly, Olsen et al. (2006) found that repeated heat stress treatments (30 °C for 2 or 4 h) of nematodes (*Caenorhabditis elegans*) throughout life had a larger beneficial effect on longevity than a single treatment early in life. However, many repeated episodes of heat stress have been shown to reduce rather than promote life extension (Lagisz et al. 2013). Moreover, the treatment did not influence longevity when applied to nematodes late in life.

Positive effects of early-life mild stress on longevity were also found in the fruit fly *Drosophila melanogaster* (e.g., Hercus et al. 2003; Le Bourg 2005) or in the Caribbean fruit fly *Anastrepha suspensa* (López-Martínez and Hahn 2014). Fruit fly males, but not females, that lived for two weeks in early life at 3 or 5 g of hypergravity and were then subjected to four heat shocks from 4 weeks of age onwards (30 or 45 min at 37 °C, twice a week for 2 weeks; five replicates for each length of heat shock) lived around 15 % longer than controls that experienced the same heat shocks but were at 1 g of hypergravity in early life (Le Bourg 2005). Positive effects of early-life mild stress on longevity in *Drosophila melanogaster* were also found when the thermal treatment was repeated more times (Hercus et al. 2003). The hormesis-induced effect on longevity was associated with a higher survival rate to heat stress and to a higher expression of the heat shock protein 70 (Hercus et al. 2003). However, there was also a reduction in fecundity (number of eggs), supporting the idea that trade-offs might occur when a trait is hormetically stimulated.

Hormetic effects induced by early-life heat stress were also found without subsequent exposure to a stressful treatment. A single heat shock treatment (37 °C for 1 hour at 4 days of age) extended mean longevity in *Drosophila buzzatii* but not in *Drosophila koepferae* (Scannapieco et al. 2007). A detailed demographic analysis showed that there was no effect on longevity in *D. koepferae* because, while the rate of senescence was slowed down (hormetic effect), the rate of mortality was increased. The heat-induced expression of heat shock proteins 70 was similar between species, but *D. koepferae* was more resistant to knock-down by heat stress (Scannapieco et al. 2007). These findings showed that species (also when closely related as in this case) can exhibit different patterns of hormesis in response to a same stressor intensity or dose of a certain chemical as shown in Fig. 2.1. Such differences may have reflected, for example, the differing life history strategies of the two *Drosophila* species (Scannapieco et al. 2007). *D. buzzatii* lived longer than *D. koepferae* under standard laboratory conditions, as well as wild ones (Sambucetti et al. 2005; Scannapieco et al. 2007). Moreover, during the first days of life, *D. koepferae* flies invest more massively into reproduction and have higher fecundity than *D. buzzatii* flies (Sambucetti et al. 2005; Scannapieco et al. 2007). Therefore, for *D. koepferae* flies, it might be more advantageous to invest more in resistance in early life (when reproduction occurs and natural selection is stronger) than in a long-lasting phenotype. However, investing lots of resource in self-maintenance might be traded off against investment in reproduction under natural conditions where resources may occur in more limited supply.

2.4.3 Birds

The first evidence of early-life conditioning hormesis in birds came from studies on poultry. Yahav and McMurtry (2001) showed that exposure of male broiler chickens (*Gallus gallus domesticus*) to 36 or 37.5 °C increased their thermotolerance (lower mortality and lower production of triiodothyronine) when exposed 6 weeks later to 35 °C compared with chickens exposed early in life to higher temperatures. Importantly, this study found that the effects were dependent on the temporal window during which chickens were treated early in life. In fact, the priming effect occurred in chickens exposed to heat stress when 3-day-old, but not in chickens treated at 1, 2, 4 or 5 days of age.

Studies on poultry may suffer from the fact that strains are artificially selected. Support for a hormetic priming effect in early life was also found in other bird species. In particular, a study on captive zebra finches (*T. guttata*) tested for the first time the hypothesis that individuals exposed to mild heat stress early in life suffer less oxidative stress when faced with high heat stress in adulthood than do individuals that were either not pre-exposed or were exposed to high heat stress in early life (Costantini et al. 2012).

Zebra finches are small passerines that live in the arid and semiarid regions of Australia. In these regions, the ambient temperature may reach very high values and large temperature fluctuations can occur during different parts of the day or season. The thermal features of Australian climate face zebra finches with problems of conservation of body water and maintenance of sublethal body temperature (Zann 1996) and, possibly, maintenance of oxidative balance. The timescale over which zebra finches experience high temperatures might therefore be very important: for example, the levels of heat stress encountered vary among individuals, depending on factors such as the season in which they are born and the prevailing weather conditions; those individuals that experience heat stress could actually be better placed to cope with subsequent high temperatures if the initial exposure has induced a hormetic conditioning response. There is therefore the potential for variation among individuals in the extent to which temperature fluctuations will cause oxidative stress later in life, dependent on their early thermal experiences. In zebra finches, the minimum mean oxygen consumption occurs at an ambient temperature of around 35 °C; increases in oxygen consumption occur below 29.5 °C (lower critical temperature) and above 40 °C (higher critical temperature) (Calder 1964). Zebra finches keep the body temperature relatively constant at ambient temperatures between 10 and 30 °C, while above 30 °C a gradual progression to mild hyperthermia occurs; the pulmocutaneous water loss (ratio of water loss to oxygen consumption) increases gradually between 10 and 30 °C, more rapidly between 30 and 40 °C, and sharply above 40 °C; the lethal body temperature calculated by Calder is 46.4 °C (Calder 1964). Keeping these thermal characteristics of zebra finches in mind, the investigators assigned randomly zebra finch siblings at the age of 42–45 days old (not sexually mature at this age) to three treatment groups, which involved exposure to different

temperature regimes during the conditioning phase in early life: controls (kept throughout at the standard housing temperature, i.e. 21–23 °C); exposure to mild heat stress (38 °C, which is close to the upper critical ambient temperature of the thermoneutral zone of the zebra finch); exposure to high heat stress (42 °C, which is above the upper limit of the zone but below the lethal limit; Costantini et al. 2012). The conditioning procedure consisted of brief periods of exposure to higher temperatures than controls for three hours every second day, for a total of 14 times over a 28-day period. The control birds were subjected to the same handling regime, but the ambient temperature was kept at the same room temperature at which all the birds had been reared. Later in life, when birds were around 177–180 days old (sexually mature at this age), half of the birds of each early exposure group were allocated to each of two adult treatment groups: control adult (room temperature) or stress adult (42 °C). Birds were exposed to the short-term thermal adult treatment for three hours every day for a total of three consecutive days. The analyses of multiple parameters of oxidative damage and antioxidant status in the blood showed that early-life exposure to mild heat stress primed zebra finches to better withstand oxidative stress when encountering heat stress as an adult. Specifically, such birds showed no increase in plasma oxidative damage on exposure to high temperatures in adulthood. In contrast, birds that had either no previous experience of heat stress or only had experience of high temperatures showed a significant increase in plasma oxidative damage; they also exhibited a bigger decrease in red blood cell thiol antioxidants than the mild conditioning group, although this effect was evident only in females (Fig. 2.3). Birds that experienced mild heat stress in early life and were then not exposed again to heat stress also showed a decrease in thiols, as well as suffered high mortality in the first three years of life (Costantini et al. 2012, 2014). This is relevant because a disruption of homeostatic mechanisms through overoxidation of thiols makes the cells less capable of efficiently controlling the activity of free radicals (Jones 2006; Sohal and Orr 2012). Hence, a cost of hormetic priming may come through a long-term impairment in the regulation of mechanisms that control oxidative balance. It might be that the resources required to maintain a molecular "memory" needed to withstand stressful episodes become a significant cost when such events do not occur (environmental mismatch), for example if these resources are taken away from other organism functions that may impact on survival (Costantini et al. 2014).

2.4.4 Mammals

We do not have yet clear proofs that hormesis has ecological relevance in wild mammals. However, studies on laboratory rodents showed that hormesis occurs in this taxon and may have long-lasting effects on behaviour and physiology. For example, Caratero and collaborators (1998) showed that mice (*Mus musculus*) exposed to very low doses of gamma radiation in early life lived longer than controls. Increased resistance to oxidative stress might explain why longevity may

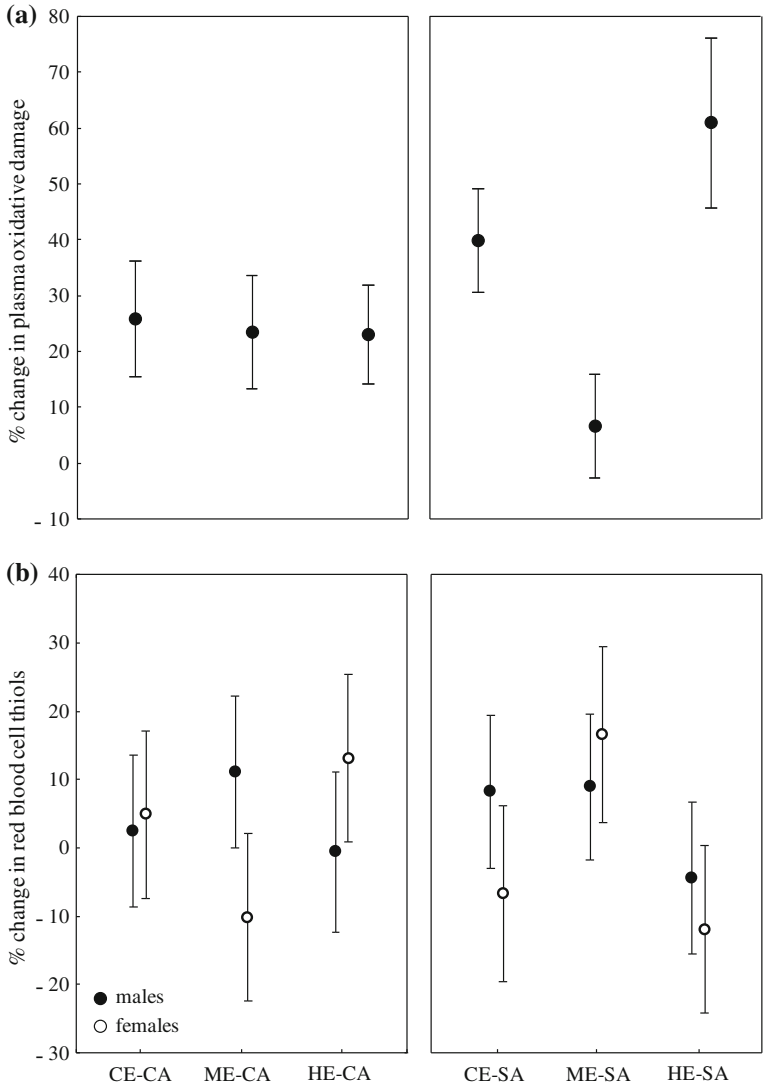

Fig. 2.3 Response of adult zebra finches to a short-term heat stress challenge (42 °C). Birds were previously conditioned to differing regimes of heat stress before sexual maturity: standard housing temperature (21–23 °C); mild heat stress (38 °C); high heat stress (42 °C). Data are expressed as the % change over the period of adult heat stress. **a** Changes in plasma levels of oxidative damage; **b** changes in thiols; treatment groups are control early-control adult (CE-CA), mild early-control adult (ME-CA), high early-control adult (HE-CA), control early-stress adult (CE-SA), mild early-stress adult (ME-SA), high early-stress adult (HE-SA). Values are shown as mean ± standard error. Reproduced with minimal alterations from Costantini et al. (2012) with permission

be increased by hormesis. This hypothesis is supported by a study from Gomez-Cabrera et al. (2008), who found that moderate exercise increased upregulation of antioxidant enzymes in rats (*Rattus rattus*).

Hormetic-like responses in mammals were also found by means of more realistic approaches. Mammalian mothers provide parental care and mother–young separations may be stressful for the pups. Although a prolonged separation from the mother may impair various physiological and cognitive functions of offspring later in life, brief episodes of separation during the first 1–2 weeks of life may induce a number of potential beneficial effects to offspring at adulthood, such as reduced stress responsiveness, enhanced spatial working memory and competitive ability for food (Meaney et al. 1991, 2001; Liu et al. 1997; Macrì et al. 2004; Tang et al. 2006; Coutellier 2013). It is not easy to tease apart the effects of maternal care provided after the pups are put back into the cages from those of stress experienced by pups while being kept separated from the mother. Some evidence suggests that the intensity of stress has downstream effects on offspring (Coutellier 2013). Stress hormones might be key candidates as regulators of these hormetic effects on development. Post-natal exposure of pups to low levels of stress hormones reduced stress responsiveness and increased cognitive capacities in rats (Catalani et al. 1993) or increased cognition in mice (Macrì et al. 2009). In contrast, post-natal exposure of pups to high levels of stress hormones have been, for example, associated with an increase in stress responsiveness and behavioural anxiety in rats (Brummelte et al. 2006; Macrí and Würbel 2007) and reduction in cognitive capacities in mice (Macrì et al. 2009).

2.5 The Compensatory Growth Paradigm

Over the developmental and growth period, environmental conditions may be very unstable/unpredictable, such as in the case of strong fluctuations in food availability, abrupt changes in weather conditions or reduced parental investment. These factors can cause scarcity of food available for young, resulting in a slowdown of the growth. Because of the link between body size and evolutionary fitness, it is expected that individuals facing a bad nutritional period (or another environmental parameter that influences growth) will compensate (compensatory or catch-up growth) when food availability becomes adequate again to meet the nutritional requirements of a growing individual. This may be done, for example, through an increase in food intake rate or in the duration of daily feeding period. However, the compensatory mechanism adopted is dependent on the pressures imposed by the species' life history. For example, compensatory growth was stronger and persistent among pre-migratory Atlantic salmons (*Salmo salar*) than among non-migratory fish (Nicieza and Metcalfe 1997). This was likely because survival rates during migration are strongly size-dependent in this fish species; hence, pre-migratory salmons needed to attain a large body size before migration (Nicieza and Metcalfe 1997).

It is sometimes more convenient to delay a life history event rather than to pay the costs of trying to being on a time schedule. Under these circumstances, compensatory growth is achieved by extending the growth period, which delays age at emergence or sexual maturity, but also comes at a lower physiological cost for the individual. In fact, achieving a large size in a short period of time may lead to long-term costs in species that go through complete metamorphosis. For example, tobacco hornworms (*Manduca sexta*) that grew faster as larvae had lower survival and capacity to cope with oxidative stress than larvae that grew slower (Harrison et al. 2013). These results suggest that any costs of an early high growth rate carried over the transition from the larval stage, through the pupae to the adult phase. The tissue remodelling typical of metamorphosis requires free radicals as promoters and regulators of metamorphosis itself; hence, it does not appear to be a time of detoxification. For example, metamorphosis in amphibians includes three major anatomical changes: complete resorption of tail and gills; de novo development of limbs from undifferentiated cells; partial but drastic remodelling of the existing larval organs into their adult forms (Damjanovski et al. 2000). Hence, while resorption of some tissues may favour elimination of damage, new tissues are made or remodelled. Production of reactive species is an inevitable consequence of these processes. Studies on metamorphosis in Japanese wrinkled frogs *Glandirana rugosa* (Hanada et al. 1997) or African clawed frogs *Xenopus laevis* (Menon and Rozman 2007) suggested that the cellular environments in the intestine and tail become progressively more oxidising during their remodelling and regression, respectively. Overall, these results supported the stressful metamorphosis hypothesis, which states that stress levels increase during metamorphosis (Campero et al. 2008). Clearly, the magnitude of costs of metamorphosis is expected to vary depending, for example, on the ecology of the species or the environmental circumstances under which a tadpole is developing. For example, growing in an environment where the risk of predation is high may force tadpoles to start their free life in the water before their development is completed. Hence, tadpoles have to keep developing in an environment that is different from the previous one. South American red-eyed tree frogs (*Agalychnis callidryas*) lay eggs in transparent jelly attached on leaves overhanging water. Wasps may raid the cluster and prey on tadpoles to feed their young. In order to avoid predation, tadpoles can wriggle free of the eggs and drop into the water below even when they are still underdeveloped. Let us now take toads that lay their eggs in desert ponds as another example. If a desert pond dries up quickly or not depends on various factors. A tadpole that is developing in one of such ponds, therefore, needs to tackle strictly the pond and adjust its rate of metamorphosis in order to complete it before the pond becomes completely dry. Conversely to larvae of other animal species, those of toad cannot develop structures that allow them to survive a dryness period. So, if the pond is drying slowly, the tadpole may also develop slowly and so it may mitigate any oxidative costs of metamorphosis. However, if the pond dries out quickly, the tadpole needs to metamorphose quickly. Clearly, this second strategy would pay the tadpole because it increases the chances of completing metamorphosis and to survive. Physiological costs might, however, be

high and these will be possibly paid in terms of a reduction in future reproductive output or accelerated rate of senescence. For example, *Pelobates cultripes* tadpoles accelerated their development in response to reduced water levels, but this resulted in upregulation of major antioxidant enzymes (catalase, superoxide dismutase and glutathione peroxidase) to mitigate oxidative stress (Gomez-Mestre et al. 2013).

Problems like those that toads have to solve may also be experienced by species that can produce resistant eggs. Damselfly species belonging to the genus *Lestes* show two main life history strategies depending on whether they live in temporary or permanent water bodies (Stoks and McPeek 2006). Temporary water bodies may dry in summer; hence, *Lestes* species living in this environment lay drought resistant eggs that remain in a diapause state across autumn and winter. After hatching, which occurs in the following spring, individuals spend 2–3 months in an aquatic larval stage and then emerge as adults in summer. It is therefore evident that larvae of these species face strong time constraints (time stress) because they must emerge before their pond dries (De Block et al. 2008). In contrast, eggs laid by species living in permanent ponds do not go through a diapause state and hatch in late summer. The larval phase lasts around 10 months through winter and to the following spring, when individuals emerge as aerial adults in early summer (De Block et al. 2008). Consequently, normal growth rates under non-stressed conditions are higher in temporary-pond *Lestes* compared with permanent-pond *Lestes* (Stoks and McPeek 2003). Moreover, compared with temporary-pond species, permanent-pond species show stronger compensatory growth to time stress. It has been suggested that this stronger compensatory growth has been co-evolved with their slower lifestyle (De Block et al. 2008). On the other hand, the shorter larval growth period and higher growth rates in temporary-pond species might give them less opportunity to further accelerate development and growth as they may be near their physiological limits (De Block et al. 2008). Hence, they might suffer higher physiological costs to compensate and, as demonstrated by De Block and Stoks (2008), these costs can carry over from larvae to adults, such as those related to upregulation of enzymatic antioxidant defences in the temporary-pond *Lestes viridis*. The extent to which metamorphosis is costly can also depend on adaptations evolved by a particular species. For example, larvae of many insect species can produce light. It has been found that bioluminescent reactions that occur in these larvae can provide auxiliary protection against oxidative damage (Barros and Bechara 1998).

Compensatory growth may also be achieved by accelerating the growth rate without changing the duration of normal growth period (Metcalfe and Monaghan 2001a, b). This second case of compensatory growth (known also as catch-up growth) is particularly interesting for the long-term effects it can have on individuals. Although in the short-term it allows an individual to achieve the body size that is supposed to be normal for its life stage and hence to have benefits, such as an adequate size for fledging, it can carry costs that emerge later in life, such as a reduction in longevity (Metcalfe and Monaghan 2001a, b). Oxidative stress may be the currency to quantify these costs.

To test the hypothesis that a reduction in antioxidant defences may be a cost of accelerated somatic growth in birds, Alonso-Alvarez et al. (2007) enlarged (six nestlings) or reduced (two nestlings) the brood size of zebra finches in order to manipulate access of nestlings to food and so their rate of growth. Nestlings from the enlarged broods accelerated their growth (especially in terms of pectoral muscle development) to compensate for the delay caused by a reduced access to food; these nestlings had red blood cells more susceptible to haemolysis when red blood cells were exposed to an in vitro free radical attack than those of nestlings from the reduced broods (Alonso-Alvarez et al. 2007). The increase in metabolic activity needed to fuel the accelerated growth may be responsible for an increase in free radicals, which might have damaged the membrane of red blood cells making them more susceptible to haemolyse. However, nestlings from reduced broods also experienced food shortage. This food reduction might also have contributed to reduce the resistance of red blood cells because of a lower access to dietary antioxidants or other nutrients that make cell membranes more resistant to oxidative damage. Dietary antioxidants that occur in the fluid component of blood might also increase the in vitro red blood cell resistance by quenching the free radicals used in the assay. Therefore, the lower resistance of red blood cells of early diet-restricted birds might also have been due to having low circulating levels of antioxidants. This scenario is supported by other studies on zebra finches, where nestling fed on a poor quality diet had later in life significant lower concentrations of circulating vitamins A and E (Blount et al. 2003) and of carotenoids (Alonso-Alvarez et al. 2006).

Catch-up growth is not only typical of species with a high pace of life like the zebra finch, but can also occur in species with a slower pace of life. In the king penguin *Aptenodytes patagonicus*, the chick has to face alone the Subantarctic winter because parents spend this time foraging in open sea. Over this period, chicks are not fed by their parents and suffer high mortality (Weimerskirch et al. 1992). Chicks slow down their growth so much that they may need around 11 months to achieve their complete growth, which is one of the longest growing periods observed in birds (Geiger et al. 2012). This scenario offers penguins opportunities for catching-up when the winter ends and food is again available; hence, they may experience physiological costs in doing so. Geiger et al. (2012) provided correlative evidence for a link between compensatory growth and oxidative stress in king penguin chicks. They found that small chicks that grew faster did it at the detriment of cell oxidative balance maintenance mechanisms as shown by higher oxidative damage and accelerated telomere loss.

Overall, these studies show that oxidative stress is a consequence of compensatory growth and that this link is consistent across taxa. These findings have also provided evidence for oxidative stress being a plausible mechanism underlying the negative effect of compensatory growth on longevity that has been shown in many experimental investigations in which growth rates were manipulated or compared among species (Metcalfe and Monaghan 2001a, b; Rollo 2002; Ricklefs 2006). However, since manipulation of growth has always been achieved by periods of dietary restriction, these studies suffer from the problem that negative effects on

lifespan may also be a consequence of early undernutrition rather than the pattern of growth per se. A recent study used brief (less than 4 % of median lifespan) exposure to relatively cold or warm temperatures early in life to deflect juvenile three-spined sticklebacks (*Gasterosteus aculeatus*) from their normal growth trajectories (Lee et al. 2013a). This induced catch-up or slowed-down growth when ambient temperatures were restored; catch-up growth caused a reduction in median lifespan of 14.5 %, while slowed-down growth extended lifespan by 30.6 %, independently of size attained or reproductive investment in adulthood (Lee et al. 2013a). Manipulations of photoperiod showed that the effects of compensatory growth on lifespan were also influenced by the time available for growth prior to breeding, being more extreme when less time was available. These data suggested that the optimal resolution of the growth-lifespan trade-off is influenced by time constraints in seasonal environments (Lee et al. 2013a). As for studies in which growth rate has been manipulated by periods of dietary restriction, the stickleback study might also suffer from the problem that thermal stress or other physiological changes (e.g., oxidative stress, cell membrane composition and fluidity) induced by ambient temperature (see Chap. 3) might have contributed to the observed effects on longevity.

Although the above studies induced increases of oxidative stress levels by inducing compensatory growth, several studies have also found a positive correlation between growth rate and oxidative damage parameters without any compensatory response. For example, free-living male and female lambs of Soay sheep (*Ovis aries*) that grew more over the first 4–5 months of life had higher plasma levels of malondialdehyde, a marker of oxidative damage to lipids (Nussey et al. 2009). Pigeon (*Columba livia*) nestlings attaining larger body mass and size after a period of 30 days had more oxidative damage and depletion of thiols than nestlings that were smaller at that age, while they also had higher non-enzymatic antioxidant capacity (Fig. 2.4; Costantini 2010). Although pigeon males and females did not differ in growth nor in oxidative stress, other studies found that male and female nestlings may adopt different strategies. In jackdaws *Corvus monedula*, growth was reduced in broods that were experimentally enlarged, and this effect was stronger in females (Salomons 2009). There was also a reduction in non-enzymatic antioxidant defences in enlarged broods, especially in males, and an increase in oxidative damage in males but not in females from reduced broods. These results suggested that males and females differently solved the trade-off between growth and oxidative stress: while males invested more in attaining a large body size at the expense of self-maintenance, females did the opposite (Salomons 2009). This might be because larger males have more chances of becoming dominant, and dominance rank influences reproductive success and survival in this particular species (Henderson and Hart 1995; Salomons 2009). However, in the studied population, it was found that dominant males have actually less reproductive success; hence, it is possible that the strategy was maladaptive in that specific context (Verhulst and Salomons 2004) and early oxidative damage may have contributed to decrease the reproductive capacity of males.

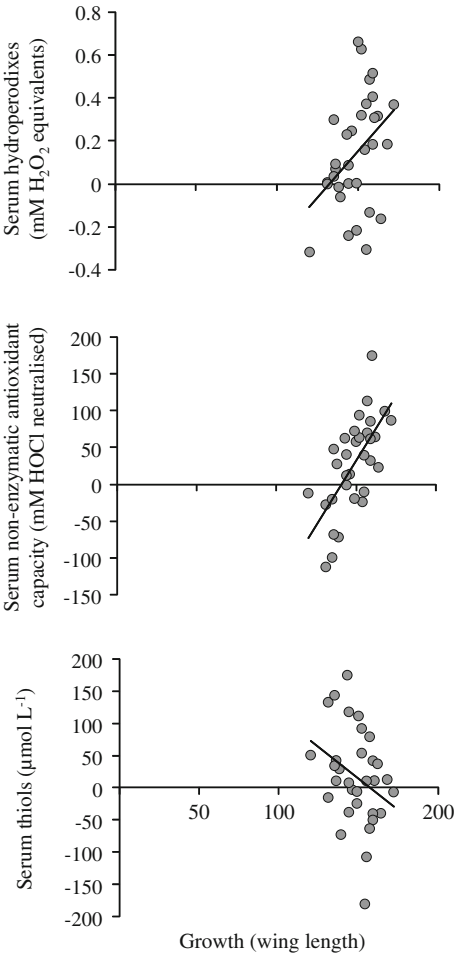

Fig. 2.4 Relationships among growth and various parameters of blood oxidative status in nestling pigeons (Costantini 2010). Oxidative damage and non-enzymatic antioxidant capacity increased, while thiols decreased in pigeons that grew more in the period 7–30 days of age (wing length is shown, but similar relationships emerge with tarsus length or body mass). Values are shown as the difference between the value at 30 and 7 days of life

Overall, these studies showed that generation of oxidative damage may be a consequence of growth. However, they do not provide direct evidence that oxidative stress can constrain growth. The role of oxidative stress as a direct constraint of growth is better exemplified in studies where it was tested the effect of experimentally induced oxidative stress on growth in fish. In particular, various experimental reports found that induction of oxidative stress through supplementation of diet with dietary oxidised lipids impaired growth and survival in various fish species, such as rainbow trout *Oncorhynchus mykiss* (Hung et al. 1981), Atlantic salmon *S. salar* (Koshio et al. 1994) or Siberian sturgeons *Acipenser baeri* (Fontagné et al. 2006).

The extent to which growth may carry oxidative costs for the individual is also influenced by factors, such as the degree of maturation of endogenous antioxidant mechanisms; the transfer of dietary antioxidants from the egg to the embryo; the

transfer of dietary antioxidants from the mother to the foetus through the placenta or to the young through the milk. Availability of antioxidants has therefore to match the growth rate in order to meet the demands and mitigate costs; hence, some degree of synchronisation is needed among these mechanisms. This is important because resolutions of life history trade-offs may differ across the developmental period, depending on the antioxidant competence of the individual.

As regards with maturation, it is well established for various physiological traits that there is some degree of plasticity in the timing of the onset of developmental events within individuals. Such plasticity has been referred to as physiological heterokairy, a term first coined by Spicer and Burggren (2003). Heterokairy can, for example, result from environment-induced epigenetic effects that modify the relative development of physiological processes (Spicer and Burggren 2003; Spicer and Rundle 2007).

In mammals, maturation of antioxidant system appears to occur mostly after birth (e.g., Allen and Venkatraj 1992; Fantel 1996). In a cross-sectional study of hooded seals *Cystophora cristata*, Vázquez-Medina et al. (2011b) found that production of superoxide anion is higher in adults than in neonatal pups, while that of weaned pups was intermediate. A similar increasing pattern was found for the activities of superoxide dismutase, while no significant differences emerged for oxidative damage to lipids, proteins and nucleic acids (thiobarbituric acid reactive substances, protein carbonyls and 8-oxo-7,8-dihydro-2′-deoxyguanosine, respectively; Vázquez-Medina et al. 2011b). The authors also hypothesised that the enhanced antioxidant protection obtained through development is a mechanism evolved to match the development of diving behaviour, which exposes seals to oxidative challenges (see Chap. 4). The hypothesis that antioxidant competence of seals increases through the transition from a terrestrial to a semiaquatic life received some support in another cross-sectional study of hooded seals (Vázquez-Medina et al. 2011a). The authors measured the complete cDNA sequence that encodes the NF-E2-related factor 2 (Nrf2), a central regulator of the antioxidant response, and compared its mRNA and protein levels in muscle samples among neonate, weaned pups and adult hooded seals, along with glutathione levels and the activity of the antioxidant enzymes catalase, glutathione peroxidase, peroxyredoxin VI, thioredoxin 1, thioredoxin reductase, glutaredoxin 1, glutathione disulphide reductase, glutathione S-transferase and glutamate–cysteine ligase. It was found that glutathione, glutathione peroxidase, peroxyredoxin VI, thioredoxin 1, thioredoxin reductase and glutaredoxin 1 increased with maturation (from neonates to adults), suggesting that the potential for peroxide removal increases with development, and that these enzymes contribute to the regulation of the intracellular redox state and the prevention of oxidative damage in seals (Vázquez-Medina et al. 2011a).

Studies on chickens (*Gallus gallus domesticus*) suggested that maturation of antioxidant machinery also occurs in birds. In chicken embryo, it has been observed a preferential transfer of vitamin A from the yolk to the embryo before day 14 of incubation, while the transfer of vitamin E mostly occurred during the last week of incubation (Gaál et al. 1995). In contrast, concentrations of reduced

glutathione and activities of superoxide dismutase and glutathione peroxidase did not show notable changes over the embryo development. The differential transfer of vitamins is likely dependent on the specific needs of the embryo. The transfer of vitamin E occurs during a period characterised by a significant increase in the content and degree of unsaturation of tissue lipids (Nobel et al. 1993). This may have adaptive value because vitamin E is a lipophilic antioxidant and unsaturated fatty acids are very sensitive to peroxidation. Similar conclusions might also be proposed for carotenoids, whose accumulation in the chicken embryo occurs in a similar way as that of vitamin E (Surai et al. 1996). In contrast, protection against oxidative damage afforded by antioxidant enzymes becomes more important after hatching, when oxygen concentration in tissues, metabolic activity and production of superoxide radical increase and concentrations of dietary antioxidants decrease, respectively (Surai et al. 1999; Surai 2002). Similarly, in various invertebrates (e.g., molluscs, crustaceans) and fish, data on development of antioxidant defences suggested that dietary and other low molecular weight antioxidants are predominant in the earliest stages of development, while antioxidant enzymes become predominant later (Rudneva 1999; Fontagné et al. 2008). It is, however, important to point out that the timing of antioxidant maturation may differ among tissues (Surai 1999, 2002), which may be because selection has favoured mechanisms that prioritise investment in tissues that are more demanding and more impacting on embryo or young survival in a certain stage of development.

As shown by the above studies on chickens, changes in oxidative status in the young individuals may also reflect physiological perturbations caused by birth or hatching. For example, the human foetus lives in an intrauterine environment that is hypoxic, with a partial pressure of oxygen of 20–25 mm Hg and a low presence of free radicals; at birth, it is exposed to a new environment with a partial pressure of oxygen of 100 mm Hg and exposure of the pulmonary epithelial cells to pressures of about 140 mm Hg in inhaled air (Robles et al. 2001). Consequently, they are at risk of oxidative damage. Studies on humans showed that, compared with the condition at birth, oxidative damage (hydroperoxides) is higher, and antioxidant defences are lower (vitamin E) or higher (Q_{10} coenzyme) 72 h later (Robles et al. 2001). Similarly, oxidative damage (hydroperoxides) was found to decrease over the growth period in nestling kestrels *Falco tinnunculus* (Costantini et al. 2006, 2007), but to increase in nestling pigeons *C. livia* (Costantini 2010) or nestling cuckoos *Cuculus canorus* (Hargitai et al. 2012), possibly reflecting among species differences in growth or in other aspects that might have contributed to mitigate, exacerbate or slow down the increase in damage. For example, for kestrels, the first days after hatching are very critical, with high mortality occurring at this time. In the first days of life, kestrel chicks rely almost uniquely on fathers for food. In contrast, pigeon chicks are exclusively fed on crop milk by both parents during the first 4–5 days of life (Levi 1969; Johnston and Janiga 1995). Crop milk contains nutrients, such as fat, proteins and antioxidants (Bharathi et al. 1997). Therefore, parents could mitigate the pro-oxidant effects of hatching by increasing the quality of crop milk. Cuckoos are brood parasites (i.e. lay their eggs in the nests of other bird species) and their nestlings evict the host's eggs or

hatchlings over the rim of the nest typically between 1 and 3 days of age. Hence, the cost of eviction might carry over the development period, increasing accumulation of damaged molecules with time (Hargitai et al. 2012).

Overall, studies that looked at maturation of antioxidant status might suffer from the problem that factors (e.g., growth rate, hormonal status) other than molecular maturation *per se* might have contributed to the observed changes in free radical production, oxidative damage or antioxidant status recorded over the development period. Moreover, the cross-sectional nature of some studies implicates that some of the increases observed in antioxidant defences might have been caused by selective disappearance of young having low antioxidant levels.

We have seen that dietary antioxidants may contribute to protection against oxidative stress in the first part of life while waiting for maturation of enzymatic antioxidant machinery. Another way of avoiding oxidative stress while growing may be to keep mitochondria uncoupled (through the action of uncoupling proteins) and so to reduce basal production of reactive species. However, adopting this strategy might come at a cost for growth because the production of energy of uncoupled mitochondria goes down and so it may not meet the energetic requirements of growth. This trade-off between oxidative stress and growth through the regulation of energy production is well exemplified in a study of common frog (*Rana temporaria*) tadpoles (Salin et al. 2012). Chronic exposure (34 days) of tadpoles to a mitochondrial uncoupler (2,4-dinitrophenol) reduced the mitochondrial generation of hydrogen peroxide, non-enzymatic antioxidant defences and thiobarbituric acid reactive substances (biomarker of oxidative damage) in tadpole homogenates (Salin et al. 2012). However, their growth rate was lower than that of control tadpoles because, while their metabolic rate was increased, their food consumption did not, hence tadpoles failed to compensate for the energy loss. These data suggested that growing in an uncoupled state might be beneficial in terms of oxidative balance maintenance; however, it might not pay in terms of growth and development if these are strongly constrained by energy limits (Salin et al. 2012).

Animal taxa that moult in order to grow are also very interesting to look at. For example, crustaceans may pass through a number of larval and immature stages between hatching and reaching their adult form, and each stage is separated from each other by a moult, in which the hard exoskeleton is separated from the soft parts to allow the animal to grow. The growth of crustaceans is more or less discontinuous, depending on the environmental context, and oxygen demands dramatically increase while moulting (Fanjul-Moles and Gonsebatt 2012). Eggs and larvae may also enter periods of dormancy/quiescence during which metabolism shifts from an aerobic to an anaerobic one. Upregulation of antioxidant enzymes during sensitive periods of development is therefore required, but it needs to be balanced against the need of using reactive species as signalling molecules to regulate cell growth and development. There are among species differences in how antioxidant enzymes and generation of oxidative damage vary across crustacean development until the adult phase (e.g., Arun and Subramanian 1998; Correia et al. 2003); hence, the role of oxidative stress as a constraint of a specific stage of

growth might also be species-dependent. The developmental trajectory of crustacean larvae is further influenced by the interaction between enzymatic and non-enzymatic antioxidants (Dandapat et al. 2003).

2.6 Conclusions

There is growing evidence in support of a role of oxidative stress as a universal cost (and possibly constraint) of growth/development strategies across the entire animal kingdom. From those species that metamorphose to those that do not do so, oxidative stress appears to be continually present in driving developmental trajectories and in underlying the costs incurred in adopting a certain growth strategy. At the same time, the adult capacity in regulating the oxidative balance is also determined by early-life experiences. Mothers may certainly influence the extent to which offspring are resistant to oxidative damage. Importantly, the interaction between environmental conditions experienced pre- and post-natally will be pivotal in programming the adult phenotype. Experiencing oxidative stress early in life may not be necessarily that bad. In fact, mild oxidative stress experiences early in life may prepare the phenotype to withstand episodes of high oxidative stress later in life through hormesis.

Environmental variation experienced in preceding generations can also influence progeny phenotype, but in a manner that is complex and difficult to predict. Mechanisms underlying transgenerational effects remain largely to be identified. However, it is increasingly recognised that hormetic effects may come through epigenetic modifications that may be transmitted to next generations.

Researchers generally focus on the effect of single ecological triggers on traits within a single dimension (e.g., morphological, behavioural or physiological phenotypes). This approach has yielded a wealth of knowledge about environmental conditions that trigger different plastic allocation strategies and reaction norms of a number of traits. We now need to understand how complex environments (in a multidimensional framework) shape the developmental pattern and the expression of suites of traits to produce adaptive (or maladaptive) phenotypes (Kasumovic 2013). It will also be relevant to address the role of oxidative stress in morphogenesis (Smith et al. 2013), given its effects on apoptosis of progenitor cells that are crucial for tissue development.

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Chapter 3
Variation in Oxidative Stress Threats and Hormesis Across Environments

Abstract Environmentally driven variation in exposure of organisms to differing oxidative stress threats is of great interest to evolutionary ecologists and physiologists. Understanding the physiological capacities of organisms to environmental stressors is key to predicting their response to changing environments. This chapter provides a synthesis of how organisms manage to withstand oxidative stress induced by a wide variety of environmental abiotic factors, such as temperature, partial pressure of oxygen or ultraviolet radiation. The chapter also discusses how hormesis primes organisms to tolerate variable environmental conditions.

3.1 The Struggle of Living in Oxidising Environments

Environmental abiotic factors, such as temperature, salinity, metal ions, partial pressure of oxygen or solar radiation, vary largely within and among habitats (Fig. 3.1), subjecting organisms to different problems and solutions of oxidative balance regulation (Freire et al. 2012; González et al. 2012; Lesser 2012; Regoli et al. 2012; Tattersall et al. 2012; Welker et al. 2013). Further environmentally driven variation in exposure of organisms to differing oxidative insults can be a product of seasonality, daily variations in sunlight exposure, or drops in oxygen availability or osmotic stress. Environmental changes can often be abrupt, which requires organisms to quickly mount an adaptive response. For example, after a tropical storm, aged trees may come crashing to the ground leaving a gap in the canopy above. This allows sunlight to reach the forest floor. Sunlight represents an important risk factor for low-light plants or animals because ultraviolet radiation can cause oxidative stress.

Species have evolved a number of behavioural and physiological adaptations to tolerate fluctuations in environmental conditions and so to maintain a stable redox state. For example, many invertebrate and fish marine species live near volcanic outflows, where warm waters poor in oxygen and rich in hydrogen sulphide merge

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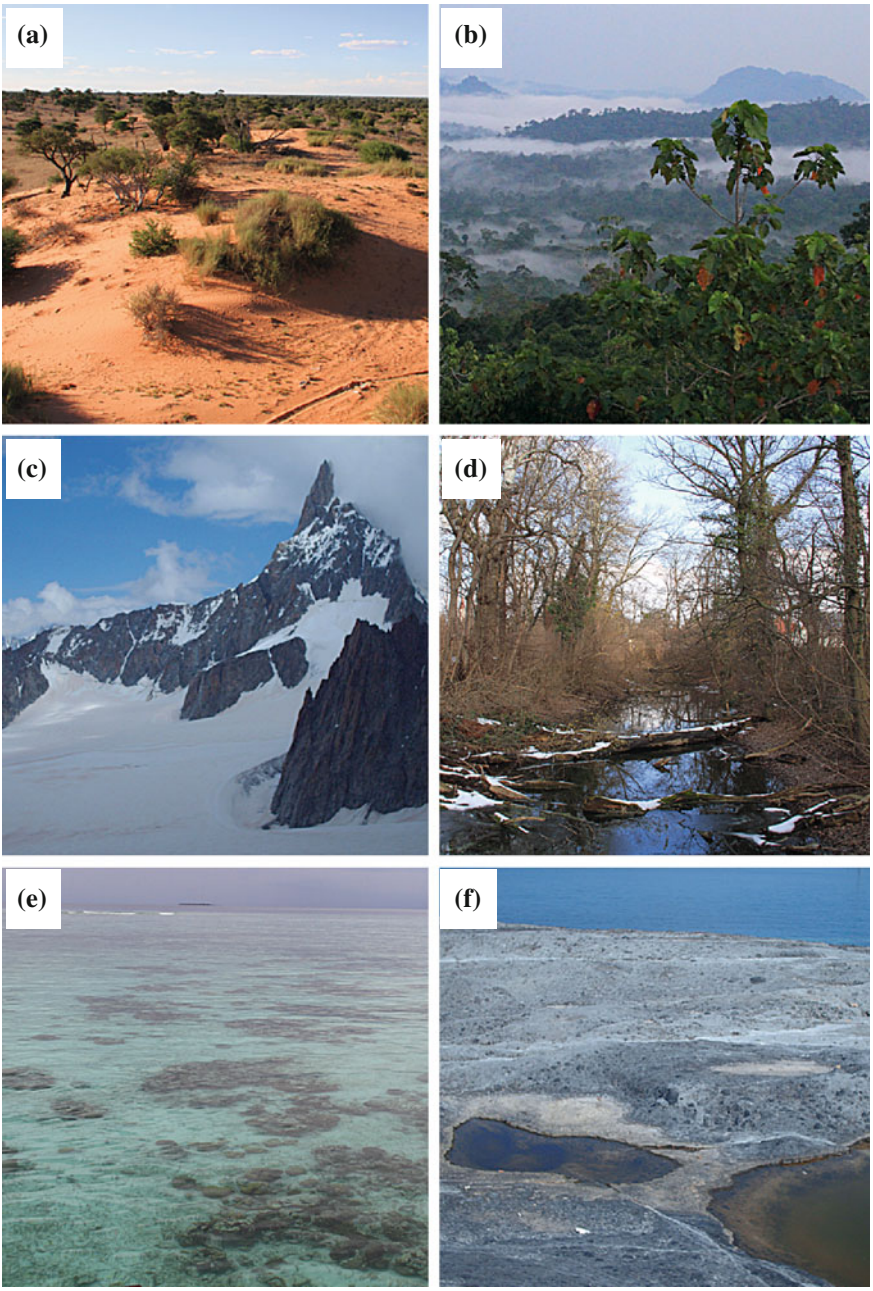
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◀ **Fig. 3.1** Examples of environments that have exposed species to different physiological demands over evolution. **a** The dry savanna (*Photograph* Kalahari, South Africa) is characterised by a scarcity of water and high summer temperatures that face organisms with problems of heat stress and water economy; **b** tropical rain forests (*Photograph* Danum Valley, Borneo) are rich in dampness, and abiotic factors tend to fluctuate less than in temperate forests; **c** at high altitudes (*Photograph* Dent du Géant, Mont Blanc Massif, France-Italy), environments are among the most challenging places on Earth because they are poor in oxygen and temperatures are low; **d** environments like flood plains are subjected to periodical fluctuations in water quantity and chemistry (*Photograph* Danube-Auen National Park, Austria); animals living in **e** coral reefs (*Photograph* Pom-Pom Island, Malaysia) and **f** intertidal environments (*Photograph* tide pools in Ponza Island, Italy) are subjected to periodical exposure to hypoxia–reoxygenation and to solar radiation; hence, tolerance of hypoxia and of ultraviolet radiation is widespread among species living in these environments. Photographs by David Costantini

with colder, oxygen-rich oceanic waters, creating very variable and extreme environmental conditions (Abele et al. 2012). Other species live in environments, like deserts, that can become really hot during the day. Species like the desert moss (*Syntrichia caninervis*) may tolerate experimental exposure to 120 °C for 30 minutes (Stark et al. 2009).

In this chapter, I examine the wide variety of environmental contexts in which oxidative stress has likely contributed to shaping variation in physiological phenotypes and life history strategies. I also discuss how hormesis possibly primes the organisms to tolerate variable environmental conditions.

3.2 Coping with Thermal Challenges

3.2.1 Thermal Relations of Organisms with Their Environments

Research on thermal relations of organisms with their environment has provided relevant examples of how phenotypes can be adjusted to the prevailing environmental conditions and why certain strategies are favoured over others. Ambient temperature has a strong effect on morphology, physiology, behaviour and seasonal timing of life history stages (Somero 1995, 2004; Hill et al. 2008; Angilletta 2009; Tattersall et al. 2009; Storey and Tanino 2012; Caro et al. 2013). Ambient temperature influences the animal's metabolic rate, cellular biophysical or biochemical processes and the physical state of cell membranes, especially in ectotherms (Somero 1995, 2004). Enzymatic translation may demand energy and building blocks (amino acids), but expression of enzymatic isoforms also requires multiple copies of a gene (Tattersall et al. 2012). Having more routes through which to respond to a thermal stressor may give more flexibility to the organism. However, this is likely to vary across species because not all species can, for example, express various enzymatic isoforms and changes in isozyme patterns may occur for some enzymes but not for others (Shaklee et al. 1977;

Tattersall et al. 2012). Such variation among species raise many questions related to selective pressures that acted on the evolution of enzymatic antioxidant mechanisms activated in response to thermal changes.

Fluctuations in ambient temperature are also a source of trade-offs. Let us take a flying insect as an example. Flight muscles of an insect have to be warm in order to work properly so the insect can fly. The insect consumes energy to generate heat that is needed to warm up the muscles. At high ambient temperature, the insect can exploit environmental heat to keep muscles warm and so has much more of its own energy available to be invested into other body functions.

The diversity in the response of animals to thermal challenges offers a unique opportunity to study the links among oxidative stress, hormesis and life history strategies in comparative physiology. Several classes of animals can be recognised, depending on whether or not they (i) produce heat by their own metabolism and (ii) regulate their body temperature (Tattersall et al. 2012). Species incapable of generating heat are classically referred to as ectotherms (or poikilotherms); in these species, the body temperature is strictly dependent on the ambient temperature. Species that warm their body using metabolically produced heat are called endotherms. Within both groups, ectotherms and endotherms, we can distinguish species that can thermoregulate their body from those that cannot do so. Ectotherms can regulate body temperature by behavioural strategies (e.g. occupying warmer environments, producing kinetic heat like sharks and swordfish). Endotherms can regulate the body temperature by behavioural or physiological means (e.g. metabolism). Endotherms that do so by physiological control are called homeotherms. It is important to highlight, however, that regulation of body temperature shows temporal or among-tissues variation. In fact, some species adopt different thermal strategies at different times of the year, such as in the case of species that hibernate, which can shift between homeothermic and ectothermic states (heterothermy). Moreover, within an individual, some tissues may show endothermy and thermoregulation, while others do not. For example, temperature profiles of penguins during dives are consistent with a model of regional heterothermy with conservation of core temperature, peripheral vasoconstriction and cooling of outer body regions (Ponganis et al. 2001). There may also be selective cooling of certain tissues, such as brain, depending on needs and on tissue requirements at particular life stages (Tattersall et al. 2012).

Homeothermic species are characterised by a thermoneutral zone, which is a range of ambient temperatures within which the resting metabolic rate is independent of ambient temperature; hence, within this temperature range, there are no regulatory changes in metabolic heat production or evaporative heat loss. Above the upper limit of the thermoneutral zone, metabolic rate increases because the organism needs to use energy to increase the rate of water evaporation from the body surface (active evaporative cooling) and, at the same time, to counterbalance cooling in order to avoid an excessive decrease in body temperature (Hill et al. 2008). If the ambient temperature increases substantially, homeotherms can enter a hyperthermic state, which is characterised by a steep rise in body temperature. The rise in body temperature allows the animal to lose heat because the temperature of

the body is then higher than the ambient temperature. Animals may also use hyperthermia to store heat and reduce the difference in temperature between body and environment in order to limit heat gain (Tattersall et al. 2012). The capacity to do so varies across species because larger species have higher thermal inertia (higher capacity to tolerate progressive hyperthermia) than smaller species. The extent to which protection against hyperthermia-induced oxidative stress is critical for species may therefore be expected to vary in relation to thermal inertia (Al-Otaiba et al. 2010) and to the evolution of behavioural strategies or thermal windows (body surfaces that promote heat loss) used to escape heat stress (Tattersall et al. 2012). All of these strategies are obviously very important for survival because when the body temperature increases beyond a specific threshold, hyperthermia becomes detrimental and heat-induced mortality occurs. Thermal kinetic energy and collisions among molecules tend to increase with ambient temperature (Tattersall et al. 2012), hence enhancing reactions between enzymes and their substrates. Activity of enzymes therefore increases with temperature. Conformational changes of enzymes, however, occur at critically high temperatures, which impair their catalytic capacity, for example, through loss in structural integrity (Somero 1995, 2004; Abele et al. 2002; Tattersall et al. 2012). There are, however, many enzymes that are catalytically more effective at low temperatures (e.g. psychrophilic enzymes), such as those of organisms living in cold environments (Gianese et al. 2001; Feller 2013).

Generally, animals become heat-stressed when they are unable to balance production and loss of heat. The level of heat stress can be mild when the ambient temperature is slightly higher than the upper critical temperature of the thermoneutral zone, but high when the ambient temperature is well above the upper critical temperature. Although ectotherm species do not have a thermoneutral region, they can also become heat-stressed and may die when the increase in ambient temperature becomes too high. Critical temperatures of ectotherms vary largely among species: some species, termed eurythermal, tolerate a wide range of temperatures, while others, called stenothermal, have narrower ranges (Hill et al. 2008). Moreover, even conspecific populations can differ in thermal tolerance (Abele 2012). In ectotherms, temperature can accelerate the rate of formation of reactive species simply by increasing the mitochondrial respiration. For example, the in vitro formation of reactive species in mitochondria of the bivalve *Mya arenaria* was found to double when the ambient temperature was increased from 15 to 25 °C (Abele et al. 2002).

3.2.2 The Good and the Bad of Cold and Heat Stress

A state of heat stress is characterised by several physiological changes beyond metabolic adjustments, such as increases in synthesis of heat shock proteins, increases in production of reactive species or oxidative damage and changes in

antioxidant status (e.g. Flanagan et al. 1998; Feder and Hofmann 1999; Lord-Fontaine and Averill-Bates 2002; Mujahid et al. 2007; Vinagre et al. 2012). For example, both gene expression and activity for the antioxidant enzymes copper–zinc superoxide dismutase, catalase and glutathione peroxidase were higher in Japanese black porgy (*Acanthopagrus schlegeli*) at 30 °C than at 20 °C (An et al. 2010). Antioxidant enzymes were likely upregulated in response to the higher production of reactive species (hydrogen peroxide in this particular study) in fish exposed to 30 °C; however, this did not prevent the increase in oxidative damage (An et al. 2010). This is particularly relevant in the light of global warming. It may only take an increase of a few degrees Celsius to induce pervasive effects on the oxidative balance of an organism. Rosa and collaborators (2012) showed that the projected near-future ocean warming of 2 °C may be already above the optimal thermal tolerance boundaries of European squids *Loligo vulgaris*. In this species, embryonic development was dramatically shortened with a concomitant negative effect on growth and survival success, an increase in oxidative damage and a consequent upregulation of antioxidant defences. Squids will probably be faced with higher demands in terms of homeostatic maintenance during development in order to increase their thermal tolerance windows. Results of this study suggested that hormetic priming during the early stages of development might increase the width of the thermal tolerance window. This is suggested from the fact that summer embryos of squids had stronger heat shock and antioxidant responses than autumn embryos. Other factors (e.g. differential seasonal investment of mothers in their eggs, larger nutritional supply of molecular dietary antioxidants in summer) may also have contributed.

Investigation of heat stress effects on oxidative balance would possibly need intraindividual repeated measures or comparisons of independent groups that are maintained under different heat stress conditions (e.g. intensity, duration and geographic gradient). Analyses of multiple tissues may also be important, although this is incompatible with non-terminal ecological research. In addition, it is essential that a combination of multiple parameters of oxidative damage and antioxidant defences is used. These issues arise from the fact that heat stress (i) results in either stimulation or inhibition of antioxidants or repair systems, depending on its magnitude; (ii) increases oxidative damage to certain macromolecules but not to others; and (iii) impinges differently on different tissues. These issues were well exemplified by a study on the heat shock response and recovery from heat stress in rotans *Perccottus glenii* (Bagnyukova et al. 2007). Moreover, the relationship between temperature and a certain parameter of oxidative stress may not necessarily be linear. For example, production of reactive species and activity of catalase increased and decreased, respectively, with a temperature increase in the intertidal mud clam *Mya arenaria* (Abele et al. 2002). In contrast, the activity of superoxide dismutase increased from 0 to 18 °C and then decreased from 18 to 30 °C, respectively.

Transient or chronic increases in oxidative stress, as well as changes in enzymatic antioxidants or heat shock proteins, were also recorded in animals exposed to low temperatures that can cause cold stress (e.g. Grubor-Lajsic et al. 1997;

Selman et al. 2000; Abele and Puntarulo 2004; Koštál and Tollarová-Borovanská 2009; Kammer et al. 2011). Cold stress can, for example, induce the organism to increase its metabolic rate to maintain a constant body temperature (Selman et al. 2000); can decrease mitochondrial membrane fluidity that may lead to an increase in reactive species production (Hazel 1995); or can expose the individual to a threat of freezing that results in ice formation in tissues, a process that may jeopardise the oxidative balance by changing the partial pressure of oxygen (e.g. by decreasing the volume of liquid available for dissolving the gas; Hermes-Lima and Storey 1992). For example, compared to gilthead sea breams (*Sparus aurata*) acclimated to 20 °C, fish maintained for 10 days at 8 °C had higher levels of liver oxidative damage (thiobarbituric acid reactive substances) and nitric oxide (Ibarz et al. 2010). Analyses of protein profiles of liver also showed that the expression of many proteins, including various antioxidant enzymes, was downregulated following cold exposure (Ibarz et al. 2010). Effects of cold stress on the oxidative balance may also emerge in the very short term. Selman and collaborators (2002) investigated whether acute cold (7 ± 3 °C) exposure (1, 10 or 100 h duration) affected protein oxidation and proteasome activity, when compared to warm controls (22 ± 3 °C), in various tissues of a small mammal model, the short-tailed field vole *Microtus agrestis*, whose thermoneutral zone is in the range 25–30 °C (Fig. 3.2). Resting metabolic rate of the vole increased significantly with duration of cold exposure. In skeletal muscle and liver, protein carbonyl levels also increased, while proteasome activity decreased, with duration of cold exposure, but they did not do so in brown adipose tissue, possibly because of upregulation of proteasome activity. Defence against oxidative damage in brown adipose tissue could have been prioritised because this tissue has a crucial thermogenic role during cold exposure in small mammals (Selman et al. 2002).

Depending on the intensity of cold stress, the animal can enter a state of adaptive or pathological hypothermia, which are distinguished by the capacity and incapacity, respectively, of the organism to rewarm to normothermia (Tattersall et al. 2012). Preserving the capacity of spontaneous rewarming is therefore important, although it is probably not cost free. However, many species have also evolved behavioural mechanisms to help rewarming, such as solar basking in reptiles (Tattersall et al. 2012). Behavioural strategies may be constrained by habitat thermal heterogeneity (e.g. availability of shelters or water bodies) and prioritisation of other activities (e.g. reproduction). Hence, oxidative costs of spontaneous rewarming and strategies of mitigating them are likely to vary across species. Threats of cold stress can also be local in terms of tissues. For example, peripheral tissues can be allowed to cool down noticeably, to near-freezing temperatures (Irving and Krog 1955; Henshaw et al. 1972). We might expect that, under these circumstances, activation of protective mechanisms is only prioritised in some tissues.

Although many findings came from laboratory studies, heat- or cold-induced oxidative stress is likely to be very relevant for organisms in the wild, since they can experience significant variations in temperature. The phenotype is programmed to deal with a wide range of temperatures, yet its flexibility may vary

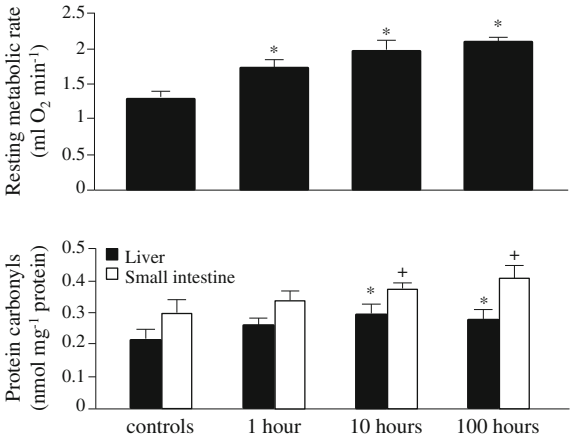

Fig. 3.2 Effects of exposure of short-tailed field voles (*Microtus agrestis*) to various levels of cold stress on resting metabolic rate and levels of oxidative protein damage (protein carbonyls). Significant differences to control values are denoted by * for resting metabolic rate, by * in the liver and by + in the small intestine. Data are shown as mean plus standard error. Reprinted with minimal alterations from Selman and collaborators (2002) with permission from Elsevier

among individuals, populations or species. The capacity to cope with unpredictable very high or low temperatures is likely to have significant evolutionary fitness consequences (examples on ectotherms in Abele 2011). Furthermore, the heat- or cold-induced oxidative stress is a possible key mediator in determining the fitness of organisms in the wild, given that the resultant tissue degradation may influence reproductive performance, growth patterns, cellular senescence and survival (Costantini 2008; Abele 2011). We certainly need experimental studies that test these hypotheses.

The timescale over which temperature extremes are experienced might also determine their impact on the organism. For example, levels of heat stress encountered in early life will vary among individuals, depending on the time of the season in which they are born and the prevailing weather conditions; those individuals that experience heat stress early in life could actually be better placed to cope with subsequent temperature extremes if the initial exposure has induced a hormetic conditioning response. There is some empirical support for conditioning hormesis priming the individual to withstand high temperatures across life (see Chap. 2). Studies on *Drosophila* fruit flies, *Caenorhabditis elegans* and *Saccharomyces cerevisiae* have shown that exposure to mild heat stress early in life can increase longevity (e.g. Shama et al., 1998; Le Bourg et al., 2001; Cypser and Johnson, 2002; Olsen et al., 2006). A recent meta-analysis on the life extension promoted by thermal hormesis showed that the response to heat stress was highly conserved across 12 invertebrate species (Lagisz et al. 2013). Results of the meta-analysis showed that heat shock temperature had the largest influence on experimental outcomes, with higher temperatures, longer duration of heat shock or

more heat shock repeats all decreasing the likelihood of any life extension; longer periods of recovery between repeated heat shocks were found to increase the likelihood of life extension; hormetic effects on longevity were stronger in young than adults. Results of the meta-analysis confirmed the importance of individual age at which the mild stress is experienced and of the strength/duration of heat stress as determinants of life extension promotion typical of hormesis. Stimulatory effects of mild heat stress have also been described in vertebrates. Studies on birds showed that early life conditioning hormesis to mild heat stress primed individuals to withstand high heat stress later in life, resulting in lower mortality (Yahav and McMurtry, 2001) and higher resistance to oxidative stress (Costantini et al. 2012) compared to individuals that were not exposed to heat stress or experienced high heat stress in early life. Hormetic effects are not limited to mild heat stress. Exposure to mild cold stress can enhance subsequent cold tolerance and survival (Lee et al. 1987; Czajka and Lee 1990) and preserve courtship behaviours (Shreve et al. 2004). For example, Yi and collaborators (2007) used three groups of young *Drosophila melanogaster* fruit flies: control (untreated at 23 °C), cold-shocked (directly exposed to –5 °C or –7 °C for 2 hours) and cold-conditioned (exposed to 5 °C for 2 hours prior to –5 °C or –7 °C for 2 hours). Cold-conditioned fruit flies had higher survival than cold-shocked fruit flies at both temperatures in the next 24 hours. It was also shown that the higher cold-tolerance of cold-conditioned fruit flies was associated with a block of apoptosis by upregulation of the anti-apoptotic protein Bcl-2 (and possibly of other factors, such as heat shock proteins; Yi et al. 2007). In another study on fruit flies, Shreve and collaborators (2004) found that mild cold stress (16 °C for 2 hours) increased courting and mating success compared to controls (23 °C), suggesting that hormetic promotion of survival may not necessarily come at a cost for reproduction. For species that have short lives, like fruit flies, having the capacity to adjust to changes in environmental temperatures may be critical for successful reproduction. However, further tests that use temperatures lower than 16 °C and apply different temperatures in the adult phase are required to fully support a role for hormesis in cold-induced enhancement of mating performance.

Thermal stress experiences may also shape the resistance of symbiotic marine species to oxidative challenges. Coral bleaching is a well-known phenomenon characterised by a loss of colouration caused by the loss of zooxanthellae, which are the algal endosymbionts of reef corals. Both field and laboratory studies showed that the increase in water temperature causes accumulation of oxidative damage, which would be a mechanism responsible for coral bleaching. Bleaching reflects a poor condition of corals, which can be characterised by reduced growth rates, suppression of sexual reproduction, impaired healing after mechanical damage, increased susceptibility to disease or mass mortality (e.g. Szmant and Gassman 1990; Meesters and Bak 1993; Brown et al. 2002a; Dunn et al. 2002; Lesser 2006). Although for certain coral species, the susceptibility to bleaching may be influenced by how much the algal genotype is resistant to thermal stress (Rowan et al. 1997), variation in bleaching susceptibility within colonies bearing genetically homogenous algal populations may also be induced by small-scale

variation in environmental conditions. In colonies of the massive coral *Goniastrea aspera* occurring at Phuket (Thailand), it was observed that during periods of maximal sea temperatures, bleaching occurred in the east but not in the west side of the colonies (Brown et al. 2002b). The genotype of algae extracted from the corals did not appear to differ between east and west corals. However, when exposed to elevated water temperatures and low irradiance in the laboratory, east but not west corals bleached in response to treatment (Brown et al. 2002b). These findings indicated that the bleaching responses could not be attributed to genetic differentiation, but were shaped by the differing local conditions corals experienced (Brown et al. 2002b). This suggests that hormetic priming (e.g. through epigenetic or maternal effects) might explain why corals containing genetically similar algae differed in resistance to bleaching. Hormesis-induced host transcriptomic changes (e.g. gene expression) likely represent one mechanism underlying the increase in thermal tolerance in corals (Bellantuono et al. 2012).

It is clear from all the studies presented above that a period of thermal conditioning (sometimes referred to as heat or cold hardening in studies on insects) may prime the system to withstand stress later in life. Thermal conditioning hormesis (sensu Calabrese et al. 2007) shows some similarity with the concepts of physiological acclimation and acclimatisation, which refer to any facultative modification in a physiological trait in response to changes in a single environmental variable in the laboratory and to changes in one or more environmental variables in the field, respectively (Wilson and Franklin 2002; Tattersall et al. 2009). In particular, the *beneficial acclimation hypothesis* states that individuals acclimated to a certain environment will have greater fitness in that environment than individuals acclimated to other environments (Leroi et al. 1994). This hypothesis has been tested extensively in the context of ambient temperatures, but with mixed support since a matching of ambient temperatures early and later in life has not always been found to confer greater evolutionary fitness (Wilson and Franklin 2002). On the basis of such studies, Zamudio et al. (1995) and Huey and Berrigan (1996) proposed the *optimal developmental temperature hypothesis*, which states that individuals raised at intermediate temperatures will have higher fitness across all temperature regimes in adult life than individuals raised at more extreme temperatures. For example, *Drosophila melanogaster* flies that were grown at 25 °C had higher walking speed as adults than flies that were grown at either 18 or 29 °C when tests were carried out at all three temperatures (Gibert et al. 2001). Note that Wilson and Franklin (2002) argued that such studies that involve potentially irreversible developmental processes cannot be considered adequate tests of the beneficial acclimation hypothesis, since acclimation involves reversible changes in physiology. However, unlike conditioning hormesis, acclimation is only concerned with the prevailing conditions and does not presume that the reversible physiological changes induced by the environment will improve future responses to stressors. In an attempt to reconcile discrepancies between theoretical predictions and empirical evidence, the concept of an optimal developmental temperature, as suggested by Zamudio et al. (1995) and Huey and Berrigan (1996), could also be viewed as a thermal hormetic response; thus,

animals reared under conditions of mild heat stress later outperform those reared at either higher (because of high stress level) or lower (no pre-exposure to stress) temperatures when tested at any temperature due to hormetically induced physiological processes (Costantini et al. 2010). Whether experimental data really do fit a conditioning hormesis framework remains unclear; this would require that the temperature experienced in early life was a mild stressor, and that the individuals exposed to this stress were then better able to cope with heat stress in later life.

3.2.3 Thermal Stress and Body Colourations

Many ectotherm species have evolved a special way of regulating their body temperature. These species have the ability to change the colouration of their skin in order to modify radiative heat loss and, possibly, to protect against ultraviolet radiation (see Sect. 3.3). A change in skin colouration is promoted by a reversible migration of pigments within chromatophores, which are specialised pigment cells located in the dermis (Bagnara and Hadley 1973; Tattersall et al. 2012). Carotenoids and melanins are two groups of pigments that many species of amphibians and reptiles use to colour themselves. Melanins, in particular, play a dominant role in rapid changes in colouration. So far, there has not been any specific experimental investigation of how oxidative stress constrains the ability of changing colour and, vice versa, of how the need to change colour to avoid hyperthermia may impact on oxidative stress. This might be relevant because, as discussed in Chap. 6, carotenoids are sensitive to passive oxidation and synthesis of melanins impacts the availability of glutathione, which is an important endogenous antioxidant.

Thermal stress may exert substantial selective pressure on body colourations because pigmentation may incur nutritional or energetic costs to the organism. For example, carotenoids are common animal pigments used for sexual signals. Under conditions of thermal stress, carotenoid saving may be important because it is possibly less costly to use them to sustain, for example, immune activity or other self-maintenance functions rather than to produce sexual colourations. Eraud and collaborators (2007) exposed male zebra finches (*Taeniopygia guttata*) to two temperature regimes (6 and 26 °C) over a period of four weeks. Simultaneously, half of the males in each temperature group were supplemented with carotenoids, whereas the other half were not. Carotenoid-supplemented males developed a redder bill, but the effect of supplementation was reduced under cold exposure (Eraud et al. 2007). Birds facing a cold stress were carotenoid limited, since supplemented males developed redder bills than the non-supplemented ones. Although cold-exposed and non-supplemented males developed duller bills, they circulated a higher amount of carotenoids at the end of the experiment compared to the pre-experimental values (Eraud et al. 2007). Overall, these results suggested that allocation of carotenoids to sustain self-maintenance mechanisms was prioritised at the expense of the ornament production. The low investment in

ornamentation might have occurred to some degree because the low temperature worked as an environmental cue, indicating that the probability of a successful breeding event would have been low under cold conditions. Accordingly, the low temperature would have triggered mechanisms that prioritised investment in self-maintenance rather than in reproduction-related traits.

3.3 Solar Radiation: The Threat Comes from Above

The organic matter that occurs in sea water may reduce the incidence of ultraviolet radiation on organisms living in shallow waters, such as corals (Zepp et al. 2008). However, reactive species can be produced through the absorption of solar radiation by this organic matter (Mopper and Kieber 2000). On the other hand, the reactive species can degrade the organic matter to low weight molecular compounds, which can be an important source of food for microorganisms (Mopper and Kieber 2000). Reactive species can also represent a threat because they might damage the cell membrane of microorganisms themselves. For example, the hydrogen peroxide produced by the degradation of organic matter has a long life in sea water and, because of its low propensity to react with other molecules, can easily pass through cell membranes (Mopper and Kieber 2000; Halliwell and Gutteridge 2007).

Ultraviolet radiation can also have more direct effects on the physiology and ecology of a species (Bancroft et al. 2007, 2008). Laboratory studies of the green sea urchin *Strongylocentrotus droebachiensis* found that exposure to ultraviolet radiation can reduce fertilisation success and influence the development time of embryos; it was suggested that oxidative stress might be a mechanism underlying the detrimental effects of radiation (Adams and Shick 1996, 2001; Lesser et al. 2006; Meng et al. 2009). These findings received support from field studies of the green sea urchin embryos, which showed that oxidative stress could be induced by radiation at depths deeper than 5 metres (Lesser 2010).

Ultraviolet radiation also influences oxidative balance in vertebrates. Exposure of zebra finches (*Taeniopygia guttata*) to ultraviolet radiation impaired pro-inflammatory immune response and induced mobilisation of stored carotenoids as suggested by the low and high levels of carotenoids found in liver and plasma, respectively (Blount and Pike 2012). However, the sunlight exposure did not induce oxidative damage, nor did it reduce intensity of sexual colouration of the bill (Blount and Pike 2012). This may have been due to a protective role of skin carotenoids against sunlight-induced damage (Alaluf et al. 2002; Sies and Stahl 2007). Although such a protective role of skin carotenoids was demonstrated in humans, it awaits evidence in wild animals.

3.4 Partial Pressure of Oxygen

3.4.1 Coping with Drastic Changes in Oxygen Concentration

The partial pressure of oxygen can drastically change in certain environments on a daily or seasonal basis, exposing organisms to potential oxidative stress. For example, cycles of dissolved oxygen in estuaries can range from anoxia (complete lack of oxygen), hypoxia (oxygen concentrations lower than normoxia) to various levels of supersaturation (200–300 % higher than normoxia) over short time periods (Ross et al. 2001). In many species, however, variations in oxygen tensions in the tissues are induced by behavioural strategies evolved to survive harsh seasons, such as in mammal species that hibernate during the cold season. Some examples of this second group of species are given in this chapter, but have been reviewed in more detail in Chap. 5, where the link between oxidative stress and activity patterns has been examined.

For individuals that enter an anoxic or hypoxic state, the increase in oxidative damage represents a real threat when cells are reoxygenated. In fact, upon reoxygenation, the production of reactive species in cells increases dramatically, increasing the need for investment in antioxidant protection. Following the hypothesis that anoxia/hypoxia-tolerant species could have biochemical adaptations to cope with free radical generation induced by reoxygenation (Reischl 1986), pioneer studies on red-sided garter snakes (*Thamnophis sirtalis parietalis*) exposed to 10 hours of anoxia at 5 °C showed that these snakes, compared to controls, had higher activity of superoxide dismutase and higher concentration of glutathione in both muscle and liver (Hermes-Lima and Storey 1993). This finding was a bit surprising because at that time, it was thought that under anoxic conditions, generation of reactive species cannot practically occur. It was in fact later discovered that in some species there is formation of reactive species under hypoxia/anoxia conditions. Therefore, the upregulation of some antioxidant defences that was found in some species has been interpreted as an anticipatory protective response to the upcoming burst of free radicals caused by reoxygenation (*preparation for oxidative stress hypothesis*, Hermes-Lima and Storey 1993; Hermes-Lima et al. 1998). Pre-treatment of antioxidants, for example, prevents post-reoxygenation injury in humans (Wang et al. 1998; Tanaka et al. 2004), while inhibition of catalase during hypoxia caused an increase in the ratio of oxidised glutathione to reduced glutathione in Nile tilapia *Oreochromis niloticus* (Welker et al. 2012). Many species, however, do not show any anticipatory response to reoxygentation following anoxia nor an increase in oxidative damage, such as in red-eared slider turtles *Trachemys scripta elegans* (Willmore and Storey 1997a, b) or in cave-dwelling salamanders *Proteus anguinus* (Issartel et al. 2009). The red-eared slider turtles had suppressed antioxidant enzyme activities when experiencing 20 hours under anoxic conditions. However, they did not suffer any increase in oxidative damage during anoxia nor reoxygenation (Willmore and Storey 1997a, b). This might be because red-eared slider turtles have high basal

antioxidant levels if compared to other non-mammalian species; these are in the range of some mammal species (Hermes-Lima et al. 2001; Hermes-Lima and Zenteno-Savín 2002). Therefore, turtles might not need to invest in upregulation of antioxidant defences. This may be explained to some extent by the diving habits of turtles, which are exposed to repeated periods of anoxia–reoxygenation that require high constitutive antioxidant defences if compared with non-diving species. Although these studies have provided relevant insight into mechanisms underlying anoxia–reoxygenation episodes, it would be important to put these studies in a more ecological framework to see whether the presence or absence of anticipatory responses may be influenced by the life history stage or the individual condition. Moreover, the use of live imaging techniques (when possible) to visualise formation of reactive species will be essential to provide robust evidence about how it changes in response to varying environmental conditions (e.g. Rivera-Ingraham et al. 2013a, b). For example, using live imaging techniques, Rivera-Ingraham et al. (2013a) found that marine platyhelminths (*Macrostomum lignano*) exposed to hyperoxic, normoxic and anoxic treatments displayed no significant differences in superoxide anion formation, whereas total mitochondrial reactive oxygen species formation was higher after hyperoxic exposure and lowest under near-anoxia conditions compared with the normoxic control group.

Selective pressures imposed by fluctuations in environmental oxygen concentration are particularly evident in coral reefs, intertidal and estuarine habitats (Nilsson and Östlund-Nilsson 2004). Marine invertebrates and fish inhabiting these environments are exposed daily to episodes of anoxia and reoxygenation because they become exposed to air during low tides and their tissues are reoxygenated when the water level increases during high tides. These species have evolved several metabolic adaptations to tolerate short-term anoxia, such as the use of fermentable fuels to produce energy and allow depression of metabolic rate (Storey and Storey 1990; Brooks and Storey 1997). Studies on the gastropod *Littorina littorea* showed that adaptation to post-anoxic oxidative stress is a further physiological mechanism evolved by intertidal species (Pannunzio and Storey 1998). However, several components of the antioxidant machinery were suppressed rather than increased over the anoxic period. Given that the anoxic period tested in Pannunzio and Storey (1998) was particularly long (6 days), it has been suggested that its duration may be relevant because short periods of anoxia normally stimulate antioxidant defences (Hermes-Lima and Zenteno-Savín 2002). These findings may also be interpreted as an example of hormetic response to anoxia, with short episodes being stimulatory, while long episodes being inhibitory to some antioxidant mechanisms. Reactive species might work as molecular mediators of such hormetic effects. There is, however, still debate about whether there is formation of reactive species in hypoxia (Rivera-Ingraham et al. 2013a, b; Welker et al. 2013). It might be that there is formation of reactive species only in some species or under specific conditions, or, simply, that this formation is transient and depends on the duration of the hypoxic state.

Similar to anoxia, the duration of the reoxygenation phase may have a different impact on damage parameters and antioxidant defences, as shown in estuarine crabs *Chasmagnathus granulata* subjected to 20 or 40 minutes of recovery from 8 hours of anoxia (de Oliveira et al. 2005). Such biphasic responses might also reflect a different production of reactive species between the anoxic and normoxic phases. For example, Dirmeier and collaborators (2002) have found in yeast that certain proteins become carbonylated during a shift to anoxia and that some of these proteins also become carbonylated during exposure to hydrogen peroxide. There was also activation of hypoxic genes. It was later demonstrated, however, that the activation of hypoxic genes is induced primarily by nitric oxide rather than by reactive oxygen species (Poyton et al. 2009). Nitric oxide may combine with superoxide anion, producing peroxynitrite, which in turn may nitrosylate proteins and induce expression of hypoxic genes (Poyton et al. 2009).

Further support for a reoxygenation-induced oxidative stress comes from studies on the effects of exposure to high oxygen concentration in sea or freshwater (hyperoxia). Results of these studies suggested that, depending on the intensity and duration, hyperoxia (like hypoxia) may act on the antioxidant mechanisms through hormetic mechanisms (Ritola et al. 2002a, b, c; Dabrowski et al. 2004; Olsvik et al. 2006). There are various examples of natural hypoxia-induced hormetic conditioning. For example, the activity of superoxide dismutase in gills and digestive glands is higher in blue mussels (*Mytilus edulis*) living in high-shore than in low-shore areas because animals in high-shore areas experience longer and more frequent periods of emersion through the tidal cycle (Letendre et al. 2008). Similarly, epaulette sharks (*Hemiscyllium ocellatum*), which inhabit the reef platforms, go through periodic cycles of hypoxia–reoxygenation because platforms repeatedly become cut off from the ocean during periods of low tides forming large tide pools (Nilsson and Renshaw 2004). During nocturnal low tides, the water oxygen can fall by 80 % due to respiration of the coral and associated organisms (Nilsson and Renshaw 2004). The intensity of the hypoxia period is, however, quite variable across the tidal cycle. This may, for example, be relevant for young developing sharks: depending on which stage of development the episode of hypoxia is experienced, it may or may not result in long-term protective effects against future episodes of oxidative stress in adult sharks. If this were the case, reproductive decisions of sharks may be influenced by tides. Females might adjust laying to the tidal cycle and select sites where offspring would experience moderate hypoxia (but also low predation pressure). For example, reproduction in this shark species occurs during periods of lower water temperatures (Heupel et al. 1999), which might limit the magnitude of hypoxia because water oxygen concentration is lower at higher water temperatures.

While periodically being exposed to decreases in oxygen concentrations and subsequent oxidative stress induced by reoxygenation, intertidal marine species may also be exposed to additional peroxidative stress caused by the photosynthetic oxygen production of benthic algae. Oxygen supersaturation favours formation of hydrogen peroxide increasing its concentration in sediments (Zika et al. 1985; Szymczak and Waite 1988). The polychaete *Nereis diversicolor* is an invertebrate

species that lives in burrows in intertidal sediments. During low tides, worms respond behaviourally by dragging air bubbles inside their burrows and exchanging for fresh bubbles when needed (Lindroth 1941). Moreover, worms may also switch to anaerobiosis and reduce their activity when exogenous peroxides become very high (Schöttler 1979). However, additional physiological mechanisms are needed to resist oxidative insults. This is particularly important during the spring spawning time because the worms have to cope with an increase in energy and oxygen demands and, therefore, take higher amounts of externally formed peroxides. Beyond keeping enzymatic antioxidant defences high, spawning worms may also rely on non-enzymatic antioxidants if there is no additional anoxic stress. When worms are incubated with peroxides under aerobic conditions during spawning, there is oxidative decomposition of haemoglobin to biliverdin, which has important antioxidant properties and may offer additional protection to that made by enzymes (Abele-Oeschger et al. 1994). However, under anoxic conditions, the biochemical decomposition of haeme is inhibited, as it requires high quantities of oxygen to decompose haemoglobin to biliverdin (Abele-Oeschger et al. 1994). There is also autoxidation of haemoglobin and consequent formation of reactive oxygen species (Abele-Oeschger and Oeschger 1995). Therefore, oxidative stress may represent a relevant constraint of reproductive investment of worms when the availability of environmental oxygen occurs in limited supply.

Beyond molecular mechanisms, many organisms also evolved behavioural strategies that might have favoured protection against oxidative stress. For example, many marine invertebrates with open circulatory systems establish low and constant oxygen partial pressure around their tissues (Abele et al. 2010). Adjustment of mantle cavity partial pressure of oxygen to lower than ambient levels through controlled pumping should prevent high oxygen gradients between bivalve tissues and surrounding fluid, limiting oxygen flux across the body surface and, possibly, any increases in oxidative stress (Abele et al. 2010).

3.4.2 The Curious Case of Symbiotic Species

Maintenance of oxygen equilibrium in tissues is not only challenged by variations in partial pressure of oxygen that occur in the environment external to the body, but also challenged by those that occur within it. This is what notably occurs in symbiotic species. Invertebrates that live in symbiosis with unicellular algae experience higher partial pressures of oxygen in their tissues than aposymbiotic species because the endosymbiotic algae produce oxygen through photosynthesis and the rate of oxygen consumption in respiration is low (D'Aoust et al. 1976; Shick and Dykens 1985; Shick 1990; Shick et al. 1996). Exposure to high concentrations of oxygen may increase the rate of basal oxidative damage generation, hence possibly influencing the rate of senescence or the reproductive strategy. However, symbiotic species do not suffer higher oxidative stress than

aposymbiotic species because they have more efficient antioxidant defences (Dykens and Shick 1982, 1984; Shick 1990; Shick et al. 1996).

Pro-oxidant effects of hyperoxic conditions in tissues may be exacerbated in symbiotic species living in environments with high solar irradiance because oxygen may undergo univalent reduction, giving rise to various free radicals. However, the toxicity of sunlight for symbiotic species seems to differ among environments (Regoli et al. 2000) and might also be dependent on conditioning processes through hormesis. For example, the Mediterranean demosponge *Petrosia ficiformis* lives symbiotically with the cyanobacterium *Aphanocapsa feldmanni*, but individuals living in submarine caves can be aposymbiotic. Aposymbiotic specimens of this species die within a week when transplanted from caves to cliffs exposed to sunlight (Regoli et al. 2000). In contrast, the mortality does not increase in symbiotic specimens transplanted to areas where solar irradiance is higher, even when they are deprived of most endosymbionts through the grazing activity of nudibranchs (Regoli et al. 2000). Therefore, the pro-oxidant effect of cyanobacteria could have prepared the antioxidant defences of their hosts to better withstand the pro-oxidant effect of ultraviolet radiation. This might be, for example, important in the Mediterranean summer. At this time, solar irradiance and water temperature are high and can represent additional stressors for sponges (Regoli et al. 2004). Therefore, it can be hypothesised that depending on the magnitude of the pro-oxidant stimulus induced by the endosymbiont, the symbiotic individual might be more or less prepared to cope with the subsequent stress induced by sunlight or ambient temperature.

Various other studies found that in species occurring naturally in both symbiotic and aposymbiotic forms, antioxidant defences are higher in the symbiotic ones (aggregating anemone *Anthopleura elegantissima*, Dykens and Shick 1982, Mitchelmore et al. 2003; maxima clam *Tridacna maxima*, Shick and Dykens 1985; Mediterranean sea anemone *Anemonia viridis*, Richier et al. 2003, Plantivaux et al. 2004). It may be speculated that the higher capacity to withstand oxidative stress conferred by the endosymbiont has favoured the evolution of this particular kind of symbiosis, because of a selective advantage conferred by the endosymbiont in environments, where the capacity to withstand environmentally induced oxidative stress has been vital.

3.5 Partial Pressure of Carbon Dioxide

Exposure to a high partial pressure of carbon dioxide (CO_2) in freshwater or sea water causes an increase in CO_2 concentration in the blood, which acidifies blood and other body tissues (Brauner and Baker 2009). Animals are equipped with systems that regulate the pH of tissues, but pH regulation is generally energy demanding (Pörtner et al. 2004; Ishimatsu et al. 2008).

An increase in CO_2 concentration in water may also face animals with a threat of oxidative stress (i) indirectly by lowering organism pH, which may trigger the

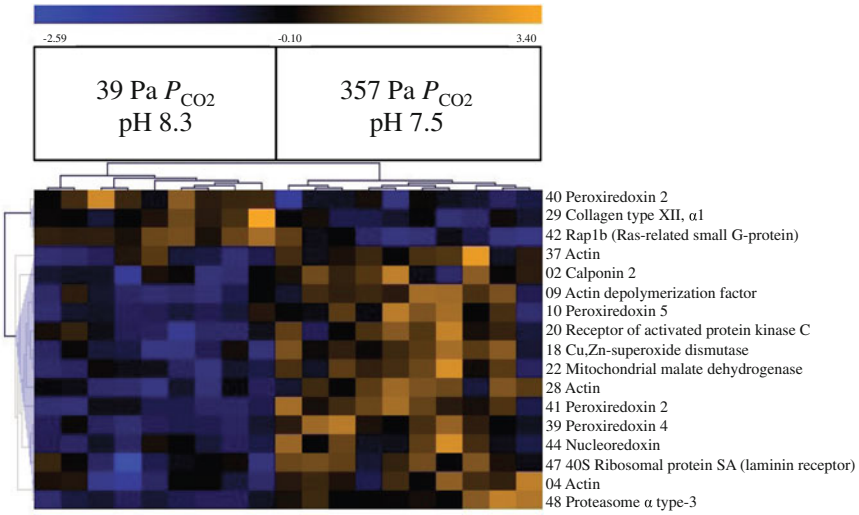

Fig. 3.3 Hierarchical clustering of identified proteins in mantle tissue of eastern oysters (*Crassostrea virginica*) in controls and conditions of elevated partial pressure of CO_2. The expression level of the protein increases from blue to orange. Reprinted with minimal alterations from Tomanek and collaborators (2011) with permission

Fenton reaction, and (ii) directly by interaction between CO_2 and reactive species to form new free radicals (e.g. nitrosoperoxycarbonate; Veselá and Wilhelm 2002). For example, eastern oysters (*Crassostrea virginica*) occur in estuarine ecosystems that are characterised by large fluctuations in CO_2 levels on a timescale from hours to months (from ca. 40 to ca. 4700 Pa; Tomanek et al. 2011). Exposure to a high partial pressure of CO_2 ($\sim$357 Pa) for two weeks resulted in a significant proteome shift in the mantle tissue of eastern oysters, compared with control conditions (Tomanek et al. 2011). Among the identified proteins, two main functional categories were upregulated in response to hypercapnia: those associated with the cytoskeleton (e.g. several actin isoforms) and those associated with oxidative stress (e.g. superoxide dismutase and several peroxiredoxins as well as the thioredoxin-related nucleoredoxin; Fig. 3.3). Although mechanisms remain to be described, results of this study indicated that exposure to high partial pressure of CO_2 may induce oxidative stress (Tomanek et al. 2011). Results of this study also provided insight into the possible stress and adaptive responses of sessile estuarine organisms to high CO_2 concentrations, which is especially relevant in the light of ongoing ocean acidification.

Exposure to high concentrations of CO_2 may trigger hormetic mechanisms. Experimental exposure to ocean conditions projected for the end of the century (approximately 1,000 μatm CO_2) caused an increase in metabolic rate and a decrease in length, weight, condition and survival of juvenile cinnamon clownfish *Amphiprion melanopus* (Miller et al. 2012). However, these effects were absent or

reversed when parents experienced similarly high concentrations of CO_2. While too stressful for young, CO_2 levels may have been stimulating the protective molecular mechanisms of parents, which they passed on to their offspring. These results suggested that hormetic programming might cross generations through epigenetic effects, which may confer to offspring a better capacity to withstand stressful conditions induced by CO_2.

In a related experiment, breeding pairs of cinnamon clownfish were held under three treatments of CO_2 (current-day control (430 µatm), moderate (584 µatm) and high (1032 µatm)) over a 9-month period that included the summer breeding season (Miller et al. 2013). Surprisingly, increased CO_2 dramatically stimulated breeding activity. Over twice as many pairs bred in the moderate (67 % of pairs) and high (55 %) compared to the control (27 %) CO_2 treatment. Moreover, pairs in the high CO_2 group produced double the number of clutches per pair and 67 % more eggs per clutch compared to the moderate and control groups. As a result, reproductive output in the high CO_2 group was 82 % higher than the control group and 50 % higher than in the moderate group (Miller et al. 2013). This hormetic effect on reproduction was not due to an effect on fish body mass, as this did not differ among groups. This study provided the first evidence of a potential hormetic effect in response to increased partial pressure of CO_2 (Miller et al. 2013). Any long-term consequences of increased reproductive output on individuals or populations remain to be assessed.

3.6 Coping with Multiple Environmental Stressors

Obviously, in a natural context, a change in a certain environmental parameter, say water temperature, is associated with changes in other parameters, such as oxygen concentration in water. The overall effect on the organism can therefore be exacerbated or mitigated depending on whether the action of different stressors is additive, synergistic or antagonistic.

Deschaseaux and collaborators (2010) examined the cellular responses of embryos of three rocky shore intertidal gastropod species (*Bembicium nanum*, *Dolabrifera brazieri* and *Siphonaria denticulata*) to temperature and salinity changes. Encapsulated embryos of each species were exposed for 72 hours to six combinations of ecologically realistic temperature and salinity levels (22° and 30 °C; 25, 35 and 45 ppt). Both extremes of salinity (25 and 45 ppt) and high temperature (30 °C) negatively affected the embryos, inducing oxidative stress and increasing embryonic mortality. Similar results were obtained in a related experiment, where eggs were exposed to six combinations of temperature and salinity (22° and 30 °C; 25, 35 and 45 ppt) until hatching (Deschaseaux et al. 2011).

Depending on the intensity, hypoxia may also prepare the organism to better cope with additional environmental stressors through hormetic mechanisms. For example, Wu and collaborators (2002) found that a priori exposure of young male locusts (*Locusta migratoria*) to anoxia (2 hours in nitrogen) had protective effects

against subsequent exposure to high ambient temperature, resulting in higher survival than locusts that did not previously experience anoxia. Under normoxia, exposure of silver catfish (*Rhamdia quelen*) to manganese increased lipid peroxidation in brain and kidney; it also increased glutathione in brain and decreased catalase activity in both tissues (Dolci et al. 2013). Moderate hypoxia was able to prevent manganese-induced lipid peroxidation in brain and to reduce it in kidney; glutathione was increased in brain, while activity of catalase was reduced in both tissues (Dolci et al. 2013). Similarly, Zambonino-Infante and collaborators (2013) showed that early life thermal experiences can shape a phenotype more resistant to hypoxia. A warmer temperature experienced during the larval stage had a positive effect on body mass and resistance to hypoxia at the juvenile stage in common soles (*Solea solea*), suggesting a potential hormetic priming of metabolic pathways. If this were the case, results of this study would suggest that early life hormetic priming might provide common soles and, possibly, other marine animal species some buffer against the effects of global warning. We need further studies that address specifically over a larger range of early life environmental stressors whether and how hormesis impacts the capacity of marine fauna to cope with environmental changes.

Hormetic effects induced by mild stressful environmental conditions are not specific to animal species. For example, Ellouzi and collaborators (2013) allocated 4-week-old plants of the European searocket (*Cakile maritima*) to four treatment groups: control, salt stress, drought stress and cadmium stress. After two weeks of recovery from this first treatment, plants belonging to each pre-treatment group were exposed to either of the following salt concentrations: 0, 100, 200, 400, 600 and 800 mM NaCl, for 1 week. Compared to plants that did not experience any stress, plants that were previously exposed to stress had lower levels of hydrogen peroxide and lipid peroxidation (malondialdehyde) after exposure to high levels of salinity. The European searocket grows in clumps or mounds in the sand on beaches and bluffs, where it can be exposed to periods of drought or intense salinity. This plant species may therefore have evolved mechanisms that are triggered during development in order to prepare the plants for the extreme conditions they will likely experience later in life.

In addition, an increase in thermotolerance can be induced by exposure to other kinds of stressful experiences. For example, Brown and collaborators (2002a) found that at elevated experimental water temperatures, reef corals (*Goniastrea aspera*) from the surfaces of colonies from shallow waters exposed in the wild to solar radiation lose fewer symbiotic algae, have lower levels of oxidative stress and have higher levels of host antioxidant enzyme copper–zinc superoxidase dismutase and of host heat shock proteins 60 and 70 than corals from surfaces of colonies less exposed to radiation in the wild. In addition, corals from highly exposed surfaces showed less chronic photoinhibition and greater Photosystem II recovery potential than coral from less exposed surfaces when both corals were experimentally exposed to high irradiance at ambient water temperature in the laboratory. In contrast, no differences were noted in algal defences (e.g. antioxidant enzymes and stress protein production, and xanthophyll cycling) either at

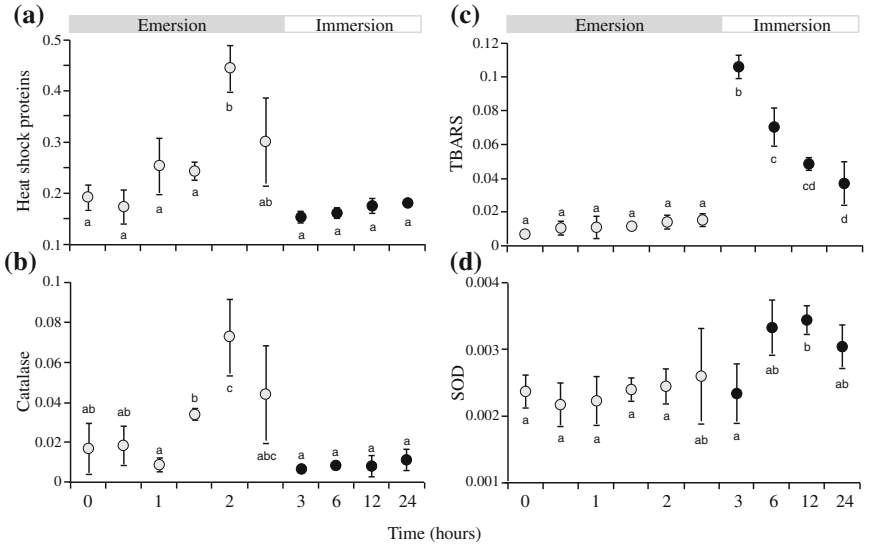

Fig. 3.4 Changes in (a) heat shock protein expression, (b) catalase, (c) thiobarbituric acid reactive substances (TBARS) and (d) superoxide dismutase (SOD) during air exposure (emersion) and reoxygenation (immersion) in the octocoral *Veretillum cynomorium*. Values are shown as mean ± standard deviation. Different letters represent significant differences between time periods. Reprinted with minimal alterations from Teixeira and collaborators (2013) with permission from Elsevier

elevated or ambient temperatures (Brown et al. 2002a). Beyond the acquisition of thermotolerance, these results demonstrated that it was the priming of the host tissues rather than of algae to be important in maintaining symbiosis under thermal stress (Brown et al. 2002a). Various protective physiological mechanisms are activated when corals are exposed to air (Fig. 3.4). It is thought that this activation has been evolved as an anticipatory protective response to the oxidative stress arising during the reoxygenation event experienced during reimmersion (Teixeira et al. 2013).

3.7 Environmentally Induced Variation in Redox State Regulation Within and Among Species

Our understanding of how variation in resistance to oxidative stress among species living in different climatic regions has contributed to shaping variation in life histories is seriously limited. Reasons are as follows: paucity of data; limited application in comparative studies of statistical control of phylogenetic relatedness; and limited consideration of life history traits. However, some general considerations can be made.

3.7.1 Invertebrates

Ultraviolet (UV) irradiance largely varies across environments, with low levels normally occurring at polar regions. Hence, polar animal species may be expected to be more vulnerable to ultraviolet irradiance than species living elsewhere. This is especially important given that the marine environments in polar regions have been experiencing increases in UV-B radiation (290 to 320 nm) as a result of stratospheric ozone depletion. They are also expected to experience increases in UV-R (290 to 400 nm; Lesser et al. 2004). Lamare and collaborators (2007) quantified the effects of UV on the rate of DNA damage (cyclobutane pyrimidine dimer production) and abnormal development in embryos of four sea urchin species: *Sterechinus neumayeri* (polar region: Antarctica), *Evechinus chloroticus* (temperate region: New Zealand), *Diadema savignyi* and *Tripneustes gratilla* (tropical region: Cook Islands). DNA damage was significantly higher in Antarctic embryos compared to the other species. Exposure to UV-R increased abnormal development, with rates generally higher in Antarctic embryos, although high abnormality rates were also associated with both UV-B and UV-A exposure. The greater sensitivity of embryos of polar species to UV-R is consistent with them having low concentrations of sunscreens and slow rates of DNA repair compared with temperate and tropical species (Lamare et al. 2006, 2007; Lesser et al. 2006). The effect of water temperature on the activity of enzymes responsible for DNA repair was negligible, suggesting that temperature compensation has a non-significant effect on DNA repair. Moreover, results suggested that production of reactive species induced by UV may be a stronger constraint for life history evolution in polar than in temperate or tropical sea urchin species.

Increased synthesis of heat shock proteins is a highly conserved response to warming. The absence of a heat shock response has, however, been observed in some *Hydra* species. Bosch and collaborators (1988) found that the freshwater cnidarian brown hydra *Hydra oligactis* was unable to synthesise heat shock proteins of any size class in response to thermal stress. However, in the related species *Hydra vulgaris*, individuals were capable of synthesising heat shock proteins of molecular masses 23, 70 and 80 kDa (Bosch et al. 1988). Presence or absence of a heat shock response was also addressed in other *Hydra* species. *Hydra oligactis* and other species lacking a heat shock response were found to uniquely occur in cold and thermally stable environments. The loss of the heat shock response in these taxa may have occurred during evolution under these cold conditions, where it would have been counterselected because thermal regimes were so stable that there was no reason to select a phenotype equipped with these costly defence systems. However, it could be that the loss of the heat shock response occurred before colonisation of cold waters; hence, this loss forced these species to occupy habitats with stable low temperatures. It should also be not forgotten that the magnitude of the heat shock response may differ among conspecific populations, with negligible responses occurring in individuals that live in environments where thermal stress is limited (e.g. *Nacella magellanica* in Pöhlmann et al. 2011).

3.7.2 Fish

Notothenioidei polar fish are extreme stenotherms that evolved for approximately 14–25 million years in the cold and thermally stable waters of coastal Antarctica, where temperatures range from +0.3 °C to −1.86 °C (Eastman 1993; Regoli et al. 2012). Hofmann and collaborators (2000) found that in one of these fish species, namely the emerald rockcod *Trematomus bernacchii*, the ability to induce heat shock proteins of all size classes following either thermal or chemical stress was absent, possibly lost during evolution in these cold and thermally stable environments. In vivo metabolic labelling experiments that involved injection of ^{35}S-labelled methionine and cysteine into whole fish previously subjected to a heat stress of 10 °C yielded no evidence for synthesis of any size class of heat shock protein (Hofmann et al. 2000). Parallel in vivo labelling experiments with isolated hepatocytes similarly showed significant amounts of protein synthesis, but no indication of enhanced expression of any class of heat shock proteins. Induction of chemical stress through exposure to the heavy metal cadmium also failed to induce synthesis of heat shock proteins. However, solid-phase antibody (western) analysis revealed that both the inducible and constitutively expressed forms of Hsp 70 chaperones are present in this species (Hofmann et al. 2000).

Polar white-blooded icefishes (those that lost haemoglobin) have constitutive characteristics that appear to make them more vulnerable to temperature-induced oxidative stress than temperate red-blooded notothenioids. Mitochondria from Antarctic icefish have higher densities of phospholipids per mg of mitochondrial proteins compared with red-blooded species (O'Brien and Mueller 2010). The high density of lipid-rich mitochondria in oxidative muscles of icefishes enhances oxygen delivery in the absence of haemoglobin and myoglobin (O'Brien 2011). However, these phospholipids are rich in polyunsaturated fatty acids, which place these fish at increased risk of oxidative damage, for example, in the case of increased water temperatures. Additionally, it was found that several tissues in icefishes have lower levels of antioxidants compared with related temperate species (Witas et al. 1984; Cassini et al. 1993; Abele et al. 2012). Heart mitochondria of icefishes were shown to be more tightly coupled than those of red-blooded fishes at 2 or 10 °C, which increased the production of reactive species in icefishes when the electron transport chain was disrupted (Mueller et al. 2011). The activity of superoxide dismutase and the non-enzymatic antioxidant capacity per mg of mitochondrial proteins did not differ between icefishes and red-blooded species, but the non-enzymatic antioxidant capacity normalised to mitochondrial phospholipid content was significantly lower in icefishes than in red-blooded fishes. It was also found that the membrane susceptibility to peroxidation was only detectable in icefishes at 1 °C and not in red-blooded species (Mueller et al. 2011). These constitutive differences contribute to make icefishes more vulnerable to oxidative stress than red-blooded temperate fishes when exposed to a thermal challenge. However, as with heat shock proteins, icefishes also appear to have lost the ability of upregulating antioxidant enzymes in response to oxidative stress.

Mueller and collaborators (2012) found that levels of oxidised proteins and lipids increased in the heart ventricle of some icefishes but not in red-blooded species in response to warming. Despite an increase in oxidative damage in hearts of icefishes, there was no activation of the antioxidant response: neither transcript levels nor activity of antioxidants increased in any tissue of any species in response to exposure to the critical thermal maximum (thermal limit above which the ambient temperature becomes lethal).

Conspecific populations may also differ in the way they regulate their redox state, depending on the selective pressures under which they evolved. Nikinmaa and collaborators (2013) analysed transcription and redox enzyme activities at a steady state and in response to an acute temperature change in three populations of three-spined sticklebacks (*Gasterosteus aculeatus*). They found that there was divergence in markers linked to antioxidant response, indicating that processes regulating the redox balance may be important targets of selection underlying adaptive divergence in this fish species.

3.7.3 Birds

Life histories of temperate and tropical birds differ remarkably. Compared to temperate species, tropical birds lay smaller clutches, grow slower as nestlings and live longer (Snow and Lill 1974; Ricklefs 1976; Cardillo 2002). Temperate and tropical birds also show physiological differences that are compatible with their lifestyles. Wiersma et al. (2007a, b) compared the basal metabolic rate (minimum metabolic rate of a quiescent, post-absorptive animal, in its thermoneutral zone and rest phase; McNab 1997) and the cold- or exercise-induced maximum metabolic rate between a large number of temperate and tropical bird species. They found that tropical birds had a reduced basal metabolic rate, a result strongly compatible with their slow life history style. Furthermore, tropical migrant birds breeding in temperate regions had a basal metabolic rate that was lower than temperate residents, but higher than tropical residents. Moreover, tropical birds had lower exercise-induced and cold-induced maximum metabolic rates than temperate species; the latter is consistent with the hypothesis that tropical birds have not been selected for high levels of thermogenesis (presumably because they are not often exposed to low temperatures) or, alternatively, the slow pace of life of tropical birds might not be compatible with a high thermogenic capacity (Wiersma et al. 2007a, b). Later, data on organ masses (heart, liver, kidneys and pectoral muscles) provided support for the hypothesis that the tropical birds had reduced metabolic rates (corrected for body mass variation) because they have smaller organs and so a lower metabolic intensity (Wiersma et al. 2012). Similar conclusions were drawn comparing tropical and temperate conspecific populations using common garden experiments. Wikelski and collaborators (2003) obtained nestling stonechats (*Saxicola torquata*) from equatorial Kenya (0° N), Ireland (51.5° N), Austria (47.5° N) and Kazakhstan (51.5° N). Birds were hand-raised and

kept in individual cages under a constant temperature of 20–23 °C and daylength conditions simulating those experienced by Austrian birds in the wild. Resting metabolic rate (corrected for body mass variation) was generally higher during moult, but differed among populations: it was lowest in the resident Kenyan birds, higher in mostly sedentary Irish birds, and highest in migratory Austrian and Kazakhstan birds. These results demonstrate that even in birds kept under common-garden conditions from early life, the metabolic rate appears to be lower in tropical than in temperate individuals.

Overall, all of these findings provide evidence of a connection between life history and species physiology and that such a connection may be underlined to some degree by the variation in organ masses. However, differences at the cellular level may also be important. Although metabolic rate is not strongly related to the oxidative balance, the metabolic machinery is an important source of production of free radicals. Moreover, given their high annual survival and low rates of metabolism, it may be predicted that tropical species have greater cellular resistance to chemical injury than cells from temperate species. To test this hypothesis, Jimenez and collaborators (2013) cultured dermal fibroblasts from 26 tropical and 26 temperate species of birds and compared the cellular resistance (dose at which 50 % of cells die) to cadmium (e.g. inhibits the systems involved in hydrogen peroxide removal), hydrogen peroxide (pro-oxidant but also source for the formation of the hydroxyl free radical), paraquat (herbicide that induces formation of superoxide), thapsigargin (inhibits Ca^{2+} pumping in the endoplasmic reticulum), tunicamycium (antibiotic that interferes with protein processing in the endoplasmic reticulum), methane methylsulfonate (DNA alkylating agent) and ultraviolet light (causes oxidative damage to various classes of biomacromolecules, especially DNA). The investigators found that cells from tropical birds were more tolerant of cadmium, hydrogen peroxide, paraquat, tunicamycin and methane methylsulfonate than cells from temperate birds, whereas for thapsigargin and ultraviolet light, tropical birds showed either lower tolerance to or no difference from temperate birds. These findings support the idea that natural selection has shaped cells of tropical bird species to be more resistant to various forms of oxidative and non-oxidative stress than cells from temperate species, providing a plausible proximate mechanism at the cellular level linking life history variation to cellular function.

Similar conclusions were drawn in a comparative study of circulating antioxidants in bird species (Cohen et al. 2008). Correlations between clutch size and antioxidants were observed in temperate but not in tropical species. Moreover, temperate but not tropical birds with higher basal metabolic rate had lower uric acid and serum non-enzymatic antioxidant capacity (Cohen et al. 2008). The authors suggested that resident tropical lowland forest bird species live in more stable environments than temperate species; hence, they might be under milder selection (Cohen et al. 2008). High constitutive antioxidant levels in temperate species might reflect both greater need for a response to a stressor or higher baseline production of reactive species in birds selected to invest more into current than in future reproduction.

3.8 Conclusions

Various mechanisms (e.g. heat shock proteins) evolved to cope with environmental stressors tend to be highly conserved in most animal taxa and differ mostly in quantity of response (e.g. amount of synthesised enzymes) rather than in the quality (e.g. typologies of antioxidant enzymes). There are, however, mechanisms that tend to be inherent in some species. Understanding how these mechanisms work and why they differ among species is the key to predicting whether organisms will be able to adjust to the global changes. For example, the rate of environmental changes may be too rapid for evolutionary adaptation to occur in species with long generation times. Hence, any increases in physiological plasticity may represent the most crucial short-term compensatory responses. Evidence suggests that hormesis favours the increase in individual flexibility and plasticity. The extent to which a change in a certain abiotic factor or in a combination of factors will impair evolutionary fitness is in fact dependent on its/their intensity and the stage of life cycle during which an individual becomes exposed. Hormetic effects may occur and program the phenotype to tolerate significant and abrupt changes in factors, like ambient temperature and partial pressure of oxygen or carbon dioxide. Hormesis might foster resistance of organisms to extreme climate events (e.g. droughts, heat waves), whose intensification is emerging as one of the most important aspects of global changes (Jentsch et al. 2007; Vincenzi et al. 2012).

Most of what we know about the relationships between ambient temperature and oxidative stress dynamics is based on studies that looked at the effects of a constant temperature. However, results of these studies might lead to poor predictions of thermal effects on animals living in environments where the ambient temperature fluctuates largely. For example, Niehaus and collaborators (2012) found that empirical models obtained at constant temperatures poorly predicted the growth and development performance of striped marsh frogs (*Limnodynastes peronii*) at fluctuating temperatures, suggesting that extrapolation from studies conducted under artificial constant thermal conditions would lead to erroneous conclusions. There is therefore the need to design ecologically relevant treatments. To this end, it is very important to record first the thermal variation in the environment of the species under study. This is also very important for studies where animals are exposed to gradually increasing temperatures in so-called ramping assays. The effect of heat stress on the physiological response may in fact depend on the rate at which temperature increases (Sørensen et al. 2013). It is therefore important to use experimental protocols that mirror the rates of temperature changes under natural conditions. Clearly, these argumentations are also valid for other abiotic parameters, like partial pressure of oxygen or solar irradiance.

Finally, it will be very important to develop studies that are built in a life history framework in order to further our interpretation of how and why individuals, populations or species differ in how they regulate their oxidative balance in response to changes in biotic and abiotic environmental factors.

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Chapter 4
Nutritional Ecology, Foraging Strategies and Food Selection

Abstract Nutrition influences many aspects of animal life history, behaviour and physiology. Food is a source of various classes of chemicals that influence the antioxidant defences and the cell resistance to oxidative stress of animals. Not only food quality but also the investment in foraging strategies can expose animals to variable degrees of oxidative challenges and antioxidant rewards. For these reasons, the link between diet and oxidative stress has been the focus of numerous ecological studies in the last decades. In this chapter, I examine the role that oxidative stress and hormesis have played in the evolution of foraging strategies, food selection and general nutritional ecology. I then discuss how the maternal strategies of nutrient or antioxidant allocation may shape the phenotype of offspring and influence their evolutionary fitness. Finally, I examine the links among hormesis, starvation-induced stress and essentiality of nutrients.

4.1 The Pervasive Nature of Food in Life

That eating throughout life is a necessity is obvious. Nutrition influences many aspects of animal life history, behaviour and physiology, such as growth rates and adult mass gain, body condition, probability of pregnancy, overwinter survival, timing of parturition, clutch size, neonatal birth mass, survival, development of sexual traits, immunocompetence, antioxidants and oxidative stress levels (Blount et al. 2003a, b; Byrne et al. 2008; Lee et al. 2008; Cohen et al. 2009; Eeva et al. 2009; Raubenheimer et al. 2009; Sorensen et al. 2009; Deeming and Pike 2013; Schneeberger et al. 2014; Stewart 2013; Wagner et al. 2013; García-Tarrasón et al. 2014). Understanding the mechanisms underlying evolution of feeding and foraging strategies and their impact on life history and physiology is, however, not so straightforward. Getting food is a complex phenomenon that includes several processes, from its selection to ingestion, digestion and absorption of nutrients. Constraints may, therefore, act at different moments, impacting on strategies of food selection and on those of nutrient processing. Moreover, costs of feeding may

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increase when animals need to intensify their foraging activity, such as when covering long distances or increasing searching time. This is important because foraging may expose individuals to predation and may increase metabolic requirements and incur other physiological challenges (e.g., oxidative stress, inflammation) related to intense physical activity (see Chap. 5).

Animals have evolved many solutions to meet their nutritional requirements. In particular, at least two general nutritional needs have been proposed to have a pervasive effect on these solutions (Hill et al. 2008): body constituents may become damaged with time, built, consumed, discarded or replaced; hence, there is continuous need of chemical building blocks; body functions require energy to work, and energy is derived from nutrients, while to differing degrees depending on the species. However, there is one additional nutritional need that has received comparatively less attention: dietary antioxidants. Moreover, there are non-nutritional factors that may have a strong effect on nutritional ecology. One of these factors may be oxidative stress. In this chapter, I examine the links among oxidative stress, hormesis and nutritional ecology.

4.2 The Oxidative Costs of Foraging

Food availability varies spatially and temporally; hence, animals may need to perform foraging trips of different durations, wait until food becomes available again or switch to other resources. There are costs that can impose constraints to the foraging activity. For example, energetic demands may be particularly high and need solutions to mitigate them (Deerenberg et al. 1998; Kullberg et al. 2002; Speakman and Selman 2003; Vaanholt et al. 2007).

Foraging activity and diet may also change with nutrient demands, such as in the case of species that shift to eating a diet with more protein while offspring feeding. A change in diet may also influence the spatial foraging distribution, especially when different food resources are distant from each other. Alternation of foraging strategies is often observed in seabirds: for example, white-chinned petrels (*Procellaria aequinoctialis*) feed mainly on fish while doing short trips along the coasts, but feed on krill and fish while performing long pelagic foraging trips (Catard et al. 2000). It follows that the oxidative balance of foraging individuals may be the result of at least three factors: the metabolic demands of the foraging trip, the antioxidant and the macronutrient content of food. Regardless of whether oxidative stress was cause or consequence, a study on Adélie penguins *Pygoscelis adeliae* provided rare evidence showing that oxidative status and foraging strategy can be related to each other (Beaulieu et al. 2010). The oxidative status of Adélie penguins was related to their spatial distribution, rather than to the duration of their foraging trip, and therefore presumably to their diet (Beaulieu et al. 2010). However, it was also suggested that a high stress level experienced before performing a foraging trip might constrain the individual to forage close to the coast rather than in open sea (Beaulieu et al. 2010).

Oxidative costs of foraging are particularly evident in diving animals. Seals cope with regular exposure to diving hypoxia by storing oxygen in blood and skeletal muscles (both very rich in haemoglobin and myoglobin, respectively) and by limiting the distribution of oxygen to all but the most hypoxia vulnerable tissues (brain, heart), through cardiovascular adjustments (Ramirez et al. 2007). However, at the end of an apnoea bout, perfusion of ischaemic tissues can potentially increase oxidant production and oxidative stress (Vázquez-Medina et al. 2012). High constitutive activity and content of endogenous antioxidants and the oxidant-mediated activation of hormetic responses against hypoxia/reperfusion-induced oxidative stress have been proposed to be the main mechanisms that allow seals (and diving birds) to cope with the physiological demands of diving (Vázquez-Medina et al. 2012). It is, however, unexplored to what degree individuals differ in the resistance to oxidative stress, which factors explain individual variation, and how this individual variation is translated in evolutionary fitness outcomes.

Changes in oxidative stress risks may also be associated to shifts in foraging strategy, such as in honeybees *Apis mellifera*. In the first 2–3 weeks of life, honeybee workers clean the hive and feed the larvae. As they get older, they shift to foraging tasks. Foraging may be highly demanding, with forager bees flying over 8 km per day to find nectar or pollen (Winston 1987). Their metabolic rate during flight increases dramatically, reaching one of the highest values ever measured in any animal species (Suarez et al. 1996). Transition from a nurse to a forager stage, therefore, likely requires the development of several adaptations to mitigate physiological demands of flight, including oxidative stress. Williams et al. (2008) showed that compared to nurse stage, naturally occurring flight elicited an increase in flight muscle content of heat shock proteins 70 (independently from induction of hyperthermia by muscle activity) in both young (had been foraging for 2–3 days) and old (had been foraging for at least 12 days) foragers; however, catalase and non-enzymatic antioxidant capacity increased only in young bees. There was no increase in protein oxidative damage in both young and old bees. Although upregulation of antioxidant defences might have been important to avoid any increases in damage, old bees apparently did not need to do so. This might have been because old bees adjusted their foraging effort to their physiological condition, so avoiding any extra effort that might have compromised their ability to regulate their physiological state. Old bees might also have prioritised other protective mechanisms, such as those that repair or remove damaged proteins. Regardless of the mechanisms, it is evident that bees need to increase their antioxidant defences while shifting to a forager life. However, their capacity of doing so may be contingent on previous experiences. In fact, despite the typical pattern of temporal polyethism, in which young workers perform tasks in the nest while older workers forage, individuals can accelerate, delay and even reverse their behavioural development in response to changes in their colony's internal and external environments (Huang and Robinson 1996). Given that a high rate of development may be associated with increases in oxidative damage and depletion in antioxidants (see Chap. 2), the timing of transition needs to be regulated in such a way to not compromise the future foraging capacity of bees.

4.3 Food Selection: Looking for Antioxidant Rewards

Food is a source of various classes of chemicals (tocopherols, carotenoids, polyphenols; for some species, also ascorbic acid) with antioxidant properties. Although the importance of dietary chemicals in the protection against oxidative stress has likely been overestimated in some taxa (birds: Costantini and Møller 2008; Simons et al. 2012; reptiles: Olsson et al. 2008, 2009; mammals: Selman et al. 2013), their multiple physiological functions (e.g., immunostimulation, protection of brain or sperm cells) may make them important candidates as molecular modulators of life history evolution (von Schantz et al. 1999; Catoni et al. 2008a; Cohen et al. 2012).

Animals normally ingest a cocktail of antioxidants and distribute them differently across tissues, depending on the chemical properties (lipophilic vs. hydrophilic) of the antioxidant and the specific requirements of a certain tissue. However, they can also be mobilised from tissues where they are stored and redirected towards sites where they are needed (Faivre et al. 2003; Costantini and Dell'Omo 2006). It is, therefore, plausible that needs of specific antioxidants are contingent upon the life cycle stage of the individual or its condition; hence, selection of food might show some degree of flexibility in order to meet the organism's requirements. It is, for example, well known that some species can select food for self-medication because that specific food contains chemicals that help the organism to combat parasites or toxins (Clayton and Wolfe 1993; Huffman 1997; Singer et al. 2009). Self-medication might also be used to regulate oxidative balance, but this possibility remains to be examined (Beaulieu and Schaefer 2013). It is also important to highlight that taxonomic differences may exist in the requirements and relevance of some dietary antioxidants for life history traits. For example, many but not all species can synthesise ascorbic acid (Chatterjee 1973; Martínez del Rio 1997); therefore, those species that can synthesise ascorbic acid might be less constrained by availability of ascorbic acid in food than those that are unable of doing so.

Food selection is clearly an important biological process. It has been documented in many species. Some recent reports have suggested that dietary antioxidant rewards might drive food selection (Carciofi et al. 2006; Catoni et al. 2008a; Schaefer et al. 2008; Beaulieu and Schaefer 2014). For example, chemicals like carotenoids or polyphenols may function as pigments, and so the intensity of the food colouration might convey to the animal to what extent the food item is rich in antioxidants (Schaefer et al. 2008a). For chemicals that do not have pigmentary properties, selection through taste, odour or physiological feedbacks has been suggested to drive food choice (Elliott and Loudon 1987; Fromentin and Nicolaidis 1996; Pierce and McWilliams 2005), while specific appetite for pigmentary antioxidants has also been shown to occur (Senar et al. 2010). The frugivorous European blackcap (*Sylvia atricapilla*) preferentially feeds on food containing anthocyanins (a group of polyphenols). Fruits rich in anthocyanins are normally black or reflect ultraviolet radiation that birds can perceive

(Schaefer et al. 2008). Blackcaps use food colour to select berries richer in anthocyanin content; however, blackcaps select the food independently of its nutritional composition or of its content in carotenoids (Schaefer et al. 2008).

Eating food rich in anthocyanins may also improve the immune function. Blackcaps supplemented for four weeks with polyphenols were more likely to mount a humoral immune response after being injected with sheep red blood cells compared to control blackcaps (54 % vs. 30 % of birds; Catoni et al. 2008b). Recent evidence also showed that captive Gouldian finches (*Erythrura gouldiae*) feed more on seeds rich in antioxidants below than within their thermoneutral zone. Moreover, this higher intake in antioxidants reduced oxidative damage of birds. Overall, these results indicated that when facing a thermal challenge (ambient temperature below thermoneutrality in this study), animals are able to take advantage of the antioxidant properties of their food to withstand oxidative stress (Beaulieu et al. 2014).

Selection of fruits rich in polyphenols may be relevant for animals involved in high-demanding activity, such as during migration (see Chap. 5). For example, birds may mitigate oxidative stress induced by a strenuous migratory flight through consumption of food rich in polyphenols (Alan et al. 2013; Bolser et al. 2013). At stopover sites, birds select foods rich in nutrients and energy, but possibly also in chemicals that help restore antioxidant defences and so prepare the body for the next flight. Beyond selection of fruits containing polyphenols, birds might also exploit nectar as a further source of antioxidants. Cecere et al. (2011) recorded 12 opportunistic nectar-feeding species at a stopover site in the Mediterranean, with *Sylvia* warblers in particular showing a marked nectarivory. Although no parameters of antioxidant status were measured, studies on other bird species found that nectar can contain polyphenols (Zhang et al. 2012). The *Leucosceptrum canum* is a plant species that produces coloured nectar (Fig. 4.1). This colour is due to the presence of a purple anthocyanidin, the 5-hydroxyflavylium (Zhang et al. 2012). A food selection experiment showed that individuals of two species of avian pollinators, the blue-winged minla (*Minla cyanouroptera*) and the oriental white-eye (*Zosterops palpebrosa*), preferentially feed on model flowers containing coloured (richer in polyphenols) rather than uncoloured nectar (Zhang et al. 2012). Whether polyphenols in nectar improve the antioxidant defences of these birds is unknown.

Preferences for foods rich in antioxidants may change with oxidative demand, such as that associated with intense exercise or consuming high-lipid diets, although only one study directly tested this hypothesis. Alan and McWilliams (2013) found, as compared to birds fed more monounsaturated fat diets, white-throated sparrows *Zonotrichia albicollis* fed diets with more polyunsaturated fats had higher oxidative damage, did not prefer diets with more vitamin E, and had similar levels of circulating antioxidants. They suggested that upregulation of the endogenous antioxidant system (e.g., antioxidant enzymes) in response to higher oxidative damage should have occurred to result in no difference in antioxidant status between diet groups. More studies are needed that directly manipulate oxidative damage and then determining if this affects dietary preferences for antioxidants.

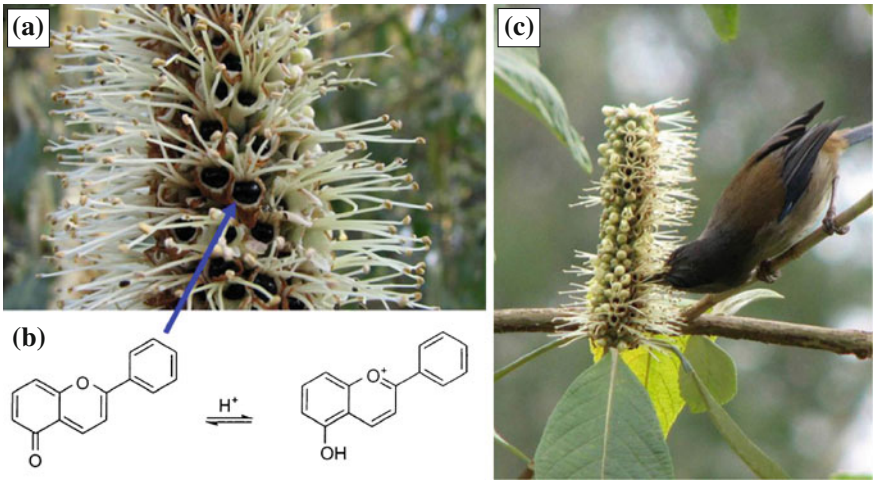

Fig. 4.1 The *Leucosceptrum canum* is a plant species that produces coloured nectar because of the presence of the pigment anthocyanidin; **a** the dark purple nectar (*arrow*); **b** the structure of the anthocyanidin. This colour has been suggested to work as a foraging signal selected because it increases the pollination efficiency through nectar visibility and palatability. **c** One avian pollinator species is the blue-winged minla (*Minla cyanouroptera*). Reproduced from Zhang et al. (2012) with permission of Wiley

Herbivore species also are notable for their capacity of selecting food. For example, abundance of herbivore insects is commonly higher on spring (or young) leaves than on mature tree leaves (Feeny 1970; Barbehenn et al. 2013). This feeding strategy is thought to increase the evolutionary fitness of insects because the higher nutritional content of spring leaves may improve their ability to grow more rapidly and to a larger body size (e.g., Hough and Pimentel 1978). However, selection of leaves may also be explained by their content in antioxidant and pro-oxidant compounds. Barbehenn et al. (2013) used larvae of the moth *Lymantria dispar* to test the effects of feeding on leaves of two contrasting host plants in the spring and summer: red oak (*Quercus rubra*), a high-quality host, and sugar maple (*Acer saccharum*), a low-quality host. On spring foliage, the combined effects of rapid consumption rate, efficient nutrient assimilation and high nutritional quality allowed larvae to grow rapidly and attain a larger body mass (Barbehenn et al. 2013). Ellagitannins, a major source of oxidative stress in the midgut of caterpillars (Barbehenn et al. 2006), were at higher concentrations in the spring than in the summer in maple leaves, but were at negligible levels throughout the growing season in oak. Production of semiquinone free radicals and oxidation of glutathione in larvae on maple remained at elevated levels in the spring and summer. In contrast, larvae that fed on oak had consistently low levels of oxidative stress (Barbehenn et al. 2013). Although feeding on leaves of different species resulted in caterpillars having different oxidative stress levels, feeding on spring leaves did not increase the capacity of larvae to cope with oxidative stress (Barbehenn et al. 2013).

Selection of food is not ubiquitous among cospecific populations or species, rather animals may just feed on what is locally available. For example, in northern and central Europe, the kestrel (*Falco tinnunculus*) is almost exclusively a vole eater, while other prey occur in its diet in seasons when the availability of voles decreases (e.g., Masman et al. 1986; Korpimäki 1985). However, individual food preferences have often been described in the scientific literature. For example, Brown (1969) and Gilraldeau and Lefebvre (1985) reported that individual pigeons selected only few seed types from a mixture of many seeds. Díaz (1996) found that the chemical composition of seeds (both seed nutritive value and seed secondary chemistry) could be important in determining food choices by avian granivores. Food preferences were also observed for various birds of prey under both captive and wild conditions (e.g., Aumann 1988; Smallwood 1989; Purger 1998; Duke et al. 1996). In particular, it has been suggested that individual feeding habits might be consistent with time in kestrels (Costantini et al. 2005). Similarly, but in a passerine species, Catoni et al. (2011) found that garden warblers *Sylvia borin* differed consistently in their food choice for carotenoids, which were the only food component whose contents changed during the course of the experiments.

4.4 Effects of Food on Oxidant and Antioxidant Status

4.4.1 Food Quality

Diet is a source of antioxidants, such as vitamin E, carotenoids and polyphenols, which may help the organism to mitigate the damaging action of reactive species (Surai 2002; Catoni et al. 2008a, b). The diet is also a source of substances that do not have antioxidant properties, but that may indirectly affect the organism's resistance to oxidative stress. For example, protein intake might affect the synthesis of some antioxidants (e.g., thiols, enzymes), whose production depends on the availability of amino acids (Li et al. 2014). The fatty acid and protein content of diet may also affect the oxidative status because the various kinds of fatty acids and proteins differ strongly in their susceptibility to oxidative damage and in how they affect the membrane composition and so its susceptibility to damage (Hulbert 2005; Pamplona and Barja 2007; Yamato et al. 2007).

Although diet certainly has an effect on the oxidative balance, the direction of this effect may be difficult to predict. For example, increases in the concentration of a given dietary antioxidant may result in no net reduction in oxidative damage because any potential beneficial effects are offset by compensatory reductions in antioxidant enzymes. For example, long-term vitamin C supplementation to laboratory mice reduced expression of several genes linked to free radical scavenging, with no net effect on various oxidative damage parameters (Selman et al. 2006). Similarly, carotenoid supplementation reduced activity of superoxide dismutase and glutathione peroxidase in characins *Hyphessobrycon callistus* (Wang et al. 2006).

Interaction between dietary and enzymatic antioxidants is, therefore, relevant for the regulation of oxidative balance.

Cohen et al. (2009) measured circulating antioxidant levels in 95 bird species and examined their associations with diet. Non-enzymatic antioxidant capacity of plasma and uric acid concentration were positively associated with invertebrate consumption and seed-to-fruit ratio, while circulating carotenoids were negatively associated with invertebrate consumption. Surprisingly, among-species variation in vitamin E was not explained by diet (Cohen et al. 2009). Results were consistent with or without control for variation in life histories and phylogeny. The authors suggested that this might have been because their diet classification better reflected the protein content rather than the micronutrient levels. Another potential reason was that circulating levels of vitamin E and carotenoids depended more strongly on among-species variation in patterns of metabolism (e.g., usage, storage, recycling; Cohen et al. 2009). However, it should not be forgotten that both vitamin E and carotenoids are lipophilic compounds that can be stored in tissues like liver. Therefore, storage might explain why it is not found any direct relationship between concentration of both compounds in diet and in blood. We also have to look at within-species variation because there may be additional factors explaining variation in antioxidants that an interspecific comparative approach does not allow to detect. The analysis of within-species patterns of variation in antioxidant levels in relation to diet may allow a more accurate analysis of mechanisms regulating absorption from food and usage of non-enzymatic antioxidants.

Conversely to vitamin E and carotenoids, vitamin C may be obtained from food or synthesised endogenously. For example, guinea pigs (*Cavia porcellus*) are mammals unable of synthesising vitamin C; hence, the availability of this antioxidant in the diet might be limiting for body functions in this or other species that lost the ability of synthesising vitamin C. Guinea pigs need 0.5 mg of vitamin C per day in order to avoid the development of scurvy (National Research Council 1978). Barja et al. (1994) fed laboratory guinea pigs for 5 weeks with three different levels of vitamin C in the diet: 33 mg of vitamin C per day, which means that animals ingested 0.54–1.1 mg of vitamin C per day (marginal deficiency); 660 mg of vitamin C per day, which is considered to be the standard optimum level; 13,200 of vitamin C per day, which was selected to see if supplementing the diet with a level of vitamin C much higher than that of a standard diet would be beneficial in terms of reduction in liver oxidative damage. Compared to guinea pigs maintained on a diet marginally poor in vitamin C content, animals fed 660 mg of vitamin C per day showed lower levels of protein carbonyls, malondialdehyde and thiobarbituric acid reactive substances and higher levels of glutathione reductase activity (Barja et al. 1994). However, the diet regime did not affect the activity of superoxide dismutase, catalase, glutathione peroxidase and cytochrome oxidase. Further supplementation with 13,200 of vitamin C per day also reduced damage to proteins and lipids, but also decreased glutathione reductase and uric acid. Moreover, both low and very high levels of vitamin C decreased body mass and unsaturation of fatty acids in cell membranes (Barja et al. 1994). These findings highlighted the protective role of vitamin C against

oxidative damage and the complexity of its effects on the organism; however, these findings are limited to animals maintained under good husbandry conditions and non-reproducing state. The use and the effects of vitamin C might differ under more demanding conditions, where the need of antioxidants is expected to be higher.

Tocopherols are another group of antioxidants that may occur in limited supply in the diet, hence potentially limiting some body functions, such as protection against oxidative damage (Giraudeau et al. 2013). de Ayala et al. (2006) provided evidence that tocopherols may actually be limiting for wild animals. They manipulated the brood size in barn swallows (*Hirundo rustica*) in order to reduce or increase competition among siblings. They then supplemented nestlings with two different physiological doses of tocopherols (decided on the basis of their content in prey and daily food intakes of nestlings) and compared them to control nestlings. Supplementation of tocopherols at the lower dose increased nestling body mass and condition, and growth of feathers compared to the other two experimental groups. The effect of tocopherols was only evident from days 6 to 10 of age, when maximum growth rate was achieved. The effects of tocopherols were, however, independent from sibling competition, suggesting that the effect of tocopherols was mostly dependent on their availability in the prey (de Ayala et al. 2006). Another supplementation trial of physiological doses of tocopherols and carotenoids, administered separately from each other, did not achieve the same results as those of Ayala and collaborators (2006). Larcombe et al. (2010) found that supplementation of tocopherols or carotenoids to nestling blue tits (*Cyanistes caeruleus*) did not improve their growth, nor their body condition. Moreover, plasma malondialdehyde (end product of lipid peroxidation) and chest plumage colouration were unaffected by the treatment. Although the antioxidant content of prey varies seasonally and among prey species, differences between the two above studies might be explained to some extent, and at least for tocopherols, by their availability in the diet. In fact, while the average α-tocopherol content of barn swallows prey was 3.93 μg g^{-1} (Ninni 2003; de Ayala et al. 2006), that of blue tit prey varied from <3.9 μg g^{-1} to ca. 28 times higher depending on the Julian date (Arnold et al. 2010). It may, therefore, be plausible that the effects of tocopherols were stronger for barn swallows because vitamin E is often in more limited supply for this species than for blue tits.

Deficiency of vitamin E has been reported to have various detrimental effects on vertebrates, such as dysfunction of the parts of the nervous system that coordinate movement or induction of myopathic lesions (Juan-Salles et al. 2003; Thomas et al. 1993). Increase in oxidative damage or reduction in damage repair systems might underlie the negative effects of vitamin E deficiency. In fact, poor supply of vitamin E may promote vitamin C deficiency, hence undermining the antioxidant protection. For example, Lebold et al. (2013) provided to zebrafish (*Danio rerio*) a diet without or with vitamin E (500 mg α-tocopherol kg^{-1} diet) for 1 year. For the last 150 days of the experiment, dietary ascorbic acid concentrations were decreased from 3,500 to 50 mg kg^{-1} diet, and the fish were sampled periodically to assess ascorbic acid concentrations. The ascorbic acid

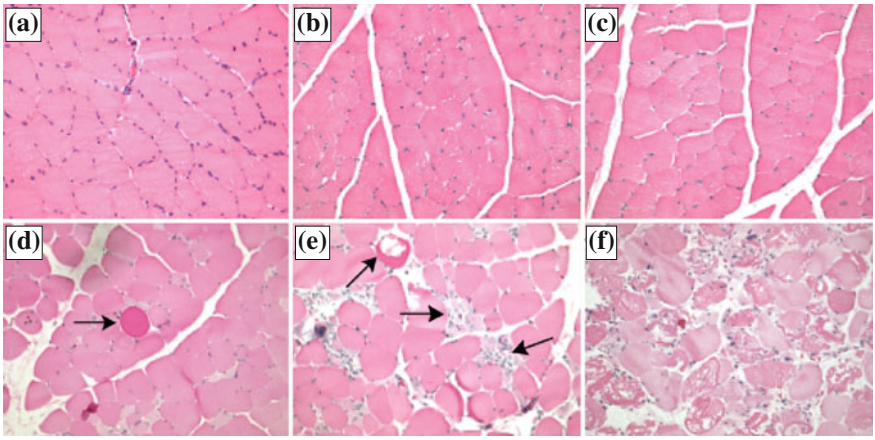

Fig. 4.2 Photomicrographs of quadriceps muscle from guinea pigs (*Cavia porcellus*) fed four different experimental diets: control, ascorbate deficient, selenium deficient and double deficient (lacking both ascorbate and selenium). Figures (**a**–**c**) show muscle from control, selenium-deficient, and ascorbate-deficient animals, respectively. Figures (**d**–**f**) show muscle from doubly deficient animals. The *arrow* in figure (**d**) points to an apoptotic cell, and the arrows in figure (**e**) point to areas of apoptotic and dying cells. Figure (**f**) shows extensive necrosis of the tissue section. Reprinted from Hill et al. (2009) with permission from Elsevier

depletion curves were faster in the vitamin E-deprived fish compared with non-deprived fish. At the end of the experiment, vitamin E-deprived fish had threefold more oxidative damage, showed sluggish behaviour and signs of degenerative myopathy of the skeletal muscle. It is premature to translate these results to a natural situation, where availability of vitamin E is unlikely to ever be exactly zero. However, results of this study suggested that it is very important to investigate the interactions among multiple antioxidants in order to have a better evaluation of the response to oxidative stress (see Chap. 1). The importance of the molecular interactions among antioxidants is, for example, supported by studies where combined antioxidant deficiencies increased oxidative muscle damage more than single deficiencies (Fig. 4.2; Hill et al. 2009) or supplementation of vitamin C enhanced vitamin E concentration, while reducing oxidative damage (Betancor et al. 2012). However, we should not lose sight of the fact that any beneficial effects of having large amounts of vitamin E available do not necessarily come through its antioxidant properties. Selman et al. (2008) showed that supplementation of vitamin E significantly increased median lifespan of mice by 15 % relative to unsupplemented controls and also increased maximum lifespan. Lymphocyte and hepatocyte oxidative DNA damage and hepatic lipid peroxidation were, however, unaffected by supplementation. Using a cDNA macroarray, Selman et al. (2008) showed that genes associated with xenobiotic metabolism were significantly upregulated in the livers of mice, suggesting that vitamin E may initially be metabolised as a xenobiotic. The absence of any significant effect on oxidative damage suggests that the lifespan extension observed was not mediated

via any antioxidant properties of vitamin E. Rather, the effect of vitamin E on longevity was likely mediated via upregulation of cytochrome p450 genes (Selman et al. 2008), possibly reflecting an hormetic effect.

Carotenoids, like tocopherols, must also be obtained by the diet; they are considered to be in limited supply for wild animals, and as such may constrain important physiological functions in which they are involved (e.g., immune response, antioxidant protection). However, two meta-analyses that evaluated the effect size of experimental supplementation trials of carotenoids or of correlative studies showed that having a higher availability of carotenoids did not result in significant increases in resistance to oxidative stress or induced very modest effects at best (Costantini and Møller 2008; Simons et al. 2012). The lack of a significant antioxidant benefit of carotenoids also emerged when the supplementation was performed over demanding phases of an animal's life. Growth and development are, for example, one such demanding period because the metabolic activity is intense, and endogenous antioxidant mechanisms are not mature yet (see Chap. 2). Hence, an increase in intake of carotenoids may carry some benefits in terms of better protection against oxidative stress. Results of various studies did not support such a scenario. For example, Costantini et al. (2007) administered carotenoids to wild nestling kestrels *Falco tinnunculus*. The supplementation started when nestlings were 7–8 days old, in order to avoid disturbance during the hatching period. Each bird was supplemented with a dose of about 4 mg of lutein plus zeaxanthin, which are the only carotenoids identified in the tissues of kestrel (Casagrande et al. 2006). The solution was orally administered according to the sketch in Fig. 4.3. Nestlings within each brood were randomly assigned to the control or the carotenoid-supplemented group. Circulating carotenoids in supplemented nestlings increased about 1.5-fold compared to the control and pre-treatment levels by the end of the supplementation period on day 17 (Fig. 4.3). The increase in serum carotenoids was within the physiological range of circulating carotenoids previously measured in free-living nestling or adult kestrels (Casagrande et al. 2006, 2007; Costantini et al. 2006). The increase in carotenoids, however, had no significant effects on oxidative damage (hydroperoxides) and on the serum non-enzymatic antioxidant capacity (Fig. 4.3), nor did it have an effect on body mass and body condition of nestlings. Therefore, carotenoid availability did not appear to limit the oxidative stress resistance and growth of nestling kestrels. It cannot be ruled out that the demands of chicks and so effects of carotenoids might vary among chicks or broods, depending on local environmental conditions or quality/reproductive investment of adults. For example, in many altricial birds, asynchronous hatching creates different castes of progeny (the so-called "structured" family; Hall et al. 2010). Older, "core" offspring and later hatched "marginal" offspring consequently experience contrasting levels of resource availability during development (Hall et al. 2010). The level of lipid peroxidation (malondialdehyde) in plasma was found to be similar between antioxidant-supplemented and control chicks, but among marginal offspring those that received an antioxidant supplement had lower plasma malondialdehyde. These data indicated that having a higher intake of antioxidants might be beneficial only under very

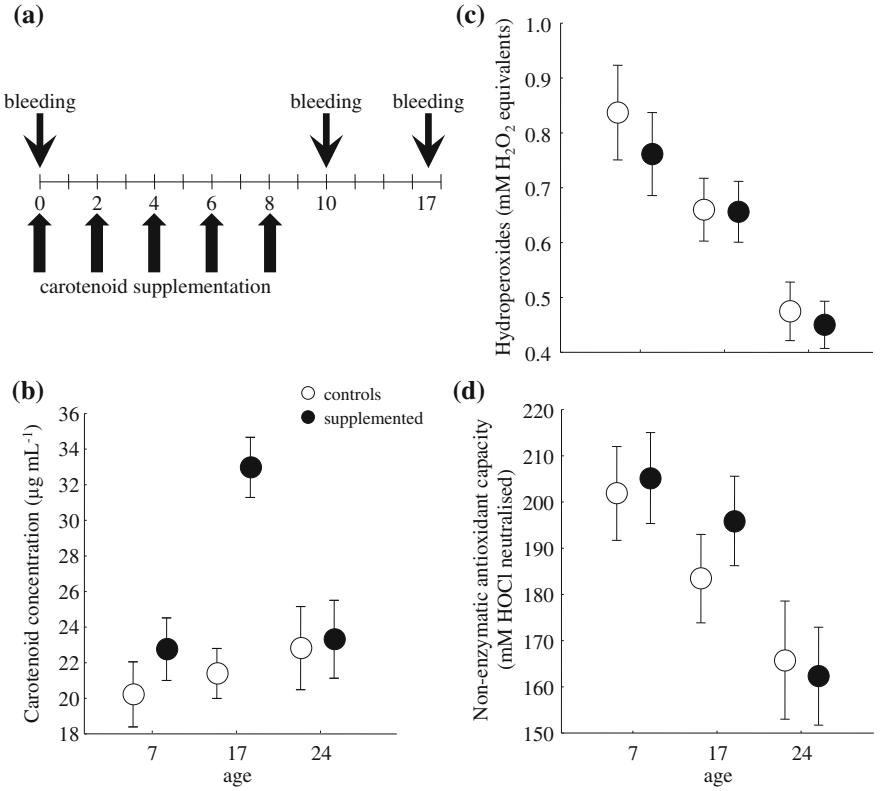

Fig. 4.3 **a** Sketch of the timing of different parts of the experiment conducted by Costantini et al. (2007). Numbers indicate the day. At day 0, nestling kestrels were 7–8 days old. **b** Circulating carotenoids increased in supplemented nestlings, but returned to basal levels at the end of the experiment; **c** supplemental carotenoids did not affect the level of oxidative damage as measured by serum hydroperoxides; **d** supplemental carotenoids did not increase the serum non-enzymatic antioxidant capacity. Values are shown as mean ± standard error. Reproduced with minimal alterations from Costantini et al. (2007) with permission

stressful conditions. Antioxidant supplementation did not affect, however, nestling survival to fledging (Hall et al. 2010).

Dietary intake of proteins or fatty acids has also an important impact on oxidative balance. Alan and McWilliams (2013) found that white-throated sparrows (*Zonotrichia albicollis*) fed a high-protein diet had higher circulating uric acid and plasma non-enzymatic antioxidant capacity but also higher production of early oxidative damage derivatives (hydroperoxides) than birds fed a low-protein diet. They then presented half of the birds from each of the high- or low-protein groups with a high intake of polyunsaturated fatty acids because these compounds are highly sensitive to peroxidation. Consumption of fatty acids did not influence the plasma non-enzymatic antioxidant capacity in the birds, but caused higher oxidative damage irrespective of previous protein intake.

The effects of single classes of antioxidants or macronutrients may also be contingent on the relative concentrations in food of other chemicals. Plummer et al. (2013) examined the carry-over effects of winter food supplements on egg production in wild populations of blue tits (*Cyanistes caeruleus*). Over three consecutive years, birds were provisioned with fat, fat plus vitamin E or remained unfed (controls). Conversely to predictions, the provision of fat in winter resulted in smaller relative yolk mass in larger eggs and reduced yolk carotenoid concentrations in early breeders. However, these effects were not seen in birds provisioned with fat plus vitamin E. Laying date, clutch size, egg mass and yolk vitamin E concentrations were not significantly affected by winter-provisioning treatment (Plummer et al. 2013). This result might be explained by birds having higher oxidative damage when feeding on fat only; such oxidative damage facilitated by the propensity of fatty acids to peroxidation would be mitigated by the presence of vitamin E. Limitation of lipid peroxidation might have occurred in the body, but it cannot be excluded that vitamin E protected oxidation of fat induced by exposure to air before being eaten by the birds, hence allowing birds to avoid ingesting lipid peroxides.

4.4.2 Food Restriction

Many animal species need to cope with long periods of fasting while incubating, waning, migrating or moulting (Mrosovsky and Sherry 1980; Cherel and Le Maho 1985; Cherel et al. 1988a, b; McCue 2010; McWilliams et al. 2004; Vázquez-Medina et al. 2011). During the fasting period, substrates of metabolic activity change from carbohydrates (phase I) to lipids and proteins (phase II) and, in the end, to an enhanced protein catabolism (phase III). Concomitantly with these metabolic changes, animals face a high body mass loss, which becomes critical in the phase III of fasting. Of the species that experience fasting during the breeding phase, seabirds are of particular physiological interest given that fasting may last up to 3–4 months in some species (Cherel and Le Maho 1985; Cherel et al. 1988a). In birds, besides body mass loss, long-term fasting may also determine several physiological adjustments, such as the increase in circulating stress hormones, plasma uric acid concentration, or stress proteins 60 and 70 or decreases in prolactin (Cherel et al. 1988a; Bourgeon et al. 2006; Spée et al. 2010; Thierry et al. 2013). Fasting can also promote production of superoxide anion, hydrogen peroxide, and oxidative damage or alter antioxidant status in a variety of mammal species, such as humans, seals or rodents (Richelle et al. 1999; Sorensen et al. 2006; Vázquez-Medina et al. 2011). However, effects of fasting on oxidative balance are quite variable, possibly depending on the phase of fasting. Geiger et al. (2012) showed in mallards (*Anas platyrhynchos*) that plasma oxidative damage decreased while fasting and then increased to pre-fasting levels when the animals were refeed. Plasma non-enzymatic antioxidant capacity also tended to decrease while fasting, but increased faster than damage while refeeding.

Northern elephant seals (*Mirounga angustirostris*) naturally experience prolonged periods (up to three months) of food and water deprivation while breeding, nursing, moulting or weaning, with no apparent detrimental effects (Ortiz et al. 1978; Ortiz et al. 2006). Moreover, elephant seals remain normothermic during their post-weaning fast and maintain a high metabolic activity (Vázquez-Medina et al. 2010). Although the fasting seals maintained high metabolic rate and consumed no dietary antioxidants, it was found that oxidative damage did not increase in fasting northern elephant seals. Vázquez-Medina et al. (2010) compared early (2–3 weeks post-weaning) and late (7–8 weeks post-weaning) fasted seals. Fasting induced an increase in the expression of the reactive species-producing protein Nox4, along with NADPH oxidase activity. In contrast, neither tissue nor systemic parameters of oxidative damage or inflammation increased with fasting (Vázquez-Medina et al. 2010). Muscle antioxidant enzymes increased 40–60 % with fasting in parallel with an increase in muscle and red blood cell antioxidant enzyme activities. These data suggested that fasting seals upregulated their antioxidant enzymes to suppress systemic and tissue levels of oxidative damage (Vázquez-Medina et al. 2010).

Findings on mallards and seals suggested that any costs associated with fasting might come through a massive use of antioxidant defences rather than through an increase in production or accumulation of cell oxidative damage. Therefore, species that experience fasting during their normal life cycle could have evolved strategies to save antioxidants in order to mitigate costs associated with their use and search. For example, a commonly observed behaviour in burrowing petrels is spontaneously deserting the egg after long fasting without any risk for the egg (Jouventin et al. 1985). The parental decision to desert the egg is related to extent of body mass loss (Chaurand and Weimerskirch 1994a, b), although this behaviour is not completely understood yet. Stress hormones could be candidates as modulators of the link between deserting behaviour and oxidative balance. Glucocorticoids increase during the fasting period (Cherel et al. 1988a), affect oxidative status (Costantini et al. 2011), and may induce behavioural adjustments to stressful events through the emergency life history stage (Wingfield et al. 1998). The observation that circulating antioxidants tend to be lower in blue petrels in lower body condition (proxy of fasting) during incubation (Costantini and Bonadonna 2010), suggesting that the loss of antioxidant capacity might be one additional mechanism that contributes to positive selection of the deserting behaviour. The strategy of deserting the nest might allow to save antioxidants; this would be compatible with the long lifespan of burrowing petrels (until 30 years) because long-lived species are predicted by life history theory to invest strongly in self-maintenance mechanisms.

Cave-dwelling organisms are another impressive example of adaptation to fasting because they have to cope with fasting periods that can extend from a month to a year (Issartel et al. 2010). A comparison of physiological and metabolic responses to 42 days of fasting followed by 10 days of refeeding between two

populations (one subterranean and one epigean) of the Pyrenean newt *Calotriton asper* showed that cave colonisation induced a decrease in metabolism together with a higher capacity to accumulate energy reserves and, therefore, to withstand unpredictable fasting periods (Issartel et al. 2010). This might also have paid in terms of higher resistance to oxidative stress given the low metabolic intensity and the stimulatory effects of fasting on self-maintenance mechanisms.

Short episodes of starvation have also been suggested to be influential for the body development when experienced in the early life. Reduction in caloric intake for a short time during development might program the individual to be more resistant against stress at adulthood through a hormetic response (Mangel 2008; Mattson 2008). Although some effects of caloric restriction were likely exaggerated by using overweight controls, some reports showed unbiased stimulatory effects of caloric restriction on various endpoints (e.g., oxidative damage) in both captive and wild animals (e.g., Noguera et al. 2011).

Starvation may also have hormetic effects on longevity. For example, glucose restriction in early life promotes mitochondrial metabolism, causing increased formation of reactive species, upregulation of catalase and hormetic extension of lifespan in adult worms *Caenorhabditis elegans* (Schulz et al. 2007). An opposite effect of early-life glucose restriction on longevity was found when worms were supplemented with antioxidants (Schulz et al. 2007), which likely caused down-regulation of endogenous protective mechanisms.

Evidence for hormetic effects of starvation was also reported in birds. A day of food shortage was found to reduce oxidative damage to proteins and DNA in chicks of a seabird species (yellow-legged gulls, *Larus michahellis*), but it did not affect lipid oxidative damage nor plasma non-enzymatic antioxidant capacity (Noguera et al. 2011). However, many seabird species may be in crisis over extended food shortage periods, experiencing poor breeding numbers and survival rates together with high stress levels (Kitaysky et al. 2007, 2010).

Caloric restriction may also enhance the organism's capacity to withstand other kinds of stressors. For example, Kailasam et al. (2011) found that rotifers (*Brachionus plicatilis*) previously exposed to caloric restriction lived longer and had higher mRNA levels of manganese superoxide dismutase and catalase than controls when exposed to a chemical generator of reactive species. While it is well established that dietary restriction tends to extend lifespan, various studies also showed that it may impair reproduction (Merry 1995; Masoro 2005). The opposite effects of dietary restriction on lifespan and fecundity, respectively, have long been thought to reflect a trade-off in allocation of resources between reproductive effort and somatic maintenance (Shanley and Kirkwood 2000). However, recent evidence suggested that survival and fecundity need not always to be traded off under dietary restriction, suggesting that hormetic effects induced by the mild stress of dietary restriction may stimulate multiple traits simultaneously. Adler et al. (2013) examined the effects of dietary restriction on survival and reproduction in both sexes and across a range of environments in the neriid fly *Telostylinus*

angusticollis. They found that the lifespan–reproduction trade-off was both context- and sex-dependent. Although dietary restriction extended lifespan in both sexes by 65 % and rendered females completely infertile, costs of dietary restriction on male fecundity were subtle and evident only in particular environmental combinations (Adler et al. 2013).

Although a short episode of caloric restriction might work as a mild oxidative stressor capable of stimulating an hormetic response, there is no definitive proof yet that it is so because only a control and a diet-restricted group are usually compared. We, therefore, need a comparison among more experimental groups in order to fully demonstrate the biphasic nature of the hormetic response to dietary restriction. It is also very important to evaluate whether an hormetic response is triggered only when overall caloric intake is restricted or when only a certain class of nutrients is restricted. In fact, restriction of intake of specific nutrients has also been shown to induce stimulatory responses (Piper et al. 2005), but increases in oxidative damage have been observed when the nutrition was inadequate (malnutrition; Feoli et al. 2006). Restriction of intake of specific nutrients in early life may have pervasive, long-lasting effects on adult antioxidant protection. For example, zebra finches (*Taeniopygia guttata*) experiencing a short period of low-quality nutrition (carotenoids and vitamins) during the nestling period had a twofold reduction in plasma levels of carotenoids, vitamins A and E at adulthood (Blount et al. 2003a). These findings were interpreted as evidence of a long-term impairment in the capacity to assimilate dietary antioxidants through a reduction in compounds like lipoproteins, which are responsible for uptake and transport of lipophilic antioxidants (Blount et al. 2003a). A lower capacity of assimilating antioxidants from diet may be expected to exacerbate trade-offs that occur from their investment in antioxidant protection, morphological development and production of sexual signals.

In the experimental testing of the effects of nutrition on oxidative balance, it is also critical to assess the effects of nutrition on growth rate and match/mismatch of early and adult diet because (1) growth has a direct impact on the oxidative balance (see Chap. 2) and metabolic rate (Criscuolo et al. 2008) and (2) setting up an adult phenotype to exploit or tolerate better certain nutrients might make it unprepared to metabolise different nutrients, which may carry metabolic costs. Finally, we need studies that assess the effects of dietary restriction over important stages of life. For example, fasting may occur while breeding, migrating or moulting. Moulting, in particular, might be very interesting to look at because metabolic demands of moulting and fasting may be conflicting. While moulting requires high consumptions of oxygen and amino acids for keratin synthesis, fasting is characterised by a reduction in energy consumption and intake of nutrients, like amino acids themselves. Moreover, the loss of feathers in birds may carry increased thermal demands because of a decrease in body insulation. Optimisation of investment among these competing functions is, therefore, required.

4.5 Antioxidants and Nutrients as Maternal Programming Tools of Offspring Oxidative Balance

4.5.1 Dietary Antioxidants

Royle et al. (1999) showed that two dietary compounds with antioxidant properties (tocopherols and carotenoids) could be deposited differentially among eggs, depending on their laying sequence. This discovery led to several hypotheses related to the importance of maternal antioxidants for the health of their offspring. Given the link between oxidative stress and testosterone metabolites, and that the level of yolk testosterone in eggs within a clutch may be negatively correlated to that of yolk dietary antioxidants, Royle et al. (2001) hypothesised that these two maternal factors have complementary, but opposing effects: testosterone induces and dietary antioxidants mitigate oxidative stress, respectively. Royle et al. (2001) also suggested that the effect of testosterone on offspring hatched from last-laid eggs would be detrimental if food were scarce due to reduced availability of dietary antioxidants.

Effects of maternally derived antioxidants are not strictly limited to the embryonic period, but persist after hatching. The hepatic concentration of carotenoids soon after hatching was found to be 29 times higher in chicks (*Gallus gallus domesticus*) from mothers that were supplemented with carotenoids than those that were not (Karadas et al. 2005). Importantly, the higher hepatic concentration of carotenoids in chicks from supplemented mothers persisted for the first week of life even when chicks were not provided dietary antioxidants (Karadas et al. 2005). Although the long persistence of maternal carotenoids in chicks might be advantageous in terms of protection against post-hatching oxidative stress (Karadas et al. 2005), a study on yellow-legged gull (*Larus michahellis*) chicks showed a higher level of oxidative damage in 9-day-old males but not females hatched from eggs enriched with carotenoids than chicks from eggs non-enriched with carotenoids (Saino et al. 2011). Moreover, chicks from first-laid eggs enriched with carotenoids had higher oxidative damage than chicks from first-laid control eggs. This pattern was opposite in chicks from last-laid eggs where oxidative damage was higher in controls. Finally, carotenoid enrichment of eggs did not affect chick oxidative damage at hatching (Saino et al. 2011). Therefore, the effects of carotenoids in chicks in the first days of life are not so straightforward to predict, likely because of the interactions of multiple factors, such as the laying order of the egg, the maternal condition, the various physiological functions of carotenoids and the species (e.g., see also Hall et al. 2010). For example, carotenoids may enhance the immune activity, which in turn may increase the basal production of pro-oxidants and damage (Costantini and Møller 2009). Moreover, carotenoids may enhance growth rate (Orledge et al. 2012), which in turn may increase oxidative damage (see Chap. 2). Hence, the role of carotenoids and, possibly, of the other dietary antioxidants in the early life of birds might be complex and important in the resolution of growth and development

trade-offs (Saino et al. 2011). Such complexity is not peculiar of carotenoids. Protective effects of maternal vitamin E supplementation on chicks (*Gallus gallus domesticus*) may last at least until 12 days of age (Lin et al. 2005). Brain oxidative damage (measured as thiobarbituric acid reactive substances) decreased as the maternal supplementation of vitamin E increased ($r = -0.909$); basal production of reactive species decreased in chicks from supplemented mothers as compared to controls; effects on activities of antioxidant enzymes were variable across tissues and doses of vitamin E administered to the mothers (Lin et al. 2005). However, effects of vitamin E on nestling were found to vary among species (e.g., de Ayala et al. 2006; Larcombe et al. 2010).

4.5.2 Nutrients

Nutrients, such as fatty acids and amino acids, used by organisms to construct cell membranes differ in their molecular susceptibilities to oxidation. Hence, animals may actively create their cell compositions in order to make them more resistant to oxidative stress (e.g., use of fatty acids, Hulbert et al. 2007; Buttemer et al. 2008). Having the capability of actively controlling nutrients may also be important when the female is depositing them into the egg. In this way, females could directly influence the susceptibility of embryo tissues to oxidative stress (Surai et al. 1996). For example, the n-6 polyunsaturate, arachidonic acid, forms between 8 and 19 % of the phospholipid fatty acids of egg yolk of the northern gannet *Morus bassanus*, the great skua *Catharacta skua*, the American white pelican *Pelecanus erythrorhynchos* and the double-crested cormorant *Phalacrocorax auritus* (Surai et al. 2001). In pelicans and cormorants, such yolk composition is consistent with the consumption of freshwater fish in which arachidonic acid may be a significant acyl constituent. In contrast, this finding is more difficult to explain for the gannet and skua because they largely feed on marine fish with a low arachidonic acid content. These results suggested that female birds may actively control fatty acid deposition in the egg (Surai et al. 1996).

Although mothers might have some control over which nutrients are passed to their offspring, a prolonged period of poor maternal diet may have detrimental consequences for the offspring (including increased oxidative stress), especially in those species like mammals where the foetus is fed by the mother over a prolonged period. These effects may come through a state of malnutrition of offspring and/or the costs induced by a post-natal catch-up growth (see Chap. 2). For example, a reduction in longevity has been shown to occur in offspring of protein-restricted pregnant rats (Jennings et al. 1999). The reduction in longevity might be explained by an accelerated rate of telomere shortening associated with: growth retardation during the foetal life followed by post-natal catch-up growth (Jennings et al. 1999); or increase in oxidative damage associated with normal growth (Langley-Evans and Sculley 2005), which were both observed in rats from protein-restricted pregnant mothers. It is clear that the temporal window during

which mothers experience diet restriction is important for offspring. For example, Tarry-Adkins et al. (2008) compared the oxidative profile among three- and twelve-month-old offspring of rat mothers from three experimental groups: controls in which mothers were always fed nutritionally adequate diets; protein restriction of mothers during gestation followed by adequate nutrition during lactation that allowed for rapid catch-up growth of offspring; and protein restriction of mothers only during lactation. Offspring from mothers, whose protein intake was restricted during lactation, had lower damage to DNA, higher activities of antioxidant enzymes (e.g., superoxide dismutase, catalase) and fewer short telomeres than the other groups (Tarry-Adkins et al. 2008). This pattern of variation in oxidative profile among groups was certainly driven to some degree by the rate of growth, which was slow during lactation for offspring of mothers protein-restricted during lactation. Conversely, mothers protein-restricted during gestation produced offspring with much faster growth rates, which apparently incurred physiological costs for offspring (Tarry-Adkins et al. 2008).

We have also some evidence that longevity and oxidative stress resistance resulting from caloric restriction of adults can be passed on to subsequent generations. Kaneko et al. (2011) found that rotifers (*Brachionus plicatilis*) under caloric restriction lived 50 % longer than those fed ad libitum and had enhanced oxidative stress resistance and increased mRNA levels of catalase and manganese superoxide dismutase. The daughters from the calorically restricted mothers lived 20 % longer than those from the mothers fed ad libitum regardless of food-rich and caloric restriction conditions for the daughters. Furthermore, the daughters from the caloric restriction-treated mothers were endowed at birth with a higher ability to resist oxidative stress and the increased mRNA levels for catalase, but not for manganese superoxide dismutase. Moreover, daughters from the caloric restriction-treated mothers laid eggs richer in catalase and manganese superoxide dismutase.

4.6 On Nutrients, Toxins, Nutritional Hormesis, Essentiality and the Bertrand's Rule

Compounds like fatty acids or proteins are normally categorised as nutrients, while compounds that elicit defensive responses like alkaloids or terpenoids are termed toxins. This classification is certainly operationally valuable for many purposes, but as highlighted by Raubenheimer and Simpson (2009), labelling compounds in this way also presents the danger that the dynamic nature of biology is obscured by a static one-to-one mapping between the chemical structure of compounds and their functional consequences. It follows that the dichotomy between toxin and nutrient may not be always realistic, while a distinction between toxic and nutritious or deleterious and beneficial may be more productive and closer to biological reality (Berenbaum 1995; Raubenheimer and Simpson 2009). The

dichotomy between toxin and nutrient was already highlighted by Ames et al. (1990), which used the term *natural pesticides* to refer to chemicals that naturally occur in food and that may have toxicological significance. Given this dual nature of many dietary chemicals, being toxic or nutritious, it has been proposed that the intake of dietary compounds may induce hormetic responses (Hayes 2007; Mattson 2008; Calabrese and Mattson 2010). It has also been suggested that the benefits of a diet rich in phytonutrients may stem more from their pro-oxidant rather than antioxidant properties. These chemicals would be responsible for the induction of a mild injury to the mitochondria, which would activate self-maintenance mechanisms through the signalling activity of reactive species produced by the mitochondria themselves (*mitohormesis hypothesis*; Tapia 2006).

The shape of an hormetic response is quite similar to that typical of the so-called essentiality or Bertrand's rule for essential nutrients (Bertrand 1912; Raubenheimer et al. 2005; Raubenheimer and Simpson 2009). Essentiality of the nutrient refers to a dose–response relationship for such substances that is characterised by negative evolutionary fitness consequences when there is not enough and when there is too much, as for example with sodium chloride or iodine (Chapman 1998; Kefford et al. 2008). The distinction between hormesis and essentiality is, however, not always so straightforward. The so-called essential elements do occur in natural habitats, and some of them are likely to be never zero in an organism. It is, therefore, difficult to define which the control level should be. If we refer to the control level as the concentration that a certain element has in the environment in the absence of human activities, this raises many difficulties because the human activity has a global impact, and elements can *per se* vary naturally across time and space (Kefford et al. 2008). If we rather take the statistical mode of the distribution within a population as the control level, it might be possible for some essential elements to show J- rather than U-shaped dose–response relationships, hence falling in the hormetic realm. This is because the detrimental effect of deficiency induced by the scarcity of a certain element would never be as much as that of toxicity induced by the element when occurring at high concentrations if we demonstrate the availability of that element to be never zero. Clearly, such a scenario cannot be generalised, while it should be limited to the nutrient, environment and species under study.

There is good evidence that non-essential dietary compounds may generate typical hormetic responses (Stipanovic et al. 1986; Calabrese and Blain 2005; Hayes 2007; Mattson 2008; de la Celorio-Mancera et al. 2011). Nutrients generating hormetic responses (sometimes referred to as hormetins sensu Rattan 2008) can work through one or more pathways related to stress responses or cellular maintenance and repair (Ali and Rattan 2006). A classic example of hormetic effects related to nutrients comes from studies on dietary antioxidants (carotenoids), which suggested that these compounds can have beneficial effects at low doses, but are detrimental above a certain threshold dose. Ethanol is another compound that many animal species get from food that can induce hormetic responses. This is well exemplified by an experiment on five species of *Drosophila* fruit flies (Holmes et al. 1980). When adult fruit flies are exposed to differing

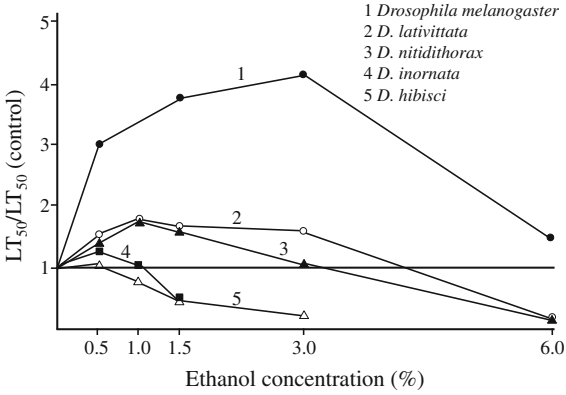

Fig. 4.4 Adult survivorship expressed as the ratio LD_{50} (lethal dose at which 50 % of test subjects exposed die) for various *Drosophila* species plotted against ethanol concentration. The thresholds for each species are indicated by the *intercept of the line* plotted for a ratio of one with the survivorship curves (Holmes et al. 1980; Parsons 1981). The width of hormetic zone for the response of longevity to ethanol differed among species and was smaller for *Drosophila* species that normally feed on food poor in ethanol content. Reproduced with minimal alterations from Holmes et al. (1980) with permission of Wiley

concentrations of ethanol, only four out of the five species showed an hormetic response, and these four species showed different threshold concentrations above which ethanol became a metabolic stressor, inducing a decrease in longevity (Fig. 4.4). Consequently, the width of the hormetic zone was smaller for *Drosophila* species that normally feed on food poor in ethanol content. These findings indicated the importance of the hormetic zone when predicting the responses of an individual, population or species to an environmental perturbation.

Not all hormetic responses to intakes of non-essential nutrients are induced by the stimulatory or toxic action of nutrients themselves. Rather they can be triggered by energy that can be produced by those compounds. Low levels of energy intake appears activating hormetic responses in a variety of organisms, including yeast, insects, rats, mice and human beings (Mattson 2008). For example, a low level of dietary energy restriction has been found to increase levels of heat shock proteins 70 in liver cells of rats (Heydari et al. 1993) and may cause increases in other cytoprotective molecules, such as antioxidants. These responses reduce levels of oxidative stress (Ugochukwu and Figgers 2007), and thereby might have a beneficial effect. High levels of food deprivation, however, are obviously detrimental. Hormetic effects induced by dietary energy restriction can also involve changes in systems that regulate cellular energy metabolism (Rodgers et al. 2005; Hyun et al. 2006; Liu et al. 1997), in particular through changes in mitochondrial activity (Tapia 2006).

The application of the hormetic model to nutrition science could also lead to a re-evaluation of the generality of the *thrifty phenotype hypothesis*, which proposes that the epidemiological associations between poor foetal and infant growth, and

the subsequent development of type 2 diabetes and the metabolic syndrome, result from the effects of poor nutrition in early life. According to the hormetic model, the long-term effects of poor early nutrition could be mediated by the environmental quality the individual will experience later in life. So, an individual that has developed under poor nutritional conditions could outperform one that has had good early conditions if both are exposed later in life to periods of lower food availability or quality (Monaghan 2008). For example, zebra finches exposed to poor nutritional conditions when chicks had greater exploratory and foraging activity as adults (Krause et al. 2009). This had no clear evolutionary fitness benefits in the laboratory but could be beneficial in the natural environment, although these finches also had a tendency to lose body condition faster during subsequent periods of food restriction (Krause et al. 2009), which does not fit the hormesis framework. However, it is important to know whether the nutrient is essential or not in order to distinguish hormesis from essentiality.

Finally, it is important to highlight that energetic constraints are not only of exogenous nature (e.g., availability of food, energy content of diet), but also of endogenous nature. Mitochondria, being the energy makers of organisms, may expose the individual to energetic constraints because any damage to mitochondria may reduce their efficiency in ATP synthesis and so the daily metabolizable energy intake of an animal. Oxidative mitochondrial damage might, therefore, increase energy demands of the individual, particularly during demanding phases of the year, such as reproduction or migration. Organisms can actively respond to mitochondrial damage, not only through up- or downregulation of specific proteins and repair systems, but possibly also through changes in mitochondria density as shown in compensatory responses to changes in physical activity in birds (Brackenbury and Holloway 1991) or to cold acclimation in marine invertebrates or fish (Guderley and St-Pierre 2002) in an attempt to meet the energetic requirements of the body.

4.7 Conclusions

Food has a pervasive role in the animal's life because it provides nutrients and energy. Animals are not passive absorbers of nutrients, rather they can prioritise absorption of specific nutrients to meet their metabolic demands. Selection has also favoured some degree of flexibility in the foraging behaviour of some species so that they can modify their foraging activity in order to look for food that provides chemicals they need, as illustrated by examples of self-medication. However, it remains elusive whether phytonutrients (e.g., the so-called dietary antioxidants) act as important antioxidants or whether their beneficial effects stem more from their pro-oxidant properties.

Many studies showed convincingly that diet composition in early life has long-lasting effects on the adult phenotype, and this has implications for mothers who may use nutrients to program the phenotype of their offspring. Importantly, food

may not be continuously available; hence, animals may become exposed to various degrees of starvation stress. There is evidence that mild starvation may stimulate self-maintenance mechanisms and that these may have cross-generational effects. However, this evidence is mostly based on a few artificially selected laboratory animals. We lack studies that analyse the effects of starvation on oxidative balance and hormesis across the life history stages of free-living animals. We also need to examine in detail the hormetic effects of starvation in order to elucidate whether such effects come through a restriction in overall caloric intake, in specific nutrients, in substrates for energy production or a combination of all of these factors.

Although supplementation studies of single dietary chemicals provided valuable insight into our understanding of the effects of diet quality on oxidative stress resistance, we now need studies that combine them in order to fully assess their biological relevance. We also need to consider that there is among-species variation in digestive flexibility, which may inform us on how and why the absorption of nutrients or antioxidants is given priority or is constrained. For example, while flavonoids effectively decreased glucose absorption in rats *Rattus norvegicus*, flavonoids did not have any effect on glucose absorption in American robins *Turdus migratorius* (Skopec et al. 2010). Beyond mechanistic explanations, it may be speculated that American robins evolved mechanisms that allow simultaneous absorption of glucose and flavonoids, because they need glucose to sustain their energy-demanding migratory flight and flavonoids to mitigate oxidative damage, respectively. If it were so, we might expect flavonoids to constrain glucose absorption more in year-round than in migratory populations of American robins.

Karasov (2011) argued that the match between digestive features and diet provides evidence of trade-offs that preclude doing well on all possible dietary substrates with a single digestive design, and this influences ecological niche partitioning. The flexibility of the digestive system varies largely among species, and having a reversible flexibility is very important because it allows successful reproduction in many environments where food resources may differ and change over time (Karasov 2011). This flexibility may also be important when the organism needs molecules to fuel its antioxidant machinery. Therefore, the analysis of among-species or individual variation in flexibility in absorption of antioxidants, amino acids needed to build up antioxidant enzymes or of nutrients that are also substrates of oxidative damage can inform ecologists about the ability and constraints of animals in responding to ecological challenges, such as changing in resource availability. This might also be very important to further our understanding of why some species have been exploiting food derived from human activities (e.g., food waste) and how they can tolerate these foods that they did not evolve to feed on (Fig. 4.5).

The extent to which nutrition may impact on oxidative balance of animals also depends on the metabolic requirements of each stage of life and on the availability of nutrients over that phase itself. The analysis of metabolic rate together with that of oxidative balance may, therefore, prove informative. Although the link between metabolic activity and oxidative balance regulation is generally low, the metabolic

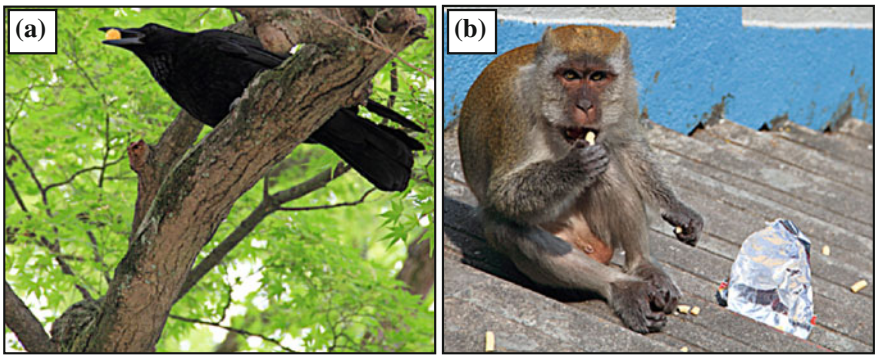

Fig. 4.5 Food wastes of anthropogenic origin are becoming important nutritional resources for many animals, rodents, mammalian carnivores and gulls being renowned consumers of garbage. There are also species that unexpectedly learned to feed on food of anthropogenic origin; for example, *Gallotia* lizards of the Canary Islands surround fearless the tourists, which feed them (Emiliano Bruner, pers. comm.). Examples abound. In picture (**a**), a carrion crow (*Corvus corone*) in Kyoto (Japan) is going to swallow a candy; in picture (**b**) a long-tailed macaque (*Macaca fascicularis*) at Batu Caves (Malaysia) is feeding on chips they obtain from tourists. Enrichment of natural diet with these foods raises interesting questions on how these species can metabolise these new foods and on which long-term effects feeding on food wastes may have on evolutionary fitness and population dynamics. Photographs by David Costantini

rate is still part of the picture and might be relevant under specific circumstances. If this were the case, having a plastic metabolic machinery might allow the organism to adapt to seasonal variation in food quality and quantity through adjustments of metabolic rate, thereby exploiting more efficiently available nutrients and mitigating any oxidative costs.

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Chapter 5
Coping with Physical Activity and Inactivity

Abstract There is an impressive variation in activity and inactivity patterns among animal species, which illustrates the difficulties of generalising findings from one species to another. Oxidative stress appears to be a ubiquitous consequence of physical effort and also a threat for those species that enter cycles of short- or long-term dormant states and arousal. Hormesis may provide an important mechanistic and theoretical framework to examine the response to and the biological effects of physical effort and preparation for arousal from a dormant state. In this chapter, I examine the differing cases where oxidative stress and hormesis have possibly contributed to shape variation in activity patterns and the solutions animals have evolved to combat the damaging consequences of oxidative stress. I also examine the way animals exploit situations causing moderate stress to shape a phenotype that is better able to cope with the challenges of an intense physical effort or that occur when the organism passes from a metabolically depressed dormant state to an active one.

5.1 Redox Biology of Physical Activity

Activity patterns in animals are influenced by a number of factors, including the animal's physiological adaptations, weather and climatic conditions, food availability and distribution, availability of local breeding sites, foraging behaviour, or disturbances caused by competitors, predators and humans (e.g., Kahurananga and Silkiluwasha 1997; Boldt and Ingold 2005; Hopcraft et al. 2005; Nevoux et al. 2013). There may be obvious benefits in moving from one place to another, such as when the recently colonised territory is richer than the previous one in terms of food or availability of breeding sites. There are, however, also energetic, time, risk and opportunity costs (e.g., McWilliams et al. 2004; Bonte et al. 2012). These costs may influence the performance of moving individuals and the evolution of activity patterns.

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Physiological costs are dependent to some degree on physical effort, which is expected to greatly vary daily and annually, as well as among sexes, individuals or species. Costs also depend on the environment. Migratory birds or seabirds while foraging may have to cope with strong oceanic winds. Diving birds and mammals daily cope with hypoxic episodes while submerged. Similarly, burrowing animals and those that live at high altitude or cross mountainous regions while migrating perform their activities under conditions of low partial pressure of oxygen (Ramirez et al. 2007).

Physical activity is certainly energy demanding, but it can also be antioxidant demanding. For example, contractile skeletal muscle cells produce reactive species during physical exercise, which may cause oxidative damage, especially when the exercise is intense or prolonged (Alessio 1993; Ji 1999; Powers and Jackson 2008). Powers and Jackson (2008) reported that the first suggestion that physical exercise results in free radical-mediated damage to tissues had appeared in 1978 (Dillard et al. 1978), and since then, there has been a growing number of studies assessing the mechanisms underlying the link between physical effort and oxidative stress. It has been, for example, shown that in skeletal muscle there may be various potential subcellular sites/mechanisms of superoxide production, such as mitochondria, sarcoplasmic reticulum or phospholipase A_2-dependent processes (Powers and Jackson 2008). Moreover, physical activity may cause inflammation and activation or suppression of immune cells (Shephard and Shek 1994; Woods et al. 1999; Murphy et al. 2008), which in turn may influence oxidative balance (see Chap. 8). For example, animals may redirect macrophages and other phagocytic cells towards muscle fibres that were injured (McArdle et al. 2004). Although this process is important for the regeneration of the tissue damaged, phagocytic cells release reactive species that might cause further damage (Zerba et al. 1990; Powers and Jackson 2008). It is here that physiological trade-offs can occur.

Although physical activity may cause an increase in metabolic rate and energy expenditure, oxidative damage does not necessarily increase in proportion in part because defences can be upregulated and because the leakage of protons from the mitochondria (and hence generation of reactive species) significantly decreases with the shift from a resting to an active state (Loschen et al. 1971; Herrero and Barja 1997). The decreased leakage of reactive species may have evolved as a protective mechanism against severe oxidative stress that aerobic organisms would otherwise experience during exhausting activities. A recent study suggested that moderate exercise may stimulate the antioxidant response (Gomez-Cabrera et al. 2008). In fact, a number of studies are showing that physical activity may result in the hormetic activation of a number of molecular pathways, involving anti-stress and anti-inflammatory responses, as well as mechanisms of damage repair (e.g., Ji et al. 2006; Gomez-Cabrera et al. 2008; Radak et al. 2008; Rattan 2008). There is, however, an impressive variation in activity patterns among animal species, which illustrates the difficulties of generalising findings from one species to another because evolution may have favoured different coping strategies.

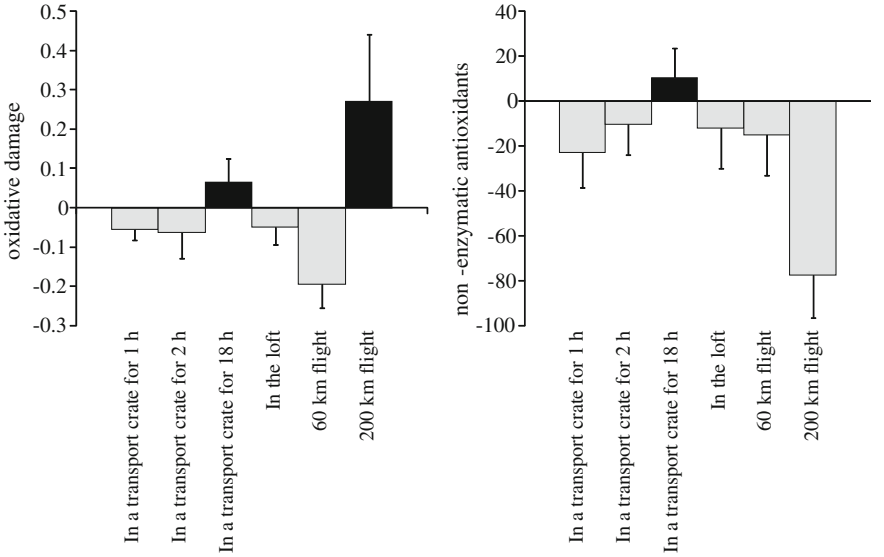

Fig. 5.1 Effects of flight effort on oxidative damage (serum hydroperoxides, mM H_2O_2 equivalents) and serum non-enzymatic antioxidant capacity (mM HOCl neutralised). Values are shown as mean + standard error of post- minus pre-treatment values. Reproduced with minor modifications from Costantini et al. (2008) and from Costantini and Lipp (2010) with permission

In this chapter, I examine the differing cases where oxidative stress and hormesis have possibly contributed to generate variation in activity and inactivity patterns and the solutions animals have evolved to combat the damaging consequences of oxidative stress.

5.2 Physical Effort, Oxidative Stress and Hormesis

Physical activity may increase the production of reactive species, potentially leading to oxidative stress. The increase in physical effort has to be sufficiently high to induce changes in redox equilibrium. For example, while pigeons (*Columba livia*) flying ca. 200 km experienced an increase in serum oxidative damage and a decrease in serum non-enzymatic antioxidant capacity compared to pigeons kept inside lofts or transport crates, pigeons flying ca. 60 km did not show any evidence of serum oxidative stress (Fig. 5.1; Costantini et al. 2008; Costantini and Lipp 2010). Rather, the effect of flight on oxidative damage appeared to be of hormetic nature because oxidative damage decreased in pigeons that flew 60 km. Further data showed that, within pigeons flying a similar distance, pigeons that suffered less oxidative damage and saved more antioxidants at the end of flight were those with lower homing time, i.e., those that came back to the loft in a

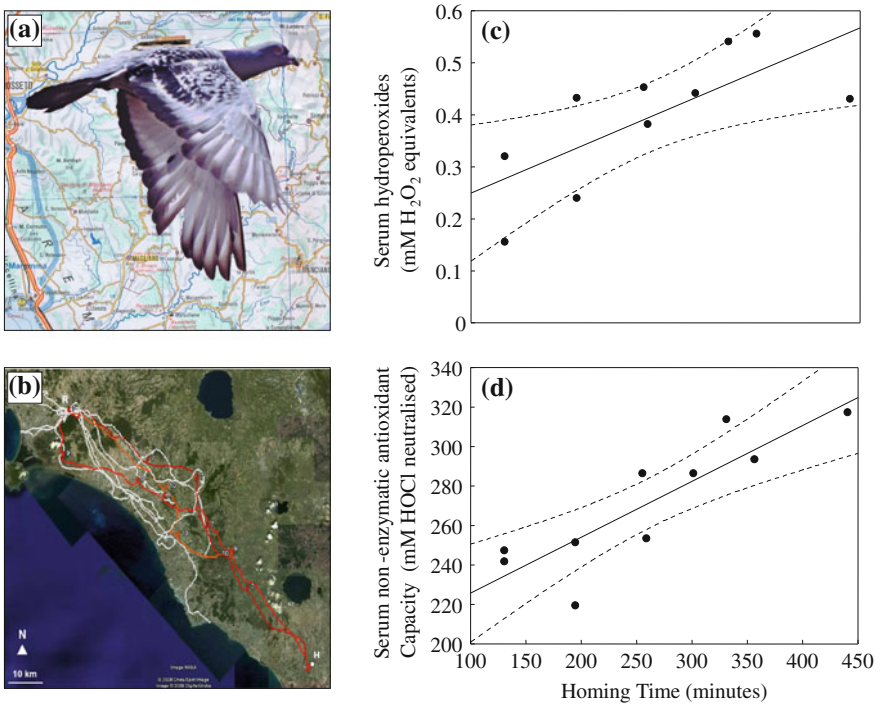

Fig. 5.2 **a** Pigeon carrying a GPS data logger on its back (Photograph courtesy of Maria Savini); **b** Example of GPS tracks of homing pigeons between the release site (*R*) and the home loft (*H*); marks indicate stops for resting and numbers refer to individual pigeons; **c** Relationship between duration of homing flight and post-flight serum oxidative damage ($r = 0.72$, $P = 0.018$); **d** Relationship between duration of homing flight and post-flight serum non-enzymatic antioxidant capacity ($r = 0.87$, $P = 0.001$). Measures of oxidative damage and antioxidant status were done in blood taken from each bird within 8 min of their return to the loft. Pigeons that came back later had a lower straightness index (homing performance) and a lower % of time spent flying. The *dashed outline* indicates 95 % confidence interval. Unpublished results of David Costantini, Gaia Dell'Ariccia and Hans-Peter Lipp

shorter time (Fig. 5.2). Studies on pigeons flying in a wind tunnel (Rothe et al. 1987) or in the wild (Schwilch et al. 1996) showed that a shift to a high and stable lipid-based metabolism may occur within 1–2 h of flight. Prolonged flights caused increased hydrolysis of triglycerides from adipose tissues to free fatty acids and glycerol and oxidation of free fatty acids by muscle activity (Schwilch et al. 1996). These metabolic changes may be determined to some degree by the time elapsed since feeding, which is an important determinant of the fuel pigeons use in flight (Gannes et al. 2001). Overall, a lipid-based metabolism may have further contributed to increasing oxidative stress in pigeons that flew 200 km. Unsaturated fatty acids are the main form of lipids stored in avian tissues (McWilliams et al. 2004) and are quite susceptible to free radical damage (Chap. 1). Increased oxidative stress might also have been consequence of an increase in the ratio of

unsaturated over saturated fatty acids, which occurs in the blood after exercise (Nikolaidis and Mougios 2004). These findings suggest that pigeons and, in general, birds might need to balance energy gain and oxidative costs, both derived from metabolising lipids.

As with the pigeon studies, Vaanholt et al. (2008) found that long-term voluntary exercise, while higher in mice (*Mus musculus*) selectively bred for higher wheel-running activity than in controls, did not influence the activity of superoxide dismutase or glutathione peroxidase. Similar results were obtained in another study, where the antioxidant status of voluntarily exercising short-tailed field voles (*Microtus agrestis*) was compared to that of sedentary voles (Selman et al. 2002). In contrast, activity of antioxidant enzymes was increased in rats that were subjected to forced swimming (Gunduz et al. 2004). These findings also suggested that, when non-experimentally forced, individuals may choose a level of physical activity that is not too demanding. So the approach of leaving individuals under captive conditions to move voluntarily might not induce a response that would occur under natural conditions, where individuals may be often forced by predictable or unpredictable social and non-social factors to increase their physical effort.

There are clearly various technical difficulties associated with experimental investigations of physical activity in captivity. First, it is important to build a system that allows proper quantification of the physical activity of experimental animals. Second, it is helpful to have a system that allows the experimenter to regulate and manipulate the intensity of physical activity in order to have different levels of treatment. Third, it is important to have a user-friendly and relatively inexpensive system that can be used by different laboratories in order to make results comparable.

Although devices already exist for quantifying running activity in mice and rats (e.g., running wheels, Gattermann et al. 2004; Judge et al. 2005), these have the disadvantage of allowing individual animals to choose their level of activity, so they may slow down the rate of running if the physiological costs become significant. The use of wind tunnels has provided a powerful tool to analyse the kinematics of flight and the effects of flight effort on the physiological status of birds (Pennycuick et al. 1997; Engel et al. 2010). Although most bird species studied so far did not require training, some species have appeared to be problematic because they required large amounts of space and extensive training sessions. A possible more user-friendly system for avian biologists may be based on the use of automated moveable perches that are positioned on opposite sides of a flight aviary and that are operated on timers such that they alternately fold away, forcing the birds to fly from one side of the aviary to the other throughout the experimental session (Nudds and Bryant 2000). Developing the approach adopted by Nudds and Bryant (2000), Costantini et al. (2012, 2013) used an improved simple automated system to manipulate the intensity of flight in zebra finches (*Taeniopygia guttata*) to test whether there is a link between the degree of oxidative stress induced by a short term major increase in physical activity (Fig. 5.3) and the biochemical integration of the blood redox system (Fig. 5.4). Moreover, by

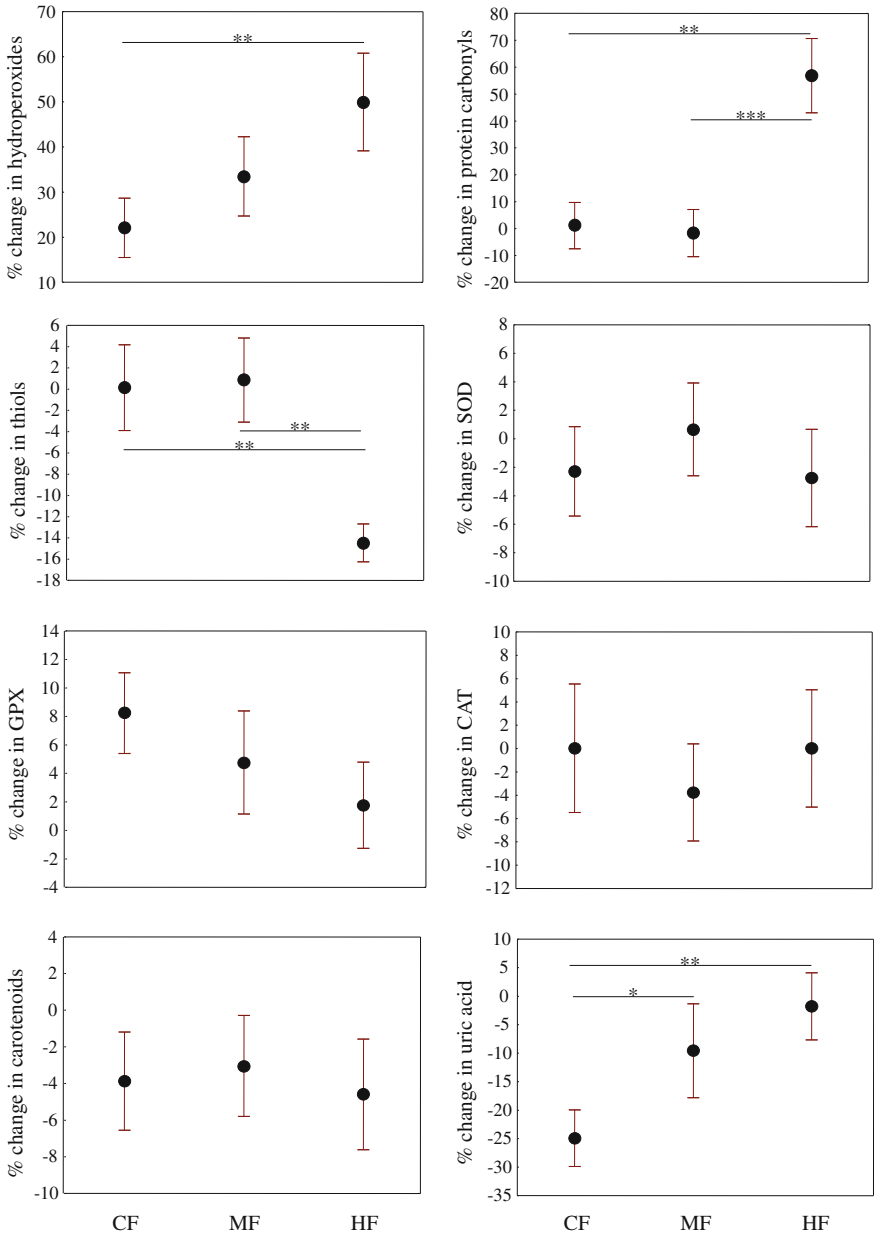

Fig. 5.3 Response to various levels of flight activity expressed as percentage within-individual change over the experimental period. Values are shown as mean ± standard error. Post hoc comparisons are based on least square means. Significant differences among groups are highlighted as follows: $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$. *CF* control flight; *MF* mild flight; *HF* high flight. *GPX* glutathione peroxidase; *SOD* superoxide dismutase; *CAT* catalase. Reproduced from Costantini et al. (2013) with permission

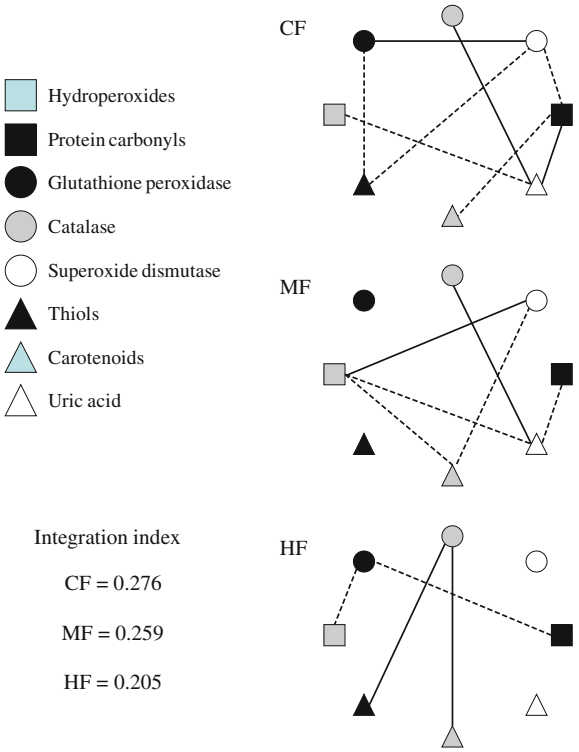

Fig. 5.4 Network model drawn to show the connections between eight oxidative status parameters measured in the blood; networks are presented separately for the three treatment groups (*CF* control flight, *MF* mild flight, *HF* high flight). Each node of the network refers to one variable. Significant Pearson correlations between standardised within-individual change values (calculated for each treatment group separately) are indicated by *continuous* (*positive*) or *dashed* (*negative*) *lines* between nodes; note the progressive reduction in the number of lines and increasing dissociation between nodes moving from CF through MF to HF. The integration index was calculated according to Pavlicev et al. (2009) and Haber (2011); it can vary between zero (corresponding to no relationships among the variables) and one (perfect correlation between all variables). Reproduced from Costantini et al. (2013) with permission

inducing intermediate as well as high levels of flight activity, the experiment allowed to test for evidence of hormesis in both the level of oxidative stress and the degree of redox integration. Birds were allocated to three experimental groups that differed in the intensity of flight activity: Control Flight, Mild Flight and High Flight. Compared to Control Flight birds, High Flight birds had a greater increase in oxidative damage (both hydroperoxides and protein carbonyls) and decrease in thiol concentration (indicating higher oxidation of thiols) over the course of the flight trials. High Flight birds had also a greater increase in protein carbonyls and a decrease in thiols than Mild Flight birds; the change in both oxidative damage biomarkers and thiols did not differ between controls and Mild Flight birds

(Costantini et al. 2013). The concentration of plasma uric acid in control birds significantly decreased over the trials compared to both other groups. The change in catalase, glutathione peroxidase, superoxide dismutase and carotenoids did not differ among groups. The multivariate analysis of variance showed that the multivariate response significantly differed between High Flight birds and both Control and Mild Flight birds, while it did not differ between these two last groups. Such differences reflected a differential index of integration of the physiological response, being similar in Control and Mild Flight birds, but lower in High Flight birds. A similar pattern was found for the covariance in changes in redox state of plasma and red blood cells: while the covariance was significant in all three experimental groups, it was lower in High Flight birds than in the other two groups.

The results of this study clearly showed that, when exposed to a short term major increase in physical activity, individuals had more oxidative stress. Results also showed that oxidative stress and integration increased and decreased, respectively, with the intensity of the flight effort, and there was no evidence of a hormetic response. The effect of the flight treatment was to cause a reduction in the correlations among the measured biochemical variables (expressed as percentage within-individual change over the experimental period) in the High Flight birds. This resulted in a lower redox integration index in these individuals. A reduced correlation among biochemical measures in the High Flight compared to the Control and Mild Flight would suggest that in those individuals, the change in one component of blood oxidative status was less dependent on that of other components. In parallel with this, a reduction in integration was also apparent in the covariation between the changes of oxidative status of plasma and that of red blood cells with the increase in flight effort. The loss in biochemical integration may reflect a failure in the homeostatic mechanisms regulating the oxidative status of the blood. The effect of the short term increase in flight activity was particularly acute for High Flight birds, which could have perceived it as an unpredictable and stressful event. Although in the wild, zebra finches can fly tens of kilometres per day, under captive conditions, the physical activity of birds is usually low (over the experimental period Mild and High Flight birds were flying around 6 and 16 times, respectively, the distance of Control Flight birds, who were probably as active as all birds had been in their holding cages prior to the experiment; Costantini et al. 2013). Therefore, the increase in the level of exercise experienced by the High Flight birds may have been too great for them to have adjusted their homeostatic mechanisms sufficiently to control their oxidative status, especially if thiol concentrations also dropped (Jones 2006; Sohal and Orr 2012). Consequently, an increase in production of free radicals might not have been tackled by the antioxidant response, leading to an increase in oxidative damage. It is also worth highlighting that oxidation of molecules involved in redox regulation and control might have altered their functionality, hence reducing their capacity of interacting with other molecules.

Another explanation for the increase of oxidative stress in High Flight birds is that they deprioritised compensatory mechanisms for oxidative stress in the short

term because for some reason it was too costly to upregulate these under the circumstances of the trials. Zebra finches could, for example, be programmed to sacrifice protection against oxidative stress during periods where allocation of resources to other specific functions (such as flight) is given priority. It is noteworthy that zebra finches whose flight activity was experimentally increased (by increasing the number of chicks that they had to feed) showed a decrease in the antioxidant enzymes superoxide dismutase and glutathione peroxidase in pectoral muscle (Wiersma et al. 2004). Although experimental zebra finches were not breeding at the time of the experiment, it could be that the manipulation of flight activity might have created conditions similar to the experiment by Wiersma et al. (2004). This would imply that mechanisms regulating blood oxidative status are conserved across those different stages of the life cycle (e.g., chick-rearing period, migration) that demand strong increases in physical activity. The fact that in the experiment by Costantini et al. (2013), enzymatic antioxidant defences did not decrease as in the study by Wiersma et al. (2004) might also suggest that the lack of an additional effort (i.e., chick rearing as in Wiersma et al. 2004) or the younger age of birds in the study by Costantini et al. (2013) could have made the overall effort less demanding for the birds.

Finally, since results also showed that there was not only an overall reduction in integration but also a change in the specific connections among variables, this could suggest that the decrease in integration was the result of an increase in modularity, i.e., the molecular groups measured were part of different pathways that under stressful conditions work almost independently from each other. Given that in an unstable environment (like that induced by the intense flight-induced stress), an organism can be faced with several environmental pressures it could be more functionally efficient to separate the processing of the stimulus and the response to it among different modules, according to the nature of the stimulus itself as suggested for metabolic networks of bacteria by Parter et al. (2007). Although the short term increase in flight activity did not induce any hormetic response (at least within the time frame of the study), it is well established that physical activity can induce hormetic responses (Mattson and Calabrese 2010). Depending on the type of flight (e.g., short with repeated takeoffs, steady-state), as well as its duration and intensity, the flight effort can be either stimulatory or detrimental in terms of energy expenditure or stress. An increase in energy expenditure has been previously demonstrated in captive zebra finches engaged in short and repeated flights accompanied by around 500 takeoffs in 8 h (Nudds and Bryant 2000). However, changes in metabolic rate were also affected by the training level of the individual bird, with trained finches showing a decrease rather than an increase in metabolism compared to untrained finches (Nudds and Bryant 2001). Similarly, plasma concentrations of malondialdehyde (an end-product of lipid peroxidation) in captive budgerigars *Melopsittacus undulatus* were significantly lower after 9 weeks of flight training than after a single exercise session (Larcombe et al. 2010).

All the aforementioned studies used the classic approach of increasing physical effort to test effects on the level of oxidative stress. Relevant results were also

obtained by limiting the activity of animals. Prevention of flight activity of male houseflies (*Musca domestica*) decreased their rate of oxygen utilisation and almost tripled their lifespan as compared to those permitted to fly (Yan and Sohal 2000). Rate of mitochondrial hydrogen peroxide generation at various ages was lower in the low-activity flies than in the high-activity flies. Oxidative damage to mitochondrial proteins, adenine nucleotide translocase, and aconitase, detected as carbonyl modifications, was attenuated; and the loss in their functional activity occurring with age was retarded in the long-lived low-activity flies as compared to the short-lived high-activity flies (Yan and Sohal 2000). Although this and many other reports found that physical effort may increase oxidative damage through an increase in production of reactive species, such effects may not occur in all taxa. For example, fruit flies (*Drosophila melanogaster*) that were allowed to fly throughout life had higher oxidative damage, while the levels of reactive species produced by mitochondria isolated from high-activity and control flies did not differ significantly (Magwere et al. 2006). They also had higher mortality rates and decreased median and maximum lifespans compared to controls. The high-activity flies had altered membrane fatty acid compositions, which likely made them prone to increased lipid peroxidation (Magwere et al. 2006). These findings suggest that selection might have built up among species variation in which mechanisms are used to regulate their resistance to oxidative stress over periods of intense physical effort. While some species may regulate, say, mitochondrial activity, others may opt to modify the molecular composition of cell membranes. An alternative explanation is that species have some degree of flexibility in the way they regulate their oxidative balance. Hence, discrepancies among studies might reflect variation in experimental conditions, which favoured use of different physiological mechanisms, regardless of the species.

A number of studies have also found that exposure to a relatively low level of free radicals as a consequence of moderate regular exercise may actually reduce oxidative stress, through the upregulation of antioxidant enzymes or molecules that repair damage to proteins or nucleic acids (e.g., Davies 1986; Hollander et al. 2001; Radak et al. 2003; Alessio and Hagerman 2006; Ji et al. 2006; Radak et al. 2008). In birds, depending on the type of flight (e.g., short with repeated takeoffs, steady-state), as well as its duration and intensity, the flight effort can be either stimulatory or detrimental in terms of energy expenditure or stress. In mammals, similarly to avian examples previously discussed in this section, moderate physical activity reduced levels of oxidative damage to nuclear and mitochondrial nucleic acids in rats (Nakamoto et al. 2007), while a high physical effort resulted in an increase in several parameters of oxidative damage, upregulation of antioxidant enzymes and depletion of non-enzymatic antioxidants (Margonis et al. 2007).

Regardless of species, it seems that it is the reactive species themselves that are important in triggering an hormetic response. Supplementation of antioxidants can prevent humans or rats from building up adaptive mechanisms to physical activity (Gomez-Cabrera et al. 2005; Ristow et al. 2009). This has been interpreted as evidence for a suppression of free radical production during exercise due to intake of antioxidants (Gomez-Cabrera et al. 2005; Ristow et al. 2009). However, other

studies did not find such detrimental effects of antioxidants (Theodorou et al. 2011; Nikolaidis et al. 2012a). For example, male budgerigars (*Melopsittacus undulatus*) provided with a diet rich in antioxidants over 8 weeks of flight training showed significantly greater improvement in the performance of repeated escape flights than males provided with a diet poorer in antioxidants (Arnold et al. 2010). Moreover, budgerigars forced to perform short flights mimicking escapes from predators had an increase in oxidative damage, which did not occur when birds were fed on a diet rich in antioxidants (Larcombe et al. 2008). Rapid acceleration during takeoff is an important component of prey escape; hence, hormetic effects of dietary antioxidants on flight performance might be under positive selection because they might increase survival probability.

We should not lose sight of the fact that studies on vertebrates found that changes in biomarkers of redox state may persist for and/or appear several days after the end of a muscle-damaging physical activity (Nikolaidis et al. 2012b). Similarly, biomarkers of redox state may fluctuate over the dormant period, as well as at arousal. The temporal window at which samples are taken may drastically change the overall picture. Repeated sampling will be therefore essential.

5.3 Costs of Migration and Strategies to Mitigate Them

5.3.1 Long-Distance Migrations

Every year, millions of animals migrate twice over long distances from the breeding to the wintering grounds and back. Migration is a ubiquitous phenomenon, found in animal groups as diverse as insects, crustaceans, fish, amphibians, reptiles, birds and mammals (Aidley 1981; Milner-Gulland et al. 2011). There are obvious benefits for migrating animals, such as moving towards environments with better climatic conditions, higher food availability or other better conditions for successfully reproducing. However, there are also costs. Migration imposes a number of physiological challenges, such as a high energetic turnover, the maintaining of body homeostasis and fasting (Gwinner 1990; McWilliams et al. 2004). For example, birds derive energy from fat and protein stores accumulated in the pre-migratory phase (Berthold 2001). Accumulating fat and proteins stores before migrating and taking stops while migrating to refuel are important for the timing and success of migration in birds (Alerstam and Lindstrom 1990; Jenni et al. 2000).

Migration is not only a matter of nutrients. Migratory animals are also potentially exposed to various sources of oxidative stress: for example, birds may need to maintain an intense and prolonged metabolic activity required to sustain the flight; may experience depletion of nutrients and dietary antioxidants because of fasting, which may exacerbate any costs of physical effort; may experience inflammation, which may increase oxidative damage levels. Moreover, migratory

species may be relatively sedentary outside of the period of migration, leading to marked contrasts in the intensity of flight activity required during the year. Bird species also differ in the migratory strategy, which might build up further variation in the physiological machinery (McWilliams et al. 2004; Falsone et al. 2009). For example, while some species migrate during the daylight, other species fly during the night and rest and forage during the daylight. The time budget dedicated to foraging or, in general, to stopover is influenced by the amount of energy stores that are still available, with fat individuals leaving the site sooner than lean individuals (Fusani et al. 2009; Goymann et al. 2010). The environmental conditions of the stopover site, where birds stop to rest and/or feed, may further contribute to the intensity of the oxidative challenge, with inhospitable areas possibly reducing the chances of obtaining resources that might be important to limit oxidative damage.

Migratory species are likely to be able to cope with flight-induced oxidative stress better than non-migratory species since they might have evolved more effective defence systems. Arctic terns (*Sterna paradisaea*), for example, migrate annually between the northern and southern polar areas. That they might make this gruelling trip more than 30 times shows that they have evolved appropriate mechanisms for this intermittent high exercise level. Seabirds, like the Arctic tern, have cell membranes particularly resistant to peroxidation. For example, the analysis of myocardial phospholipids revealed significantly lower peroxidation index values in the Procellariiformes than in the Galliformes, due mainly to the proportionately higher monounsaturated fatty acids and reduced polyunsaturated (particularly n-6) in the seabirds compared to the fowl (Buttemer et al. 2008).

So far, there is no clear demonstration that migration presents animals with substantial oxidative threats. However, there is some indirect evidence suggesting it may be so. Costantini et al. (2007) analysed the blood oxidative balance in garden warblers (*Sylvia borin*) and barn swallows (*Hirundo rustica*). Birds were trapped while migrating at a ringing station located in Ponza, a small island located around 500 km off the African coast from which birds departed for the migratory flight towards the breeding European grounds. The low number of recaptures (less than 5 % of trapped birds, M. Cardinale personal observation) at Ponza indicates that most birds spend less than one day or usually only few hours on the island and that birds trapped in Ponza at maximum spent few hours after a non-stop flight. Therefore, the parameters of oxidative status measured in both garden warblers and barn swallows should have mirrored the physiological status of birds that incurred a large energy loss during a previous non-stop flight (Costantini et al. 2007). Estimates done on the neighbouring Ventotene Island suggested that birds landing in these islands had completed a 14–16 h non-stop flight (Pilastro et al. 1995; Schwilch et al. 2002). Results of the study showed that garden warblers with higher nutrient stores had higher plasma non-enzymatic antioxidant capacity and a better balance between intermediate oxidative damage products and circulating antioxidants (Costantini et al. 2007). Interestingly, such a relationship was much less evident in barn swallows, a species with relatively low metabolic activity (Hails 1979; Bruderer et al. 2001; Pennycuick 1990). Metabolism during

flight in hirundines, such as barn swallows and swifts, is 49.3–72.6 % lower than other birds of similar size (Hails 1979). Moreover, unlike most other passerines, hirundines rarely show regular flapping flight (Bruderer et al. 2001), which demands one of the highest metabolic rates of any form of vertebrate endurance locomotion (Pennycuick 1990). Although these data did not provide proof that animals may experience oxidative stress while migrating, it is important to highlight that the level of energy stores (fat and muscle) is a good proxy of migration effort and predictor of the likelihood of successfully achieving the migratory flight. Hence, birds in good condition may preserve their antioxidant defences. If oxidative stress constrains future reproductive investment (see Chap. 7), still having a good antioxidant status at the end of migration would then pay in terms of higher reproductive success. Oxidative stress might therefore be one important mechanism underlying the trade-off between the migratory effort and the development of traits in preparation for breeding or the reproductive success. This might be particularly important in those species where males, once arrived on the breeding grounds, compete for territories. Birds may, for example, adopt behavioural strategies while flying (e.g., decrease in flight speed) in order to minimise energy expenditure (Hambly et al. 2004). Such behaviours have been favourably selected because they might also help avoiding increases in oxidative damage to tissues, which would compromise survival and future reproductive success.

Further studies provided some evidence in favour of a link between migration and oxidative balance. For example, Ninni et al. (2004) found that late arriving male barn swallows had higher levels of circulating carotenoids and depleted a larger amount of carotenoids shortly after settling in the breeding area. This pattern was also consistent across two seasons. Carotenoids are accumulated in fat and used in times of high physiological demands as shown in garden warblers before the autumn migration (Metzger and Bairlein 2011).

We should also recognise that, while migration effort may increase oxidative stress, generation of oxidative damage might in turn reduce muscle efficiency, hence reducing the chances of completing the migration. Many animal studies provided compelling evidence that reactive oxygen species contribute to muscle fatigue induced by prolonged muscular contractions (Powers and Jackson 2008; Reid 2008; Westerblad and Allen 2011). For example, induction of oxidative stress through knockout of manganese superoxide dismutase impaired mitochondrial activity and lowered endurance capacity in energetically demanding activities (Williams et al. 1998; Kinugawa et al. 2005; Lustgarten et al. 2009, 2011). Dietary antioxidants like vitamin E may prolong endurance to muscle fatigue (Novelli et al. 1990), although discrepancies exist in the literature about the anti-fatigue effects of dietary antioxidants (Powers and Jackson 2008).

Another consequence of oxidative stress that might impair migratory performance is a reduction in cognitive abilities. Brain oxidative stress may reduce homing abilities and memory. For example, Rosa et al. (2007) investigated the effects of intense and exhaustive exercise on male mice, undergoing 10 days of intense and exhaustive running program on cognition and its possible relationship with brain oxidative stress. There was a remarkable memory reduction in exercised

animals in comparison with the control group, and this memory reduction was associated with the increase in the brain oxidative stress. Concurrent vitamin C and E supplementation fully prevented the memory decrement induced by the intense physical activity and partially recovered both the increased brain lipid peroxidation and protein oxidation (Rosa et al. 2007). As with physiological adaptation to physical effort, brain functions (e.g., spatial learning, memory) and neurogenesis may be enhanced by exposure to mild doses of free radicals through hormetic effects (Anderson et al. 2000; Ang et al. 2006; Pereira et al. 2007). Consistent with the concept of mitohormesis, transiently increased levels in exercise-induced reactive species trigger an adaptive response promoting maintenance of homeostasis (Ristow et al. 2009). There is potential for hormesis to play a relevant role in individual physiological response to migratory effort, considering that migration may be performed under very variable environmental (e.g., weather) and individual (e.g., energy and antioxidant stores) conditions that may expose to differing intensities of free radical production.

One of the strategies animals may have evolved to mitigate any oxidative costs induced by strenuous migratory activity is to select food rich in dietary antioxidants while refuelling at stop-over sites. Although food selection might have been primarily evolutionary shaped by the needs of macronutrients, such as fat and sugars, that are used to produce energy, such a selection might also have conferred an indirect advantage in terms of intake of antioxidants, which would have strengthen evolution of this behaviour. For example, birds might mitigate oxidative stress through consumption of food rich in polyphenols (Bolser et al. 2013). Studies on rodents found that polyphenols may enhance cognitive abilities and resistance against oxidative stress because polyphenolic compounds are able to cross the blood brain barrier and localise in various brain regions important for learning and memory (anthocyanins in Andres-Lacueva et al. 2005). For example, administration of polyphenol extracts from blueberries (*Vaccinium ashei*), blackcurrants (*Ribes nigrum*), boysenberries (*Rubus loganbaccus* × *baileyanus*) or cranberries (*Vaccinium macrocarpon*) to rats improved muscle strength and balance or enhanced neuronal functioning and restored the brain's ability to generate a neuroprotective response to stress, although effects differed among plant extracts (Shukitt-Hale et al. 2005). Similarly, administration of a polyphenol-rich extract of wild blueberries *Vaccinium angustifolium* to mice increased their learning and memory and reduced their brain oxidative damage, respectively (Papandreou et al. 2009). Given that polyphenols are also accumulated in avian brain (greenfinch *Carduelis chloris*, Mullen et al. 2010), there is potential for polyphenols to play in birds similar roles as those described in mammals.

5.3.2 Vertical Migration

Huge numbers of marine organisms daily move from deeper to surface waters and back. This vertical migration faces organisms with metabolic requirements

induced by the physical effort and with the need of dynamically adjusting their physiological system in order to tolerate the changes in water parameters such as temperature and partial pressure of oxygen. Euphausiacea crustaceans (the so-called krill) are renowned for performing the deepest migrations; interestingly, they also exhibit very high antioxidant enzyme activities (Tremblay et al. 2012). There are among species differences in expression of antioxidant enzymes with depth. For example, the tropical krill *Euphasia eximia* shows higher activity of superoxide dismutase at the surface than in the deeper waters, while in the sub-tropical *Nematoscelis simplex* and the temperate *N. difficilis* the activity of superoxide dismutase is higher in deeper than in shallower waters (Tremblay et al. 2012). These study systems offer interesting opportunities to test the effects of environmental hormetic conditioning and oxidative stress on life history strategies. It might be informative to examine those species that reproduce at the sea surface and then larvae migrate into the deep waters, being so exposed to differing environmental conditions. It is here that oxidative stress might play an important selective role for larvae. For example, palolo worms of the *Eunicidae* family are sea annelids that have a unique spawning behaviour. In preparation for spawning, they generate a tail containing eggs and sperm (epitoke). At spawning, all epitokes are released simultaneously and make their way to the surface, where fertilisation of eggs occurs. The larvae hatch quickly and then migrate to the ocean floor. They have to withstand any costs of migration straightaway after hatching, an important temporal window for selection to operate, where being prepared to tolerate oxidative stress might be vital.

5.4 Quarrelsome Families: Competition Among Siblings

In species with parental care, offspring have evolved behaviours, sometimes quite extravagant, that are used to solicit food and care from their parents either selfishly at the expenses of their siblings or honestly conveying that they are in need of food (Rodríguez-Gironés et al. 1996; Godfray 1991). A prediction of life history theory is that such behaviours carry costs for the young, and these would prevent a runaway escalation of solicitation behaviours (e.g., Macnair and Parker 1979; Parker et al. 2002). Costs induced by competition among siblings would be related to factors such as emission of continuous and loud vocalisations to beg food or fights that may increase the physical effort of young. Any costs would also be strengthened by the inability of the young to compete and so to fail to get enough food or heat in the first period of life, when capacity of thermoregulation is still immature. However, evidence in favour of physiological costs of sibling competition has been elusive for years. For example, studies looking at energy expenditure found that begging may have low metabolic costs in chicks (e.g., McCarty 1996; Abraham and Evans 1999), at least when tested aerobically and not anaerobically (Weathers et al. 1997). In the last few years, there has been growing

interest in oxidative stress as a potential cost of sibling competition, and some evidence was published in favour of this hypothesis.

In many altricial bird species, hatching asynchrony results in different casts of offspring (structured family), with core and marginal offspring being the earlier and later hatched, respectively. Marginal offspring consequently may suffer more the competition with their older and larger siblings. Hall et al. (2010) found that an experimental increase in antioxidant availability did not influence the begging behaviour of red-winged blackbird (*Agelaius phoeniceus*) chicks, but increased their growth rate, irrespective of hatching order. Furthermore, antioxidant supplementation reduced and increased the level of plasma oxidative damage (malondialdehyde) of marginal and core chicks, respectively. Selection appeared to favour the strategy of investing antioxidants to boost traits (growth) that would increase the capacity of successfully competing with siblings. It was unclear why antioxidants decreased oxidative damage in marginal but not in core chicks, although their growth rates and begging behaviours did not differ.

In another study on an altricial bird species, Boncoraglio et al. (2012) found that barn swallows (*Hirundo rustica*) nestlings with higher plasma oxidative damage had poorer begging performance in terms of posture and duration, two traits of begging that are known to influence parental food allocation in barn swallows (Boncoraglio and Saino 2008). The relationship between begging bout duration and oxidative damage was stronger under food deprivation conditions, possibly reflecting a contribution of nutritional stress. However, there was no relationship between begging and plasma non-enzymatic antioxidant capacity. Results of this study also suggested that, given the individual consistency of oxidative damage over the nestling period, behavioural begging may integrate information on oxidative damage nestling and so signals offspring quality to their parents (Boncoraglio et al. 2012). Parents may consequently exploit this information to adjust their care allocation strategies in order to optimise fitness.

Moreno-Rueda et al. (2012) also found that begging may entail some oxidative costs in birds. Magpie (*Pica pica*) chicks experimentally solicited to spend more time begging suffered more oxidative damage, but such a relationship emerged only when body mass gain and immune response were controlled for in the models. However, there was no relationship between begging duration and superoxide dismutase, glutathione peroxidase or glutathione reductase, respectively. Hence, it is possible that by reducing growth and immune response, two processes that generate pro-oxidants, nestlings avoided oxidative damage (Moreno-Rueda et al. 2012).

There is also indirect evidence in favour of an oxidative cost of competition. This evidence comes from studies where the oxidative stress of nestlings was compared among different brood sizes or where the brood size was enlarged or reduced. These studies found that oxidative damage was higher in larger broods of wild Eurasian kestrels *Falco tinnunculus* (Costantini et al. 2006) and wild common starlings *Sturnus vulgaris* (Costantini et al. 2010; Bourgeon et al. 2011); antioxidant defences were lower in larger broods of captive zebra finches *Taeniopygia guttata* (Alonso-Alvarez et al. 2006), wild common starlings *Sturnus vulgaris*

(Costantini et al. 2010; Bourgeon et al. 2011) and wild great tits *Parus major* (Costantini et al. 2010). However, oxidative damage was found to be lower in larger broods of wild great tits (Costantini et al. 2010), and blood oxidative damage or antioxidant defences were not influenced by the manipulation of brood size in wild red-winged blackbirds *Agelaius phoeniceus* (Hall et al. 2010) and wild great tits (Losdat et al. 2010), respectively. These studies were done in a way that makes it difficult to determine to what degree behaviours such as begging or fights among siblings contributed to shape oxidative balance because there were additional sources of variation, such as parental effort in chick rearing or nestling growth rate, that likely affected the oxidative status of individuals.

Although oxidative stress might be a cost of competition, it could also be hypothesised that competition is antioxidant demanding; hence, oxidative stress might also contribute to constrain the intensity of begging itself. Great tit nestlings hatched from eggs laid by carotenoid-supplemented females and raised in small broods begged more intensely than nestlings from eggs laid by females that were not supplemented (Helfenstein et al. 2008). Similarly, supplementation of vitamin E to yellow-legged gull (*Larus michahellis*) chicks enhanced the total number of calls, especially in smaller chicks (Noguera et al. 2010). However, supplementation of a cocktail of vitamins (including C and E) and minerals to red-winged blackbird nestlings did not influence their begging behaviour (Hall et al. 2010). Strategies of competition may, however, be many, so antioxidants may be used to grow faster rather than to beg stronger (Hall et al. 2010).

5.5 Oxidative Stress Risks Through the Transitions from Dormancy to Arousal and Back

Oxidative stress is a threat for organism functions not only during phases of intense physical activity but also when the organism passes from a metabolically depressed dormant state to an active one. This is what happens in species that hibernate, aestivate or enter a short-term torpor. Many species have evolved the ability to suppress basal metabolic rate and enter a hypometabolic or dormant state during periods when food occurs in very limited supply or environmental conditions become too energetically and physiologically demanding to manage. This is what occurs in seasonal environments characterised by cold or hot periods and dryness, or in environments that are continuously cold like Polar Regions. Intertidal organisms also daily experience a transition from an active and fully aerobic state during high tides to an inactive and metabolically depressed state during low tides. Many species may also enter short-term periods of metabolic suppression, such as during torpor in mammals or birds. Metabolic suppression can therefore come in varying degrees, ranging from a 20–30 % reduction in metabolic rate for a few hours during nightly torpor in small birds and mammals to over 95 % reduction in metabolic rate for many weeks during seasonal hibernation in mammals or diapause

in insects, through to a virtually ametabolic state that allows desiccated seeds, spores and cysts of many plant and animal species to remain viable for many years (Storey and Storey 1990; Hand and Hardewig 1996; Storey 2001; Staples and Brown 2008). For all of these species, problems come up both during the dormant state and, in particular, at arousal. For example, many species that have colonised regions that are seasonally or perpetually cold go into hibernation. Over the dormant state, they have to face with temperatures that fall substantially below 0 °C. Lipids are the primary substrates for energy production while hibernating (Heldmaier et al. 1999), and, consequently, there is more availability of substrates prone to be peroxidised. Hibernating species have evolved various mechanisms to avoid the lethal intracellular freezing, but oxidative damage to tissues may occur and jeopardise anti-freezing mechanisms (Costanzo and Lee 2013). Similarly, species that aestivate in warm environments have to withstand stresses induced by heat and lack of water. During the transition from a dormant to an active state, cells are reoxygenated and the metabolic rate increases. Consequently, there is also an increase in the production of reactive species, which raises the need for the organism of investing in antioxidant protection to avoid severe damage. Several studies found that antioxidant defences may be upregulated during a dormant state or at arousal (Fig. 5.5) in a diverse group of species, from terrestrial snails and frogs to snakes and squirrels (Hermes-Lima and Storey 1993; Hermes-Lima et al. 1998; Carey et al. 2000; Hermes-Lima and Zenteno-Savin 2002; Orr et al. 2009; Salway et al. 2010). It is thought that this is a strategy selected to prepare the organism to withstand the oxidative burst induced by the increase in oxygen consumption and the tissue reoxygenation experienced during the dormancy–arousal transition (Hermes-Lima et al. 1998). The need to have antioxidant defences high during dormancy might be one reason that explains why uric acid concentration increased in channelled apple snails (*Pomacea canaliculata*) during aestivation and that levels of allantoin (the product of non-enzymatic oxidation of uric acid) increased 24 h after arousal (Giraud-Billoud et al. 2011, 2013). Urate is certainly a product of nitrogen metabolism, but it also has antioxidant properties (Chap. 1). Hence, its accumulation over the aestivation may be favoured because it may give a selective advantage in terms of better protection against oxidative stress.

That keeping antioxidant defences up at arousal might be protective against oxidative stress was, in particular, demonstrated by a study on male Syrian hamsters *Mesocricetus auratus* (Bertuglia and Giusti 2003). Plasma hydroperoxides did not change during a period of hypoxia, while they increased by 90 and 72 % within 5 and 15 min of reperfusion, respectively, and began to decrease significantly after 15 min of reperfusion. The increase in plasma hydroperoxides was, however, not observed in hamsters that were injected a bolus of superoxide dismutase 30 min before the reoxygenation (Bertuglia and Giusti 2003).

Various species are very flexible in their aestivation behaviour, being able to emerge rapidly from their subterranean burrows, thereby permitting opportunistic feeding and breeding when availability of water and food increase. To do so, these species have to prioritise protection of skeletal muscle to avoid atrophy, which would compromise the post-aestivation activities. For example, estivating green-

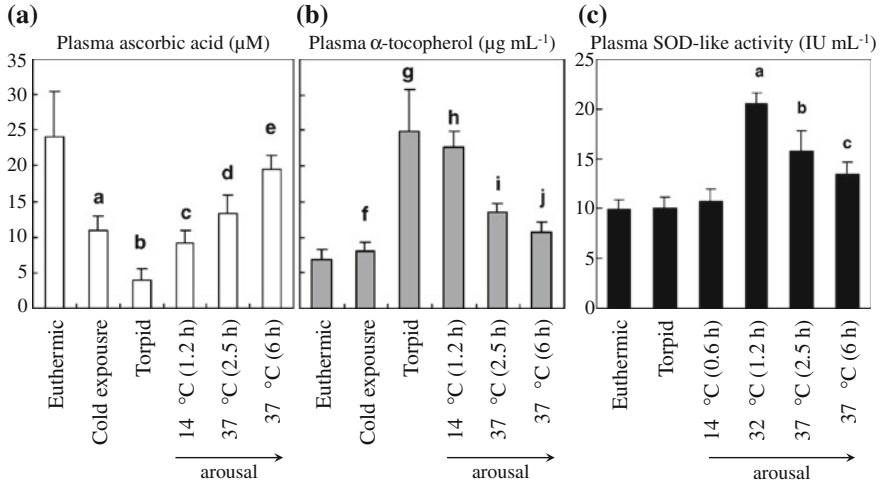

Fig. 5.5 Changes in antioxidant levels during arousal from torpor in Syrian hamsters (*Mesocricetus auratus*). **a** and **b** Blood was taken at the indicated points during arousal from torpor. Sampling of blood at 37 °C was performed 2.5 and 6 h after the onset of arousal. Hamsters that did not hibernate, but were acclimatised under the conditions of cold (5 ± 1 °C) and 2:22-h light/dark cycle for 7 weeks, were used as cold control animals. *a* $P < 0.01$ compared with the euthermic or 37 °C (6 h) groups, $P < 0.05$ compared with the torpid group; *b* $P < 0.01$ compared with the euthermic, 37 °C (2.5 h) or 37 °C (6 h) groups, $P < 0.05$ compared with the cold exposure group; *c* $P < 0.01$ compared with the euthermic or 37 °C (6 h) groups; *d* $P < 0.01$ compared with the euthermic or torpid groups; *e* $P < 0.01$ compared with the cold exposure, torpid or 14 °C groups; *f* $P < 0.01$ compared with the torpid or 14 °C groups; *g* $P < 0.01$ compared with the euthermic, cold exposure, 37 °C (2.5 h) or 37 °C (6 h) groups; *h* $P < 0.01$ compared with the euthermic, cold exposure, 37 °C (2.5 h) or 37 °C (6 h) groups; *i* $P < 0.01$ compared with the torpid or 14 °C groups, $P < 0.05$ compared with the euthermic group; *j* $P < 0.01$ compared with the torpid or 14 °C groups. **c** Upregulation of superoxide dismutase (SOD) in mid- to late arousal from torpor. Blood was taken at rectal temperatures of 14, 32 and 37 °C (1.2, 2.5 and 6 h after arousal induction, respectively). *a* $P < 0.01$ compared with all other group; *b* $P < 0.01$ compared with the euthermic, torpid, 14 or 32 °C groups; *c* $P < 0.01$ compared with the euthermic, torpid or 32 °C groups. Values are shown as mean plus standard deviation. Reprinted with minor modifications from Okamoto et al. (2006) with permission from Elsevier

striped burrowing frogs (*Cyclorana alboguttata*) keep their mitochondria quiescent in order to reduce free radical production, maintain uncoupling proteins active, upregulate the activity of superoxide dismutase 2 and prioritise combustion of lipids to spare proteins in their muscle (Hudson et al. 2006; Reilly et al. 2014). Muscles of different function use different biochemical regulation mechanisms to avoid increased damage at higher temperatures during aestivation, and protective priority is given to jumping muscles, which have important post-aestivation locomotor functions (Young et al. 2013). This is further supported by a study showing that, while mitochondrial production of reactive species is strongly reduced in muscle, it is not in cardiac muscle fibres (Reilly et al. 2014).

Various studies showed that not all antioxidant defences are kept upregulated while in a dormant state. In 13-lined ground squirrels (*Spermophilus tridecemlineatus*), for example, some of the change in catalase activity during hibernation was interpreted to be an effect of an alteration in energy metabolism (Page et al. 2009). Moreover, some studies showed that the upregulation of antioxidant defences may not occur or simply is not maintained throughout the dormancy period. For example, superoxide dismutase expression or activity did not differ between active and hibernating ground squirrels (Page et al. 2009). Similarly, a comparison of antioxidant status of six organs between two-month estivating and 10-day awakened Couch's spadefoot toads (*Scaphiopus couchii*) showed that the activities of several antioxidant enzymes and the concentration of glutathione were generally lower in estivating toads (Grundy and Storey 1998). Moreover, products of lipid peroxidation (conjugated dienes, lipid hydroperoxides) were higher in tissues of estivating than awake toads, suggesting accumulated oxidative damage to lipids during dormancy (Grundy and Storey 1998). These results suggested that not all species evolved anticipatory response mechanisms. For example, red-eared slider turtles (*Trachemys scripta elegans*) had suppressed antioxidant enzyme activities when experiencing 20 h under anoxic conditions, simulating a period of torpor. However, they did not suffer any increase in oxidative damage during anoxia nor reoxygenation (Willmore and Storey 1997a, b). This might be because red-eared slider turtles have high basal antioxidant levels compared to other non-mammalian species or in the range of some mammal species (Hermes-Lima et al. 2001; Hermes-Lima and Zenteno-Savín 2002). Therefore, turtles would not need to invest in upregulation of antioxidant defences. This might be explained to some extent by the diving habits of turtles, which are therefore exposed to repeated periods of anoxia–reoxygenation that require high constitutive antioxidant defences if compared with non-diving species. However, the duration of the dormant state might be also important. Toads were estivating for 2 months, while turtles were dormant for only one day. Therefore, the response to the dormancy–arousal transition might be biphasic, with short-term periods of inactivity being stimulatory, while long-term periods being inhibitory of the individual resistance to oxidative damage.

Suppression of antioxidant defences observed in turtles would not be compatible with the idea of an increase in resistance to oxidative stress through hormesis. Therefore, some species could have evolved other strategies to control an excessive increase in oxidative stress during arousal. It has been suggested that some species can avoid profound tissue hypoxia, thus making the transition less stressful in terms of oxidative burst (Page et al. 2009). For example, in the Arctic ground squirrel (*Spermophilus parryii*), the torpid state is associated with both high arterial partial pressure of oxygen and low expression of the hypoxia inducible factor-$1-\alpha$ (Ma et al. 2005). It was also suggested that some species might mostly rely on repair systems that would be activated to detoxify the body from accumulation of oxidative damage products generated at arousal (Hermes-Lima and Zenteno-Savín 2002). Uncoupling proteins and other less classical antioxidant mechanisms may also be important. For example, the proteus *Proteus anguinus* has exceptional anoxia tolerance, although it

does not present the ordinary responses mediated by antioxidant enzymes recorded in many other species (Issartel et al. 2009). However, the expression of a particular kind of uncoupling protein was found to be increased by 170 % during reoxygenation (Issartel et al. 2009).

5.6 Conclusions

There is strong evidence supporting a link between physical effort, oxidative stress and hormesis. Oxidative stress represents a ubiquitous response to physical activity, and hormesis may provide an important mechanistic and theoretical framework to examine the response to and the biological effects of physical effort. Studies on laboratory models showed that hormetic responses to physical activity can occur in various tissues, such as bone, brain, heart, intestine, liver or skeletal muscle (e.g., Caillaud et al. 1999; Ogonovszky et al. 2005; Pereira et al. 2007; de Lira et al. 2008; Rosa et al. 2008; Stranahan and Mattson 2010). A unifying feature that may underlie the qualitatively similar hormetic response to physical effort is oxidative stress. Most work to date has involved laboratory rodents. We now need to translate these paradigms to natural animal populations in order to assess why some activity patterns are favoured over others under specific circumstances and how oxidative stress induced by physical activity may constrain performance and life history decisions. There is much scope for novel experimental work in which the effects of physical activity are assessed during different life stages and in different species or environments. The merger of biochemical and genomic tools may greatly further our understanding of the biological consequences of physical activity. Genome analysis may inform us about the evolution of mechanisms that deal with costs associated with different modes of locomotion. For example, whole-genome sequencing of two distantly related bat species (the black flying fox *Pteropus alecto* and the mouse-eared bat *Myotis davidii*) showed that a high proportion of genes in the DNA damage checkpoint–DNA repair pathway were found to be under positive selection in the bat ancestor (Zhang et al. 2013). This may indicate that the necessity of controlling oxidative stress has represented an important need in the evolution of flight (Zhang et al. 2013).

Reactive species may be a threat or a promoter of physiological compensatory responses (hormesis) not only during phases of intense physical activity but also when the organism goes into a dormant state or passes from a metabolically depressed dormant state to an active one. Evidence from species that hibernate or aestivate suggests that organisms may upregulate mechanisms that limit oxidative damage while in the dormant state or at arousal. Strategies differ among species, which limits generalisations. There has been little attempt to put these mechanisms in a more general framework in order to assess their impact on survival perspectives and life history traits under differing circumstances. We therefore need studies that go beyond the mechanistic explanations if we really aim to understand

the biological relevance of oxidative stress in species where the dormant–arousal transition is a central stage of their life. For example, we need studies that examine the extent to which variation in the physiological responses to the dormant–arousal transition is functionally significant and, if heritable, to which extent this variation may be a target of natural selection.

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Chapter 6
The Costs of Make-up in Sexual Selection and Social Signalling

Abstract Scientists have long been fascinated by the evolution of body colourations and, in particular, of sexually selected ornaments. Males may develop carotenoid- and melanin-based ornaments or achromatic morphological sexual signals. Females may also produce pigmented or non-pigmented patches that are important in social competition with females and in the context of sexual selection. The development of these signals may impose costs to both males and females, but the underlying physiological mechanisms are not well understood yet. The development of colour signals can also be costly in contexts beyond that of mate choice, such as in the case of quality signalling in young or in warning signalling (e.g., Batesian and Müllerian mimicry). Recent research has focussed on how the building up of body colourations induces oxidative stress and how oxidative stress itself may constrain investment in signalling. This chapter examines the many hypotheses that link carotenoid- and melanin-based colourations to the capacity of the bearer to withstand oxidative stress. It also examines the role of oxidative stress in the development of colour signals in young and of conspicuous body colourations that are used by some species to signal their unprofitability to predators.

6.1 Visual Sexual Signalling in Males

Production of secondary sexual signals is a key component of reproductive investment of males in sexually reproducing species (Darwin 1871; Andersson 1994). A biological signal can be defined as "*any act or structure which alters the behaviour of other organisms, which evolved because of that effect, and which is effective because the receiver's response has also evolved*" (p. 15 in Maynard Smith and Harper 2003). The scenario gets complicated when different secondary sexual traits occur simultaneously in a same species. Some contrasting hypotheses have sought to explain the evolution of multiple signals (Zuk et al. 1992; Møller and Pomiankoski 1993; Johnstone 1996; Candolin 2003; Lozano 2009): the *multiple messages hypothesis* states that each sexual trait signals a different quality

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of the male; the *redundant signal hypothesis* states that each trait provides a signal of the individual overall quality, so errors in assessment of signals by the receiver may be reduced; the *unreliable signals hypothesis* states that a female may need to look at multiple traits because one trait could be an unreliable indicator of overall male quality; the *sexual interference hypothesis* proposes that additional male signals evolve as a way for males to hinder female mate choice by interfering with the propagation and reception of other males' sexual signals; hence, females have responded by evolving the ability to glean meaningful information from male signals.

Regardless of the mechanisms that explain evolution of single or multiple sexual signals, it is well established that conspicuousness of ornaments varies largely among males. It is thought that this variation underlies differing qualities of males and that this information is translated in the expression of secondary sexual signals. Females may use this information to select a mate that will bring to her and to her offspring some evolutionary fitness benefits (Fisher 1915; Kirkpatrick and Ryan 1991; Andersson 1994). Fitness benefits of having a high-quality partner are clear, whether these be in terms of the genetic benefits to the offspring, contribution to parental care, provision of a high-quality development or rearing environment or avoidance of infectious diseases. It follows that males in better condition or genetic quality would be expected to express more exaggerated ornamental traits than lower-quality individuals. For condition-dependent signals to work in evolutionary terms, their production should carry costs that only high-quality males would be able to afford. They also need to be honest; that is, the information they convey needs to be reliable; otherwise, the ornament would be quickly counter selected if males were attempting to cheat (Zahavi 1975; Zahavi and Zahavi 1997). For example, Pike et al. (2007c) showed that male sticklebacks (*Gasterosteus aculeatus*) maintained on a low-carotenoid diet allocated more carotenoids to their nuptial colouration in an attempt to maintain sexual attractiveness. Not only were they not chosen by females in mate choice trials, but also their capital investment in sexual colouration was paid in terms of higher oxidative damage and lower survival (Pike et al. 2007c). Hence, stickleback females were able of making adaptive mate choice decisions on the basis of male colourations.

In order to provide mechanistic support for the Zahavi's (1975) handicap principle, Hamilton and Zuk (1982) suggested that male secondary sexual traits indicate heritable variation in parasite resistance. It was later suggested that increases in energy expenditure or predation risk might also be two currencies underlying the costs of ornament production (Andersson 1994). It has been later realised that expression of ornaments may be sensitive to oxidative stress. Hence, it has been hypothesised that oxidative stress may be one important agent linking the expression of sexual ornaments to genetic variation in fitness-related traits, thus promoting the evolution of female's mate choice and male's sexual ornamentation (von Schantz et al. 1999). Since then, research has focussed on how the building up of secondary sexual traits based on carotenoids or melanins induces oxidative stress and how oxidative stress itself may constrain investment in signalling.

6.1.1 Carotenoid-Dependent Secondary Sexual Traits

Carotenoids are lipophilic pigments that animals are unable to synthesise de novo; hence, they must rely on dietary sources (Hill and McGraw 2006a, b). The quantity of carotenoids that is available in food may further be limited by factors like parasites or interactions with other nutrients that may limit their intestinal absorption (e.g., Tyczkowski et al. 1991; Surai 2002).

Carotenoids form the basis of many yellow, orange and red sexual ornaments in the animal kingdom, including fish, reptile and bird species (Hill and McGraw 2006a, b). Depending on the species, carotenoids are used for colouring the feathers or areas of skin, such as legs, bellies, flanks, feet and eye rings (Hill and McGraw 2006a, b). In birds, carotenoid pigmentation is more common in the bare parts than in the plumage, and the yellow colourations appear to be more widespread than the red ones (Olson and Owens 2005).

Interest in carotenoid-based signals originated with the classic studies on guppies (*Poecilia reticulata*) published in the early 1980s (Endler 1980, 1983). These studies showed that in some, but not all, populations of this small fish species, females preferred to mate with males that had bright carotenoid-based orange spots on their skin. This preference may have originated because it enabled females to obtain high-quality mates. Rodd et al. (2002) proposed an alternative hypothesis: the origin of the female preference for bright males is a pleiotropic effect of a sensory bias for the colour orange, which might have arisen in the context of food detection. This would imply a sensory bias (Rodd et al. 2002) that may have been favourably selected because of the adaptive benefits it carries: feeding on orange food may provide benefits in terms of intake of antioxidants, as well as mating with bright males may carry genetic or resource-based benefits for the female if the male colour signals honestly its healthiness.

Research on carotenoid-based sexual ornaments (especially in birds) has since then expanded considerably. The main reason for this growing interest in carotenoid-based colourations was that, given their limited availability in the environment and their multiple physiological uses (e.g., immune stimulation, antioxidant protection), the allocation of carotenoids to the production of sexual traits would give rise to conflicting resource allocation trade-offs that only healthy males would be able to solve (Lozano 1994; Møller et al. 2000; Hill and McGraw 2006a, b).

It was suggested that carotenoid-based colourations might convey to the female information about the capacity of the male to withstand oxidative stress (von Schantz et al. 1999). The hypothesis was built up on the assumption that carotenoids are important antioxidants. Therefore, diverting carotenoids from antioxidant protection to production of sexual colouration would expose the organism to oxidative stress that only high-quality males may cope with. In 2004, however, Hartley and Kennedy (2004) proposed that the antioxidant role of carotenoids be reconsidered because unlikely to be so important as previously thought. Hartley and Kennedy's idea was later supported by meta-analysis (Costantini and

Møller 2008; Simons et al. 2012) and a comparative study of 36 species (Cohen and McGraw 2009), which found that the role of carotenoids as antioxidants is weak to non-significant in studies on nestlings or adult birds, a taxon that so far has received the greatest attention from evolutionary ecologists. For example, earlier correlative or supplementation studies on nestling kestrels (*Falco tinnunculus*) proposed to revaluate the antioxidant role of carotenoids in birds given the absence of a significant effect of carotenoids on oxidative stress during a such stressful phase like that of growth and development (Costantini et al. 2006, 2007a). The importance of carotenoids as antioxidants was also questioned in studies on lizards, although some variation may occur between different male colour morphs (Olsson et al. 2008, 2009). Mixed results were also found in fish (Wang et al. 2006; Pike et al. 2007c).

Hartley and Kennedy (2004) proposed two alternative hypotheses to explain why carotenoid-based signals are so widespread and seemingly associated with mate quality and reproductive advantage. First, since carotenoids are prone to oxidation and consequent bleaching, they may signal the availability of other, more potent and limiting antioxidant resources, rather than being involved in physiological trade-offs themselves (later named the *protection hypothesis* by Pérez et al. 2008). Second, if carotenoids are wholly or partly the resource being advertised, then they are signalling other qualities, such as their contribution to cell signalling, gene activation, regulation of immune activity, tissue repair or embryonic development (named the *alternative function hypothesis* by Metcalfe and Alonso-Alvarez 2010). A third hypothesis proposes that carotenoids might exert detrimental effects on cell membranes, potentially acting as pro-oxidants (*toxicity hypothesis*) when occurring in the body at high doses (Zahavi and Zahavi 1997; El Agamey et al. 2004; Costantini et al. 2007b) or under certain contexts (Simons 2013; Beamonte-Barrientos et al. 2014). Consequently, individuals producing carotenoid-based traits would reveal their capacity to endure this handicap (Metcalfe and Alonso-Alvarez 2010). More recently, Johnson and Hill (2013) proposed that the oxidation of carotenoids for the purpose of ornamentation in birds and reptiles is coupled to the inner mitochondria membrane, so that ornamental colouration would more generally reflect the efficiency of cellular respiration instead of resistance to oxidative stress only.

Although evidence in favour of a link between carotenoid-based colouration and capacity to withstand oxidative stress in adults is still weak (see next sections), many studies have found positive effects of carotenoid supplementation on traits as diverse as escape flight (Blount and Matheson 2006), parental care (Pike et al. 2007b), sperm quality (Helfenstein et al. 2010), song (Van Hout et al. 2011; see also Baldo (2012) for a link between oxidative damage and song) or immunity (Simons et al. 2012). Hence, either the antioxidant action of carotenoids is so specific that the available methods to assess oxidative stress have not allowed investigators to grasp an antioxidant effect of carotenoids or should we look at other physiological functions (not only as enhancers of immune activity) that carotenoids may have. There are also other possibilities. For example, there may be a loss of integration between sexual ornament and the rest of organism's traits (Badyaev 2004). This would imply that

mechanisms underlying the elaboration of a sexual trait would work as a module, hence almost independently from other physiological mechanisms, such as those regulating the oxidative balance. A main consequence might be a limitation of costs for the male (Badyaev 2004), which would result in a weak association between carotenoids and oxidative status parameters. This would mean that a greater expression of sexual ornamentation and usage of carotenoids would not induce a corresponding increase in the oxidative cost. Alternatively and specifically for carotenoid-based plumage colourations, we have to consider that they are complex traits because the colouration is dependent on several distinct and partially developmentally independent components, such as type and quality of carotenoids, size and symmetry of patch area (Badyaev et al. 2001). Therefore, there may be significant differences in selection pressures on the various components of colouration. Differential selection would be evident in distinct fitness components (e.g., fecundity, survival) operating on different traits of ornamentation as shown in studies on the house finch *Carpodacus mexicanus* (Hill 1992, 1993; Badyaev et al. 2001). Another important implication of the complexity of a carotenoid-based plumage trait is that costs of production of its components are likely to differ among individuals and environments (Badyaev et al. 2001). For example, while some carotenoids are deposited unchanged through a metabolically cheap route, other carotenoids are metabolically converted in other forms through a more costly metabolic route (Hill 1996; Stradi et al. 1997). Although biotransformation of carotenoids is genetically controlled, the metabolic costs associated with it might be relevant for the overall energetic budget and might vary across individuals and contexts.

In summary, whether the biochemical pathways underlying the development of distinct ornament components differ, it may result that different molecular components of oxidative balance were related to distinct traits of an ornament. If this were the case, selection pressures operating on distinct ornament traits would also operate differently on the cited oxidative balance components, possibly limiting biochemical integration among them (e.g., conflict between sexual and natural selection). This implies that general patterns of association between ornaments as a whole and oxidative stress may be difficult, if not impossible, to detect. Therefore, it might be important that experimental investigations of the link between oxidative stress and carotenoid-based colouration consider the nature of the ornament, the nature of the biochemical parameter of oxidative status and the biochemical pathway that links a certain oxidative status parameter to ornament development.

6.1.1.1 The Protection and the Sparing Hypotheses

The protection hypothesis is supported by studies on fish or birds, where experimental provision of non-carotenoid antioxidants enhanced carotenoid-based signals. Pike et al. (2007a) fed male sticklebacks a diet containing a fixed amount of carotenoids and either low or high, but biologically realistic levels of the colourless antioxidant vitamins C and E (Fig. 6.1). High-antioxidant diet males produced a more intense nuptial colouration, but not a larger pigmented area, and

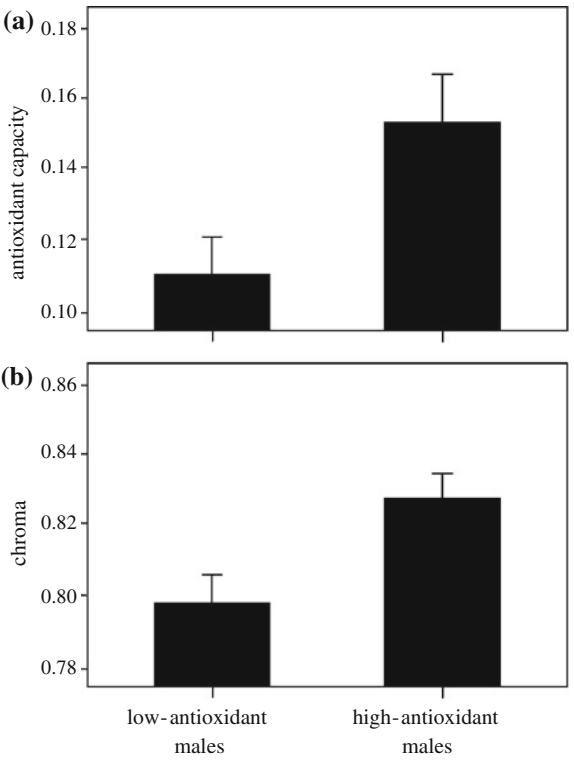

Fig. 6.1 **a** Antioxidant capacity of fish homogenate (measured in Trolox equivalents) and **b** nuptial coloration chroma for three-spined male sticklebacks (*Gasterosteus aculeatus*) on the high- and low-antioxidant diet treatments. Data are shown as mean plus standard error. Reprinted from Pike et al. (2007a) with permission from the Royal Society

were preferred over size-matched males of the opposite diet treatment in mate choice trials (Pike et al. 2007a). Moreover, the signal chroma was positively correlated with a measure of non-enzymatic antioxidant capacity performed on the homogenate of the whole fish.

Similarly, Pérez et al. (2008) modified the availability of the non-pigmentary antioxidant vitamin E in the diet of wild yellow-legged gulls (*Larus michahellis*) before egg laying by means of a supplementation experiment. Afterwards, while gulls were incubating, it was found that supplemented males had higher levels of circulating carotenoids and a larger, but not more coloured, red bill spot than control birds. The non-enzymatic antioxidant capacity of plasma was also enhanced by the vitamin E supplementation; however, the level of oxidative damage was not affected.

Similar to the above studies, administration of melatonin (hormone with antioxidant properties) enhanced the bill colouration of male zebra finches (*Taeniopygia guttata*) compared to control birds, and this effect was stronger when melatonin was supplemented in combination with carotenoids (Bertrand et al. 2006). Although melatonin may directly react with reactive species, it can, like other hormones, also regulate the activity of various antioxidant enzymes and modulate several functions, which might indirectly have an impact on carotenoids (Costantini 2010).

The protection hypothesis did not receive support from a study on wild-caught captive male greenfinches *Carduelis chloris* (Karu et al. 2008). Supplementation of carotenoids enhanced feather chroma of greenfinches, while supplementation of vitamin E did not. The basal diet of greenfinches was, however, already rich in vitamin E, which might have masked any effect of the supplementation regime (Karu et al. 2008). Similarly, a study of nestling great tits did not provide support for the protection hypothesis (Marri and Richner 2014).

Although supplementation of antioxidants seems to provide some general support to the protection hypothesis, a recent meta-analysis showed that the relationship between trait redness and oxidative stress state in birds (antioxidant defences or oxidative damage) is not significant (Simons et al. 2012). This result was consistent for both plumage and skin colourations.

Finally, it is also important to highlight the similarity of the protection hypothesis with the *sparing hypothesis* (Svensson and Wong 2011; see also von Schantz et al. 1999). Animals have many different classes of antioxidants, and if one is in excess, it may lead to the sparing of another, without changing the overall balance (von Schantz et al. 1999). For example, carotenoids may be spared from their antioxidant activity by increased levels of other antioxidants, and, conversely, other antioxidants may be spared by an increased supply of carotenoids (von Schantz et al. 1999). Hence, an increase in colour redness might not reflect a better protection of carotenoids against oxidation, but a higher availability of other antioxidants that would lead the organism to spare carotenoids and to mobilise them towards ornaments. Experimental investigations with a more in-depth biochemical approach are needed to assess whether carotenoids are better protected or spared when availability of other antioxidants increases.

6.1.1.2 The Alternative Function Hypothesis

This hypothesis states that carotenoid-based colourations might signal other qualities of male. Of these qualities, much attention has been given to the immunostimulatory role of carotenoids, hence to the capacity of carotenoid-based colourations of signalling the male immunocompetence (Lozano 1994; Hill 1999). The link between carotenoids and immunocompetence also provided a mechanistic explanation for the Hamilton and Zuk's parasite model (Hamilton and Zuk 1982) and for the Folstad and Karter's *immunocompetence handicap hypothesis* (Folstad and Karter 1992; Peters 2007). Carotenoids would improve the immune activity because they might protect immune cells against oxidative damage induced by the oxidative burst, maintain the redox environment of immune cells or influence tissue repair mechanisms and gene regulation through the action of retinoids, which are derived from carotenoids (de la Fuente 2002; Chew and Park 2004).

The alternative function hypothesis has received much support from several experimental studies and a meta-analysis. For example, male zebra finches supplemented with carotenoids produced significantly larger immune responses than control birds to a phytohemagglutinin injection, which induces a non-specific

response of T lymphocytes and macrophages (Blount et al. 2003; McGraw and Ardia 2003), as well as to injection of sheep red blood cells, which induces a humoral response to T-dependent antigens (McGraw and Ardia 2003). Moreover, supplemented zebra finches were more likely to be chosen in a mate choice experiment than control birds (Blount et al. 2003). Captive male blackbirds (*Turdus merula*) immunised with a suspension of sheep red blood cells displayed a significant decrease in bill colour, suggesting that activation of the immune system removes carotenoids stored in the beak in order to enhance the immune response (Faivre et al. 2003). Experimental results of these pioneering studies later received partial support by a meta-analysis of bird studies, which found that only the intensity of the response to a phytohemagglutinin injection was significantly and positively associated with trait redness, as well as with concentration of circulating carotenoids (Simons et al. 2012). However, carotenoid-based colour intensity was not associated with antibody production against experimentally induced agents, parasite load or white blood cell counts (Simons et al. 2012). It is worth highlighting that the relationships between circulating carotenoids and immunocompetence or oxidative status were not dependent on whether or not a bird species exhibited carotenoid-dependent colouration (Simons et al. 2012). Although this finding suggested that carotenoids may serve the same physiological functions in species having or not having carotenoid-dependent colourations (Simons et al. 2012), relevant variation among species exists for other aspects (e.g., in how species acquire and metabolise carotenoids).

6.1.1.3 The Toxicity Hypothesis

Beyond potential detoxification functions, carotenoids may also be detrimental. For example, it was shown that high but still physiologically relevant concentrations of metabolites of β-carotene can enhance neutrophil-induced genotoxicity (i.e. damage to DNA) by inhibition of myeloperoxidase (mammalian enzyme involved in the immune oxidative burst) in combination with a subsequent increase in the formation of hydroxyl radicals (van Helden et al. 2009). Hence, depending on the concentration (i.e. hormetic effect), carotenoids might be stimulatory or toxic. The imbalance in the relative concentrations of antioxidants could explain the increased oxidative damage caused by carotenoid supplementation. For example, the synergistic protection provided by carotenoids and vitamins C and E depends upon a balance among these compounds (Palozza 1998). It is also known that an increase in the concentration of one of them can disturb the balance, causing a decrease in the effectiveness of the antioxidant machinery (Wefers and Sies 1988). An increase in carotenoid concentration may, for example, cause the formation of carotenoid radicals or adducts at a level that impedes the tocopherol/ascorbate pool from coping with oxidative stress successfully, leading to pro-oxidant effects (Young and Lowe 2001). Moreover, carotenoids at high concentrations tend to aggregate or crystallize out of solution (Gruszecki 1999).

Such aggregates have been directly observed in cell membranes, and they are thought to affect the properties of the membrane profoundly by increasing its fluidity and permeability, ultimately causing pro-oxidant effects.

The idea that individuals producing carotenoid-based traits would reveal their capacity to endure the handicap of having high oxidative stress or other carotenoid-dependent toxic effects is intriguing, but so far, there have been a few attempts to test this hypothesis. Studies on bird species found that carotenoids may have toxic effects. A study on captive Eurasian kestrels (*F. tinnunculus*) tested the effects of carotenoid supplementation lasted 28 days on serum oxidative status and carotenoid-based colourations (Costantini et al. 2007b). The Eurasian kestrel is a raptor species that displays sexual dimorphism in the carotenoid-based colouration of the skin, with males being more orange/red than females (Casagrande et al. 2006). Compared to controls, supplemented birds had an increase of around 90 % in serum carotenoids, 82 % in serum hydroperoxides (Fig. 6.2), 16 and 15 % in the red and yellow colorimetric components of tarsi colour and a 6.2 % loss in body mass. Hence, the increase in colour intensity was accompanied by an increase in the production of oxidised molecules. Conclusions from this study are, however, limited to a captive context, where the dietary regime and the physiological status of birds may not accurately reflect those of wild birds. Moreover, there is no evidence yet of pro-oxidant activity of carotenoids in wild birds, and correlations between skin colouration components and oxidative damage in wild kestrels are variable in strength and sign (Casagrande et al. 2011a). However, baseline serum carotenoids of captive control kestrels (95 % confidence interval 34.96–46.48 μg mL^{-1}) were within the range of those recorded in wild adult kestrels measured during the courtship phase (95 % confidence interval 27.81–46.80 μg mL^{-1}; Casagrande et al. 2006). In contrast, circulating carotenoids of supplemented birds (95 % confidence interval, e.g., 2 weeks after the supplementation started was 56.56–82.14 μg mL^{-1}) were about twofold higher than the levels recorded in wild birds (see above). Carotenoid concentrations did not appear to be unnatural because they may be up to 60–80 μg mL^{-1} in wild kestrels (Casagrande et al. 2007, 2011a). Although there is not clear demonstration yet of how kestrel females use information content of skin colourations to select their mates, colouration of tarsi was shown to be positively associated with the number of vertebrate prey delivered by the male to the nest per time unit and with the territory quality as calculated on the basis of home-range size, habitat extension and prey availability (Casagrande et al. 2006). Hence, the skin colouration might function as an indicator of male's foraging capacity. This is very important in the kestrel, as well as in many other birds of prey, because males are responsible for provisioning the female with food during the incubation phase, and both the female and offspring for some time after hatching (Village 1990).

The toxicity of carotenoids may also occur in granivorous species, which have diets rich in carotenoids. Huggins et al. (2010) tested the physiological costs associated with the accumulation of high levels of carotenoid pigments during moult, a natural process in bird species with ornamental feather coloration. Male

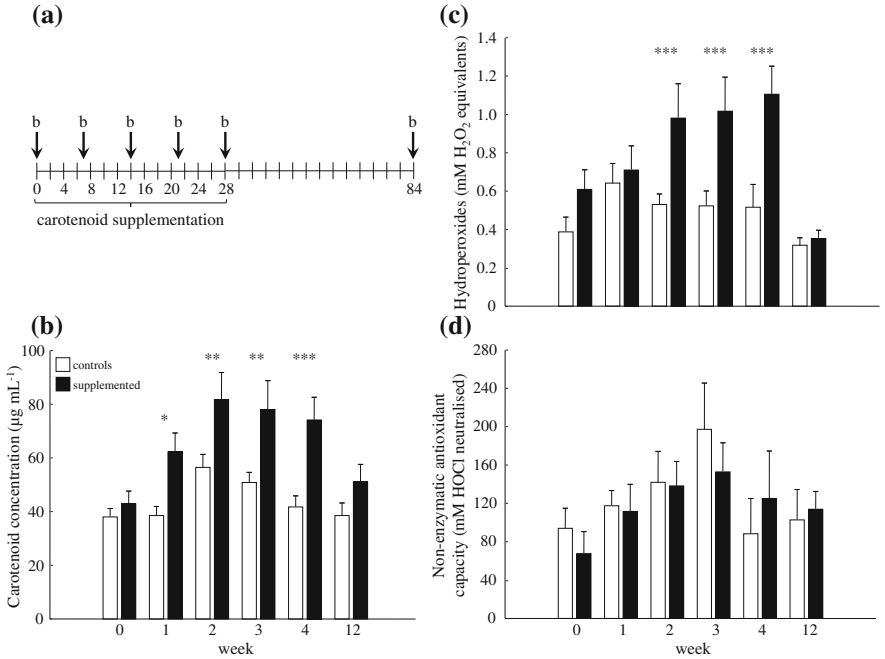

Fig. 6.2 **a** Sketch of the timing of different parts of the experiment carried out by Costantini et al. (2007b). Numbers indicate the day. Carotenoids were supplemented daily for 4 weeks to captive adult kestrels (*Falco tinnunculus*); a sample of blood was taken six times over a 3-month period. **b** The serum carotenoid concentration increased rapidly and reached a physiological threshold 2 weeks after the beginning of the supplementation period. **c** The serum oxidative damage (hydroperoxides) induced by carotenoid supplementation increased progressively but returned to baseline levels 2 months after the end of treatment. **d** Carotenoid supplementation did not significantly increase the non-enzymatic serum antioxidant capacity. Data are shown as mean plus standard error; *b* bleeding, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Reprinted with minor modifications from Costantini et al. (2007b) with kind permission from Springer Science + Business Media B.V

American goldfinches (*Carduelis tristis*), a small passerine bird that accumulates high levels of carotenoids in feathers, were maintained on physiologically high- and low-carotenoid diets through the time of natural moult. High intake of carotenoids had no measurable effect on aspartate amino transferase (indicator of hepatocellular damage), but caused an increase in creatine kinase (indicator of skeletal muscle breakdown) and a reduction in vertical flight performance (indicator of skeletal muscle integrity; Huggins et al. 2010). Any toxic effect of carotenoids may, however, have a different meaning in kestrels and American goldfinches: while in the kestrels, the colour changes dynamically depending on the changes in individual condition, the plumage colouration of the American goldfinch gets fixed after moulting.

6.1.1.4 The Inner Mitochondria Membrane Carotenoid Oxidation Hypothesis

This hypothesis predicts that several carotenoid oxidation reactions yielding ornamental pigments occur on the inner mitochondrial membrane (Johnson and Hill 2013). Three of these reactions are proposed to occur within the ubiquinone biosynthesis cluster. Johnson and Hill (2013) argued that ubiquinone and highly oxidised ornamental carotenoids share a stereochemically conserved binding region, suggesting that these two molecules may have shared similar pathways in the past. Hence, carotenoids and ubiquinones may cooperate as redox participants in anti-radical reactions or independently in helping to maintain membrane or supra-complex stabilisation during times of high-energy demand. Johnson and Hill (2013) further proposed that the hepatic inner mitochondrial membrane in birds provides a sufficient and sustainable oxidative environment with potentially compatible enzymes necessary for the sequential oxidation of dietary carotenoids to more highly oxidised forms.

There is evidence showing that carotenoids preferentially concentrate in the outer and inner membranes of mitochondria in many cell types, but these results vary and are strongly dependent on species and tissue sampled (Johnson and Hill 2013). For example, in avian hepatocytes and lymphocytes, carotenoids are highly concentrated within their mitochondria following supplementation (Mayne and Parker 1986).

The inner mitochondria membrane carotenoid oxidation hypothesis is based on the potential role that redox reactions of carotenoids may play in mitochondria. It implies that metabolic transformation of carotenoids (e.g., from reduced to oxidised forms) plays a biochemical role in protection against oxidative stress. In order to demonstrate this role, it is essential to show that oxidative non-enzymatic chemical modification (damage) to a target molecule occurs. Costs of biotransformation of carotenoids might also be related to consumption of energy and substrates needed for the reaction to work. Costs might also arise from the carotenoid-induced impairment of mitochondrial functionality and consequent stimulation of antioxidant mechanisms (Amengual et al. 2011; Lobo et al. 2012). However, species might differ in the tolerance of carotenoids that occur in mitochondria (Jonhson and Hill 2013). We know very little about how these mechanisms work and whether they are peculiar of some species or are highly conserved across animal taxa. Clearly, studies that test the inner mitochondria membrane carotenoid oxidation hypothesis in a sexual selection framework are needed.

6.1.2 Melanin-Dependent Secondary Sexual Traits

Melanins are pigments that animals synthesise from the aromatic amino acids phenylalanine and tyrosine (Hearing 1993). Melanins occur in two chemically distinct forms: eumelanin is a brown-black polymer of dihydroxyindole carboxylic

acids and their reduced forms and pheomelanin is a cysteine-containing red-brown polymer of benzothiazine units. Melanic traits are under control of the pro-opiomelanocortin gene, which is responsible for the synthesis of melanocyte-stimulating hormones and the adrenocorticotropic hormone (Lin and Fisher 2007). A meta-analysis of a small number of studies also provided some evidence for condition-dependent signalling and environmentally derived variation in melanic traits (Griffith et al. 2006).

Melanocyte-stimulating hormones and the adrenocorticotropic hormone regulate the activity of the enzyme tyrosinase, which favours eumelanogenesis to pheomelanogenesis when its activity is high (Ozeki et al. 1997; Benathan et al. 1999). Melanogenesis is also regulated by the availability of thiol groups, such as free cysteine and cysteine-containing peptides (Ozeki et al. 1997; Benathan et al. 1999). In fact, dopaquinone (from hydroxylation of the amino acid tyrosine) can react with thiol groups to synthesize pheomelanin or, in the absence of thiol groups, undergo a cyclisation that leads to the synthesis of eumelanin (García-Borrón and Olivares Sánchez 2011). Given that thiol compounds like glutathione play an important role in the regulation of cell redox status and protection against oxidative damage, it has been hypothesised that melanin-based ornaments would signal the individual oxidative stress level (Galván and Alonso-Alvarez 2008; Galván and Solano 2009; Grunst et al. 2014). The property of melanic traits to signal individual condition would be favoured by the differentiation in colour between eumelanic (black and grey) and pheomelanic (yellowish, reddish, chestnut and brown) traits (Toral et al. 2008). This is because production of pheomelanic traits needs glutathione. Hence, melanin-based ornaments might signal the ability to cope with oxidative stress because using glutathione in melanogensis might indicate that the individual has sufficient alternative antioxidant resources to counteract the decrease in glutathione itself (Galván and Alonso-Alvarez 2008; Galván and Solano 2009). This hypothesis received some partial support from correlative and experimental studies in birds and mammals.

Experimental inhibition of the γ-glutamyl cysteine synthetase (rate-limiting enzyme in glutathione synthesis) in wild great tits (*Parus major*) nestlings through the subcutaneous injection of DL-buthionine-*S*,*R*-sulfoximine (BSO) reduced, as expected, the concentration of glutathione inside red blood cells, but also increased the size of the eumelanin-based black breast stripe (Fig. 6.3) and of plasma non-enzymatic antioxidant capacity (Galván and Alonso-Alvarez 2008). Administration of diquat (herbicide that increases basal production of free radicals) in captive red-legged partridges (*Alectoris rufa*) increased the size of eumelanin-based traits and reduced that of pheomelanic ones (Galván and Alonso-Alvarez 2009). Diquat in partridges decreased red blood cell glutathione (Galván and Alonso-Alvarez 2009) and circulating carotenoids (Alonso-Alvarez and Galván 2011), but also oxidative damage measured as thiobarbituric acid reactive substances in erythrocytes (Galván and Alonso-Alvarez 2009).

In a study on a granivorous bird species, administration of BSO to captive wild-caught greenfinches (*C. chloris*) increased the blackness of the tips of tail feathers and the red blood cell glutathione and increased oxidative damage as assessed by

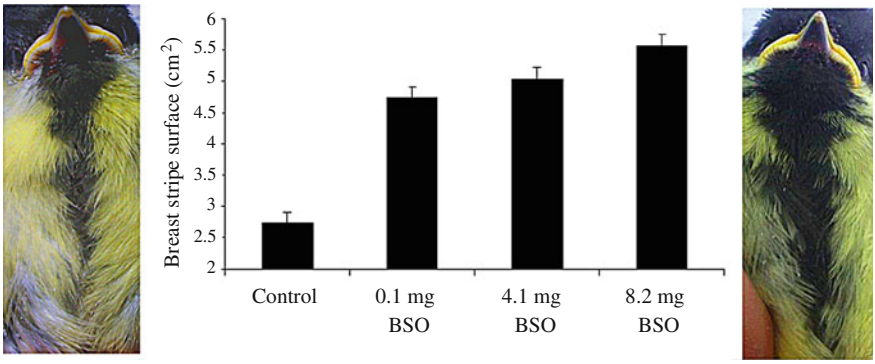

Fig. 6.3 Great tit (*Parus major*) nestlings treated with different doses of a glutathione inhibitor (DL-buthionine-S,R-sulfoximine, BSO) throughout their development showed larger breast stripes, which indicates that a reduction in glutathione levels was translated into signal expression. Least squared means + standard error are shown. Pictures on left and right sides of the figure illustrate a control and a highest BSO dose nestling, respectively. Reprinted with minor modifications from Galván and Alonso-Alvarez (2008)

quantifying the plasma concentration of malondialdehyde, an end product of lipid peroxidation (Hõrak et al. 2010). Converse to studies on great tits and greenfinches, a study on house sparrows (*Passer domesticus*) found no evidence that bib size was related to glutathione levels, but confirmed that it was negatively related to dietary calcium (Stewart and Westneat 2013).

The link between oxidative stress and melanogenesis is not peculiar of birds (examples in mammalian species: Benedetto et al. 1981; Arck et al. 2006; Wood et al. 2009; Galván et al. 2012). For example, wild boars (*Sus scrofa*) with the highest levels of pheomelanin in their hair tended to have lower levels of glutathione in their muscles (as expected whether glutathione is consumed for pheomelanin synthesis; Galván and Solano 2009) and had the highest levels of muscle oxidative damage measured as thiobarbituric acid reactive substances (Galván et al. 2012).

6.1.3 Testosterone and Ornaments

Identification of proximate mechanisms that regulate the expression of secondary sexual traits is crucial for our understanding of how such traits are regulated, which selective pressures may affect their function and evolution and which costs make these sexual signals honest. In 1992, Folstad and Karter proposed that one cost that would make the signal reliable is the suppression of the immune system (*immunocompetence handicap hypothesis*) caused by elevated levels of testosterone that occur in males during the reproductive season. This hypothesis was built up on two lines of evidence: testosterone can regulate the expression of traits as

diverse as skeletal muscle, reproductive organs or body colourations (Wingfield et al. 2001) and testosterone may be immunosuppressive; hence, it may increase the susceptibility to pathogens (Grossman 1985). Therefore, only high-quality males may afford to display sexual ornaments fully without suffering large loads of pathogens. The immunocompetence handicap hypothesis has, however, received equivocal support. A meta-analysis of experimental studies testing the effects of testosterone on immunity or ectoparasite load found a significant suppressive effect of testosterone on immunity, in support of the hypothesis, but this effect disappeared when multiple studies on the same species were controlled for (Roberts et al. 2004). The meta-analysis also showed no effect of testosterone on direct measures of immunity, but it showed that testosterone increased ectoparasite abundance in several studies and, particularly, in reptiles (Roberts et al. 2004). These results provided some support to the previous critics on the immunosuppressive activity of testosterone (the *immuno-redistribution hypothesis*, Braude et al. 1999). The immuno-redistribution hypothesis predicts that, when a male is under stress, immune cells are redeployed to target tissues, where they are most useful. This would explain why some studies found a decrease in immune cells in the blood. Implicit in this hypothesis is that testosterone is not necessarily immunosuppressive; hence, there would be no cost for the signaller. Kurtz et al. (2000) suggested a way to fit the immuno-redistribution into a handicap model: redistribution of immune cells would give rise to a trade-off between different components of the immune system, when more tissues compete for them, such as under stressful conditions that cause tissue damages (Kurtz et al. 2000). The extent to which this trade-off is costly has not been, however, clearly addressed yet. Poiani et al. (2000) also suggested that testosterone, like other hormones that may affect the expression of the same secondary sexual trait, may have either immunosuppressive or immuno-enhancing effects (*integrated immunocompetence model*), a hypothesis that would be compatible with the hormetic responses of immune cells to differing levels of hormones that have been reported in several studies (see Chap. 8).

Another mechanism that would make having high testosterone levels costly is the generation of oxidative stress (von Schantz et al. 1999). For example, testosterone may increase metabolic rate and, by stimulating the production of pigmented ornaments, cause allocation of carotenoids to ornaments, hence subtracting them from antioxidant protection. On the basis of these assumptions, it was coined the *oxidation handicap hypothesis* (Alonso-Alvarez et al. 2007). However, evidence in its favour is also controversial. Male zebra finches that received implants filled with an antiandrogen (flutamide) had lower blood antioxidant defences than control birds; however, antioxidants of birds that received implants of testosterone did not differ from those of controls (Alonso-Alvarez et al. 2007). Importantly, the hormonal treatment did not affect the carotenoid-based bill colouration. Note, however, that the sample size was small. In another study, male captive red-legged partridges (*A. rufa*), whose testosterone levels were experimentally increased, showed a stepper shading of periorbital ring colour during reproduction (Alonso-Alvarez et al. 2008). These birds also had higher levels of circulating carotenoids compared to control birds,

which was explained by a reallocation of carotenoids from signalling to antioxidant protection in order to withstand the pro-oxidant action of testosterone (Alonso-Alvarez et al. 2008). This explanation would be supported by the fact that testosterone also induced a transient elevation in levels of erythrocyte glutathione (Alonso-Alvarez et al. 2008), a non-enzymatic intracellular antioxidant synthesised by the body and used as a cofactor of activity of glutathione peroxidase (Halliwell and Gutteridge 2007). Such a transient increase might suggest a hormetic response in testosterone-treated partridges, which may explain why oxidative damage did not differ between controls and testosterone-treated birds. Note, however, that a measure of reduced glutathione should be combined with that of oxidised glutathione in order to have a more reliable estimate of the effects of an oxidative challenge (Isaksson et al. 2005).

The oxidation handicap hypothesis received some support by a study on free-living red grouses *Lagopus lagopus scoticus* (Mougeot et al. 2009). Parasite-free males were allocated to four different experimental groups: controls; receiving an implant of testosterone; infected with larvae of the parasite *Trichostrongylus tenuis*; and receiving both an implant of testosterone and the parasite infection. Testosterone implants increased comb area (a secondary sexual trait in red grouses), but also increased the lipid peroxidation as measured by malondialdehyde, a finding consistent with a handicap mechanism; the parasite challenge also increased plasma lipid peroxidation, especially when combined with the testosterone implant, and reduced the comb area in birds that were not given a testosterone implant (Mougeot et al. 2009).

Mechanisms are, however, far from being clear because testosterone was found to promote oxidative damage, but also to stimulate antioxidant defences (possible hormetic effect), to reduce or to not affect metabolic rate (reviewed in Costantini 2010). Two studies on captive birds highlighted that incongruence among studies could also be explained to some extent by the differential effects that metabolites of testosterone (17β-estradiol and 5α-dihydrotestosterone) have on oxidative stress (Casagrande et al. 2012a, b). Testosterone can be metabolised into either estradiol by the enzyme P450-aromatase or dihydrotestosterone by the enzyme 5α-reductase, but there is no transformation of the secondary metabolites into each other (Pfaff et al. 2002; Ball and Balthazart 2008). Casagrande et al. (2012b) showed that estradiol administration by implants increased oxidative damage and reduced non-enzymatic antioxidant capacity of plasma in captive Eurasian kestrels, but did not affect circulating carotenoids nor body colourations (Fig. 6.4). In contrast, dihydrotestosterone, which binds to the same receptor of testosterone, did not affect the oxidative status of birds nor their circulating carotenoids, but increased redness of the sexual signal. Finally, changes in oxidative damage or antioxidant status of plasma were not correlated with either skin redness or circulating carotenoids. These results highlighted the importance of looking at estradiol in studies where testosterone is manipulated. Conversion of testosterone into estradiol by P450 aromatase has been shown to explain several physiological and behavioural patterns in male birds (e.g., Balthazart and Ball 1998; Soma et al. 1999; Rosvall et al. 2012). The occurrence and rate of aromatase activity vary

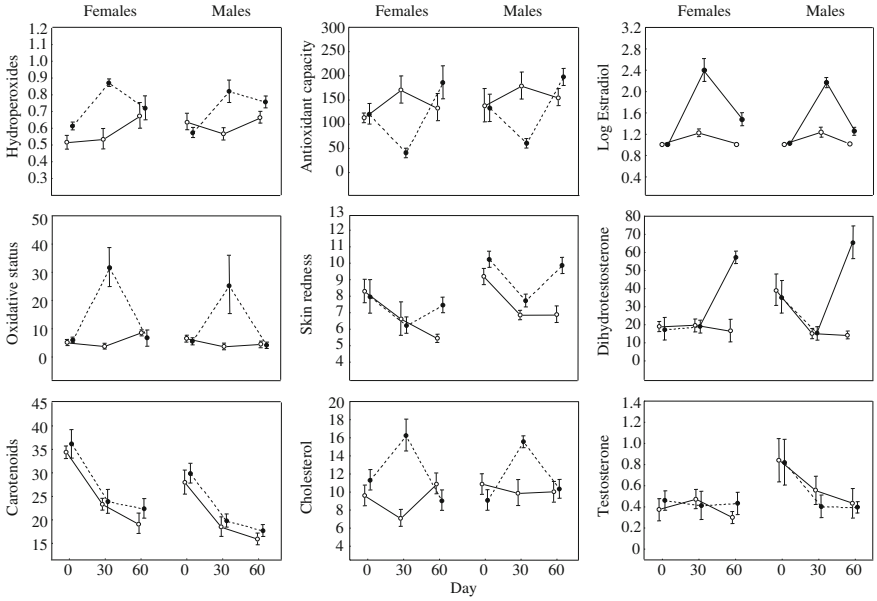

Fig. 6.4 Effects of manipulation of sexual steroids in captive adult kestrels (*F. tinnunculus*) on the variables: plasma hydroperoxides (mM H_2O_2 equivalents); plasma oxidative status (ratio of plasma hydroperoxides over plasma non-enzymatic antioxidant capacity); plasma carotenoids (μg mL^{-1}); plasma non-enzymatic antioxidant capacity (mM HOCl neutralised); skin redness (a*); plasma cholesterol (mM); estradiol (pg mL^{-1}); dihydrotestosterone (pg mL^{-1}); and testosterone (ng mL^{-1}). The graphic shows values before the start of the experiment (day 0), after manipulation from day 0 to day 30 of estradiol (day 30) and manipulation from day 30 to day 60 of dihydrotestosterone (day 60). Open circles refer to control birds (*straight line*), while *filled circles* (*dashed line*) refer to experimental birds. Data are shown as mean ± standard error. Reprinted with minor modifications from Casagrande et al. (2012a, b) with kind permission from Springer Science + Business Media B.V

among species (Fusani et al. 2001, 2003), testosterone concentration (Fusani et al. 2000, 2001) and/or time of year and sexual context (Hutchison et al. 1986; Sharp et al. 1986; Foidart et al. 1998; Soma et al. 1999). The context-dependent variation in aromatase activity might help explain some of the inconsistencies reported in the effects of testosterone on the oxidative balance (Casagrande et al. 2012b).

In another experiment, it was analysed whether testosterone metabolites or the testosterone itself influences the oxidative balance (Casagrande et al. 2012a). The effect of hormones was tested while challenging the bird's immune system in order to evaluate whether any effects of hormones on oxidative stress are dependent on whether the organism is already coping or is not coping with a stressful event. The experiment was carried out on captive diamond doves (*Geopelia cuneata*), a species showing a red-orange orbital ring, whose redness and size are increased by testosterone and dihydrotestosterone (Casagrande et al. 2011b), but are decreased by an immunological challenge through a reduction in androgen levels (Casagrande

and Groothuis 2011). Immunisation with sheep red blood cells increased oxidative damage in all experimental groups, regardless of hormone treatment, and decreased plasma non-enzymatic antioxidant capacity in all groups, except for birds that were treated with testosterone. The ratio of oxidative damage over antioxidant capacity was increased in both immune-challenged controls and estradiol-treated birds, while it did not differ between immune-challenged birds treated with androgens and non-challenged birds (Fig. 6.5). Moreover, the response of males and females to treatment did not differ. These findings did not give support to the oxidative handicap hypothesis in this species because there was no pro-oxidant effect of testosterone or dihydrotestosterone, even when the bird's immune system was challenged. Rather the data showed a slight buffering effect of testosterone on the plasma non-enzymatic antioxidant capacity in birds while mounting an immune response. This might have been due to a hormonal stimulation of antioxidant mobilisation from storage tissues towards sites, where they were needed. Interpretation of hormonal effects on oxidative balance is, however, not straightforward. Both oestrogens and androgens can induce or, in response to other molecules, show hormetic responses (Calabrese 2001a, b). Moreover, the action of sexual hormones varies across tissues, which limits inferences of studies on sexual pigmentation because they are mostly based on oxidative stress parameters measured in the blood.

6.1.4 Achromatic Morphological Sexual Signals

Males of many species did not need to evolve body colourations to attract females. Rather, they rely on the size of some secondary sexual traits. Males of collared flycatcher (*Ficedula albicollis*) and pied flycatcher (*Ficedula hypoleucea*) have a depigmented area on the forehead that has been evolved as a sexual signal (Potti and Montalvo 1991; Saetre et al. 1997; Török et al. 2003; Qvarnström et al. 2006). Of the several aspects of male quality, the forehead patch size has been shown to correlate with parameters of blood oxidative status in both flycatcher species (Markó et al. 2011; Moreno et al. 2011), suggesting that depigmented areas may also convey some information about the male oxidative status.

Pied flycatcher males with a larger forehead patch size were found to have, respectively, lower and higher levels of oxidative damage and non-enzymatic antioxidant capacity while chick feeding (Moreno et al. 2011). Similarly, collared flycatcher males with a larger white forehead patch had higher level of plasma non-enzymatic antioxidant capacity (Markó et al. 2011). In addition (1) collared flycatcher females having higher plasma non-enzymatic antioxidant capacity mated with males having larger forehead patches and (2) smaller collared flycatcher females mated with males suffering more oxidative stress (Markó et al. 2011). Similarly, pied flycatcher females paired with attractive males were more successful in reproduction while having reduced oxidative damage (Moreno et al. 2013a, b). Overall, results of these studies suggested that: high-quality males of both flycatcher species might be able to invest in the expression of sexual signals,

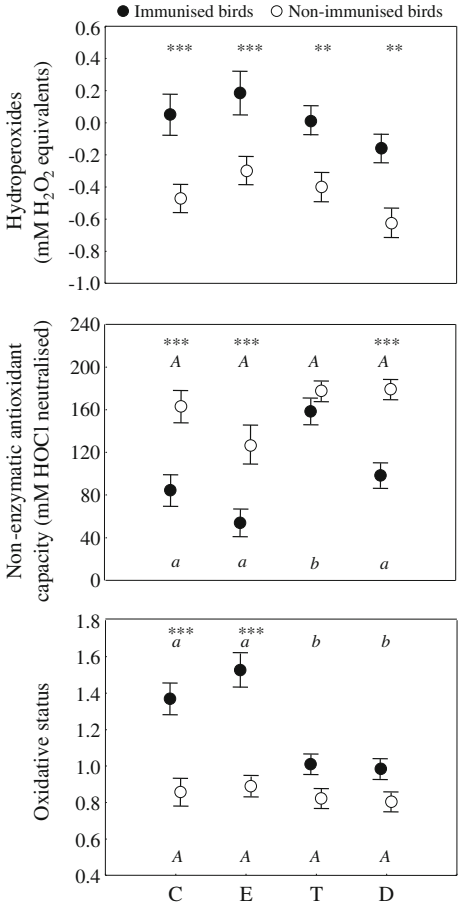

Fig. 6.5 Variation in plasma oxidative damage (hydroperoxides), plasma non-enzymatic antioxidant capacity and plasma oxidative status (ratio of plasma hydroperoxides over plasma non-enzymatic antioxidant capacity) in relation to hormonal manipulation treatment (*x*-axis) and immunization (*filled symbols* immunised; open symbols: controls). Values have been Box–Cox transformed. Letters refer to results of post hoc tests among control and the three hormone treatments within immunised or non-immunised birds. Capital letters refer to non-immunised birds. Data are shown as mean ± standard error; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, *C* control birds, *E* birds implanted with oestrogen, *D* birds implanted with dihydrotestosterone, *T* birds implanted with testosterone. Reprinted with minor modifications from Casagrande et al. (2012a, b) with permission from Elsevier

while maintaining a balanced redox state in the blood; assortative mating for the oxidative stress resistance in the collared flycatcher, where higher quality females mate with higher quality males; females may suffer less oxidative damage when mating with high quality males.

6.2 Visual Sexual Signalling in Females

Female ornamentation has received comparatively less attention than that of males in the context of sexual selection or of social interactions. In the last couple of decades, there has been growing interest in elucidating the adaptive functions of female colourations in sexual signalling (Cunningham and Birkhead 1998; Amundsen 2000; Kraaijeveld et al. 2007). Most of the work has been done on birds. Research has not focussed only on body colourations, but has also assessed the role of sexual signals external to the body, such as the pigmentation of eggshell (Moreno and Osorno 2003; see the *extended phenotype hypothesis*, Dawkins 1982).

6.2.1 Body Colourations

Production and maintenance of pigmented or non-pigmented areas of body demand resources that are costly to find and use for the ornament production (Searcy and Nowicki 2005). Moreover, females may, like males, produce badges of status (Dawkins and Krebs 1978), whose size or conspicuousness may be associated with testosterone levels, aggressiveness and status of dominance (Senar et al. 1993; González et al. 2001). There might also be metabolic costs derived from social interactions, such as when spending lots of resources to maintain a role of dominance. To test whether social interactions impose costs on signalling, Moreno et al. (2013a, b) assessed the relationship between blood oxidative damage and the white forehead patch in female pied flycatchers. Females use the white forehead patch to signal their status to other females during aggressive interactions when competing for breeding sites (Moreno et al. 2013a, b). The investigators painted white patches on females that did not have a natural patch, while females having a natural patch received white paint covering exactly their plumage patch (Moreno et al. 2013a, b). The authors then compared the reproductive investment and oxidative damage level (plasma malondialdehyde) of these females to those of females that had or did not have a natural patch. Addition of a badge to those females that did not have one caused an increase in oxidative damage, but did not influence any reproductive traits (Moreno et al. 2013a, b). These results suggest that maintenance of this badge of status demands an oxidative cost that can be afforded by some females without sacrificing reproductive investment. The balance between costs and benefits of expressing a badge of status may be dependent upon density-dependent mechanisms, i.e. the costs of competition might be severely high when density of females in a certain context is high (Moreno et al. 2013a, b). Similarly, Blas et al. (2013) proposed that the integument carotenoid-based colouration may play a significant signalling role in wider competitive contexts (e.g., social interactions) than those enforced by sexual selection. Blas et al. (2013) found that the physiological regulation of signal expression is

different in breeders and floaters (i.e. non-breeding individuals) in the black kite (*Milvus migrans*), which is a highly social bird of prey. The investigators also proposed two non-mutually exclusive hypotheses to explain results: the lower colouration displayed by floaters reflects underlying physiological limitations and mediates the access to breeding resources through social competition (*constraint hypothesis*) and young floaters have evolved mechanisms to restrain colour expression and thus signal their competitive inferiority, avoiding physiological and social costs (*restraint hypothesis*; see also Williams 1966; Curio 1983).

Carotenoid-based colourations also appear to convey information about the antioxidant status of the female. For example, female skin colouration and circulating antioxidant capacity both increased across the mating to the chick-rearing period in wild kestrels (Casagrande et al. 2011a). It is, however, difficult to explain this temporal covariation in the context of the sexual selection theory since it is likely that the signalling function is less important in kestrels while chick rearing than while mating. In kestrels, like in other birds of prey, males are responsible for provisioning the female with food during the incubation, and both the female and offspring for some time after hatching. It might be speculated that males adjust their investment in chick rearing upon the information they gather from the female colourations as, for example, females do when mate colours are manipulated (Alonso-Alvarez et al. 2012).

6.2.2 Egg Pigmentation and the Extended Phenotype

Moreno and Osorno (2003) proposed that eggshell colouration of bird eggs may constitute a secondary sexual trait. Eggshell colouration would signal the female's quality to the male, which in turn would adjust its reproductive investment. Eggs may occur in various colourations, depending on the species. In particular, Moreno and Osorno (2003) based their hypothesis on the role of blue-green colourations as signals of the female's antioxidant capacity. The egg green-blue colour that occurs in many species is based on the deposition of the biliverdin pigment (Kennedy and Vevers 1973; Miksik et al. 1996). Biliverdin is derived from oxidation of bilirubin, a bile pigment derived from the haeme catabolism. Biliverdin is deposited in the eggshell through the shell gland (Solomon 1997). In the body, the enzyme biliverdin reductase reduces biliverdin back to bilirubin (Jansen and Daiber 2012). Moreover, evidence from mammalian models suggests that biliverdin may have antioxidant properties (Kaur et al. 2003).

In pied flycatchers (*Ficedula hypoleuca*), it was found a negative covariation between egg colour and plasma non-enzymatic antioxidant capacity in those females whose nest construction effort was increased through removal of nest material. In control females, there was no association between egg pigmentation and female antioxidant status (Morales et al. 2008). These results were interpreted as evidence that pre-laying stressful conditions could exacerbate the antioxidant cost of depositing biliverdin into eggshells (Morales et al. 2008). Deposition of

biliverdin in the eggshell may be expected to be constrained by the availability of other antioxidants if its deposition becomes too demanding or if colourless antioxidants protect it from bleaching. For example, in blue-footed boobies (*Sula nebouxii*) the eggshell colour declines with laying order. However, those females that were carotenoid-supplemented after laying their first egg laid a second egg with an enhanced colouration compared to control females (Morales et al. 2011).

Two main problems have been argued against the role of eggshell colour as signal of female quality. First, an effective engagement of eggshell colour in sexual signalling has not been convincingly demonstrated (i.e. the capacity of males to discriminate clutches by colour; Reynolds et al. 2009). Second, although the antioxidant properties of biliverdin were described in some mammalian models (Kaur et al. 2003), they were not in avian species (Reynolds et al. 2009). There has not been any study yet that looked in depth at the biochemical role of this compound in birds. So far, studies did not provide any data about whether deposition of biliverdin in eggshell is associated with an increase in female oxidative damage. Moreover, for those species that breed inside a cavity or an artificial next-box, it is unclear how males may perceive the supposed signal of egg pigmentation.

As with biliverdin, many bird species deposit in eggshell protoporphyrin as a brown or red pigment, either as a background colour or as spotting (With 1973). Protoporphyrin shows both a pro-oxidant and antioxidant activity (Williams et al. 1994; Afonso et al. 1999; Shan et al. 2000). There may, therefore, be a link between eggshell pigmentation and female oxidative status. The role of protoporphyrin-based colouration of eggs as a sexual signal is under debate. Protoporphyrin-based pigmentation provides structural overcompensation for shell thinning caused, for example, by a deficit of dietary calcium (Gosler et al. 2011). If it also works as a signal of the female resistance to oxidative stress for the male still awaits experimental investigation.

6.3 Beyond Sex: Signalling in Social Contexts

6.3.1 Signalling in Females

Beyond the context of mate selection, body colourations may mediate social interactions (see also Sect. 6.2.1). There is also potential for the trait to influence the physiological status if the trait alters interactions with other individuals (e.g., acquisition of dominance status, competition for resource). Vitousek et al. (2013) provided support for the presence of a social mechanism that links plumage traits with the oxidative state of their bearer during trait advertisement, long after the completion of signal development. They found that naturally darker barn swallows (*Hirundo rustica*) had lower levels of plasma oxidative damage. Importantly, females manipulated to display darker ventral plumage during reproduction rapidly decreased oxidative damage, adopting the physiological state of naturally

darker individuals (Vitousek et al. 2013). Although the mechanisms underlying the link between colourations and social interaction have not been described yet, the pattern of physiological changes observed in female barn swallows could result from darker plumage conferring a social advantage (e.g., darker females receiving fewer challenges from conspecifics during the early reproductive period; Vitousek et al. 2013). It might also be that males paired with females manipulated to display darker ventral plumage invested more into reproduction, hence reducing the oxidative cost of females. This second explanation would, however, imply a link to reproductive strategies rather than to social interactions with other individuals in the population.

6.3.2 Signalling in Young

Interactions among family members are some of the most widely studied social behaviours in animals (Morales and Velando 2013). Although we can think that interests of family members are the same, interests are actually not identical and give rise to three main forms of evolutionary conflicts: those between parents and offspring (Trivers 1974); those among siblings, which all compete for the same resources (O'Connor 1978); and those between parents (Lessells 1999). The hypothesis of parent–offspring conflicts, in particular, proposes that a conflict arises over the amount of parental care, where the offspring tries to elicit more care than would be optimal for the parents. One way for offspring to elicit parents to provide more care is by showing signals that convey honestly their quality. Whether nestling colourations reveal honestly their quality, as it does for adults, remains poorly known yet. There is some evidence suggesting that they may play a significant role in explaining the solution of parent–offspring conflicts. This may, for example, be important for parents to decide how to allocate their limited supply of food towards offspring that are more likely to survive. That parents assess chicks' ornaments was first demonstrated by Lyon et al. (1994). They altered the appearance of half of the chicks in each brood of coots (*Fulica americana*) by trimming the orange tips off their body feathers. Parent coots fed ornamented chicks preferentially over non-ornamented chicks, resulting in higher growth rates and greater survival for ornamented chicks. Lyon et al. (1994) also showed that the experimental manipulation of chick plumage did not influence their begging behaviour. It can be concluded that the ornamental plumage of these chicks was the trait under selection by parental choice. Parental favouritism for nestlings with brighter colourations was later described in various other bird species (e.g., Götmark and Ahlström 1997; Saino et al. 2000; Tschirren et al. 2005).

Parental favouritism for brighter offspring raises the question of what offspring colours would signal to their parents. For example, carotenoid-pigmented traits of nestlings have been shown to be associated with fitness-related traits like body mass, stress level or immunocompetence (Saino et al. 2000, 2003; Casagrande et al. 2007; Loiseau et al. 2008; Dugas and McGraw 2011; Sternalski et al. 2012;

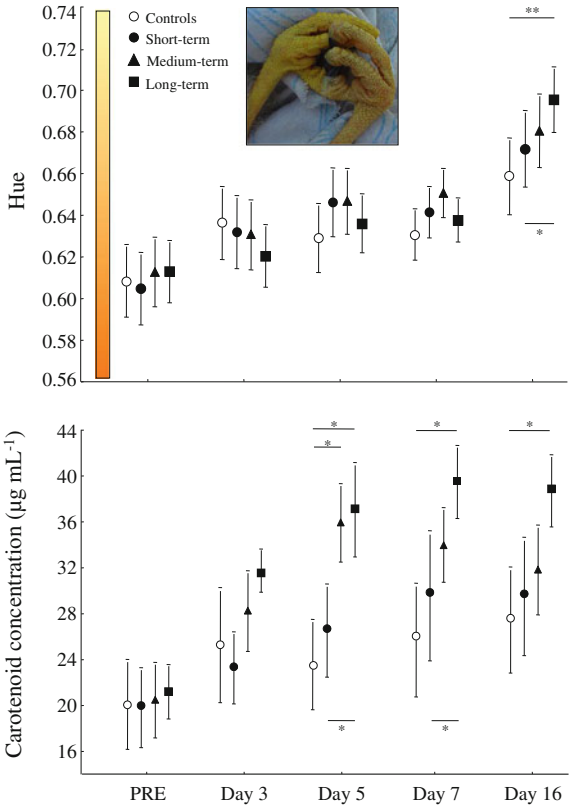

Fig. 6.6 Effects of carotenoid supplementation on skin hue and serum carotenoid concentration in wild nestling kestrels (*Falco tinncunculus*). The picture shows the differences in skin colour between a supplemented (*brighter*) and a non-supplemented (*paler*) nestling, respectively (Picture by David Costantini). On day 1 (PRE), nestlings were fed with a one-day-old mouse containing either no carotenoids (controls) or 36 mg of carotenoids (all the other treatment groups). On day 3, medium- and long-term nestlings were given a mouse containing carotenoids, while controls and short-term nestlings were given a mouse free of extra-carotenoids. On day 5, only the long-term supplemented chicks were given carotenoids. All chicks were sampled again on day 7 and 16, but none were supplemented with carotenoids. Data are shown as mean ± standard error. * $P < 0.05$, ** $P < 0.01$; PRE refers to measures made before the start of carotenoid supplementation. Reprinted with minor modifications from Casagrande et al. (2007) with kind permission from Springer Science + Business Media B.V

Martínez-Padilla et al. 2013). As with sexual ornaments, colourations of nestlings may also signal some components more directly related to the oxidative balance. For example, differences in components of the antioxidant machinery among melanic and light morphs were found in nestlings of the two birds of prey: the booted eagle *Hieraaetus pennatus* and the Eleonora's falcon *Falco eleonorae* (Galván et al. 2010). However, differences between morphs were sex- and species-dependent (Galván et al. 2010). In another study, the degree of black and reddish

pigmentation in barn owl (*Tyto alba*) nestlings was positively and negatively correlated, respectively, with the in vitro resistance of red blood cells to haemolysis induced by a free radical (Roulin et al. 2011).

A temporal gap appears to exist in the response of nestling skin colourations to a change in carotenoid availability. Casagrande et al. (2007) found that circulating carotenoids and skin colouration had a different time course in the response to different terms of carotenoid supplementation in kestrel nestlings (Fig. 6.6). Carotenoids are absorbed in the gut and pass relatively rapidly into the bloodstream. An increase in the serum levels of carotenoids was already detectable 96 h after supplementation. In contrast, changes in carotenoid-based colouration of tarsi skin (hue) emerged after around eleven days since the first supplementation. At this time, the effect of supplementation on serum concentration of carotenoids already disappeared, indicating a quick reduction in circulating carotenoids consequently to their use in physiological functions (e.g., immune response) or storage in other tissues (e.g., skin; Casagrande et al. 2007). It is not known whether the existence of a time gap between a change in the physiological status and in the body colouration would also have occurred in response to other experimental manipulations, such as immune stimulation or induction of physiological stress. Regardless, these results suggested that there might be an intrinsic limitation of colour expression (Casagrande et al. 2007). This would undermine the idea that skin colouration, at least in kestrel nestlings, plays a relevant signalling role in the context of family conflicts because of the slow response of skin colouration to any changes in nestling physiological status. However, the evaluation of offspring quality by parents may also be long term; hence, the delay in the change of skin colouration might not necessarily prevent its signalling role. This may be especially true in species, like the kestrel, that provide prolonged parental care. Finally, it cannot be ruled out that the sensitivity of spectrophotometers used to quantify skin colouration might not be sufficient enough to detect any subtle changes in colour intensity that instead parents would be able to see.

6.4 Warning Signals

As an anti-predatory strategy, many animal species have evolved conspicuous body colourations that are used to signal their unprofitability to predators (e.g., Batesian and Müllerian mimicry). Assumed for years to not vary among conspecific individuals in order to facilitate predator learning and avoidance, it is now becoming evident that individual variation in warning signals is high, and the development environment may significantly contribute to build up such variation (Mappes et al. 2005; Stevens and Ruxton 2012). Aposematic species are often warningly coloured early in life, but their chemical defences may be weak or absent because they have yet to be acquired through the diet (e.g., alkaloids acquired from dietary arthropods) or synthesised endogenously (e.g., Daly et al. 1994; Nishida 2002; Saporito et al. 2009). Hence, investment in production of

body colourations during growth might expose individuals with poor chemical defences to a higher risk of predation. A strategy to avoid this state of being conspicuous yet poorly defended against predators could be to invest lots of resources in growth rather than in production of body colourations (Flores et al. 2013). However, there may be costs associated with this strategy, such as oxidative stress (see Chap. 2). Although oxidative stress experiences in early life play a relevant role in shaping the adult phenotype, we know very little about how oxidative stress may modulate expression of warning colourations.

Poison dart frogs (family *Dendrobatidae*) are renowned for their toxicity and brightly coloured bodies. Flores et al. (2013) reared larvae of green and black poison frogs (*Dendrobates auratus*) on a relatively low or a high food supply and tested the hypothesis that (1) individuals with more resources should grow larger while reducing their investment in warning signals at metamorphic completion (2) oxidative stress might influence resource allocation trade-offs. The investigators found that, when food was abundant, frogs grew larger, and investment in signalling, and hence conspicuousness, diminished (Flores et al. 2013). Signal luminance and levels of oxidative stress were positively correlated in high-food froglets, but were negatively correlated in low-food froglets, suggesting a resource allocation trade-off when food availability is relatively low (Flores et al. 2013). Hence, oxidative stress might have constrained investment in growth and signalling of resource-limited froglets.

6.5 Conclusions

Pigmented and achromatic traits may impose costs that only high-quality males would be able to sustain. Similarly, females may also develop these costly traits and use them in social interactions and, possibly, in a context of sexual selection. The development of colour signals can also be costly in contexts beyond that of mate choice, such as in the case of quality signalling in young or in warning displays.

The importance of oxidative stress as a constraint to the conspicuousness of male colourations is, however, still under debate, in particular for carotenoid-based colourations. A shortcoming of hypotheses arises to some degree from a poor understanding of specific cellular mechanisms in which carotenoids and melanins are involved. This makes difficult to generate robust predictions and hypothesis-testing designs. It is also sometimes forgotten in studies on sexual ornaments and oxidative stress that experimental designs should be drawn in order to falsify sensu Popper (2002) rather than to confirm the hypothesis. Moreover, it is not uncommon that hypotheses on the ornament-oxidative stress link are considered a priori valid regardless of the strength, sign and significance of the results obtained. Clearly, this makes the hypothesis dogmatic rather than scientific because it is not open to falsification (Popper 2002).

Evidence in favour of a role of body colourations in signalling the male's resistance to oxidative stress in birds is weak. A recent meta-analysis showed that

the relationship between redness of plumage or skin colourations and oxidative stress state in birds (antioxidant defences or oxidative damage) is low and not significant (Simons et al. 2012). So far, evidence in support of the alternative function hypothesis appears stronger than other hypotheses given the significant relationship between the intensity of the response to a phytohemagglutinin injection and the level of ornament redness and the concentration of circulating carotenoids (Simons et al. 2012).

Regardless, we certainly need to explore in more detail whether carotenoid-based ornaments advertise resistance to oxidative stress. If they do so, we also need to elucidate which mechanisms link carotenoid-based ornaments to resistance to oxidative stress, especially if carotenoids are not the antioxidant resource traded-off. The strength of the relationship between sexual colouration and oxidative stress might differ among species, depending on how strong the selection pressure on the choosy sex is for the accuracy of the evaluation of mate's qualities. In this context, among-species variation in longevity may be important. For example, the relationship between sexual colouration and oxidative stress may be stronger in short-lived species, where there is obligate paternal care or when rearing offspring in the presence of a mate that invests weakly in chick rearing or in absence of a mate would reduce evolutionary fitness. This is well evident in studies on zebra finches, showing that a lower investment of a pair member comes at a cost for the survival of the mate (Monaghan et al. 2012). The increase in intensity of ornament colour induced by stress hormones might be interpreted as a terminal investment in an environment where long-term survival prospects are poor (Cote et al. 2010). For the opposite reasons, selection for the ability to judge the quality of potential mates might be relaxed in long-lived species, where the investment in reproduction is not capital and chances of fixing an initial mistake in the mate choice may be higher. However, the reliability of the sexual signal appears to increase with age, suggesting that individual age should be taken into consideration when assessing the signalling role of body colourations (Alonso-Alvarez et al. 2009).

It will also be important to consider in future studies the concept of morphological integration because it may provide a useful framework for understanding the development and evolution of sexual ornamentation (Badyaev 2001, 2004). Finally, there are many other notable examples of secondary sexual traits (e.g., size of antlers and horns; Vanpé et al. 2007; Ciuti and Apollonio 2011), which await investigation in the framework of signalling of oxidative status.

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Chapter 7
The Role of Oxidative Stress and Hormesis in Shaping Reproductive Strategies

Abstract The cost of reproduction is a central paradigm of life history theory. It states that high investment in current reproduction reduces survival or future fecundity. Reproduction is a demanding phase of animals' lives, since they must produce, and, in species with parental care, protect and provision, their young. In sexually reproducing species, considerable resources are also invested in mate choice strategies, which combine to shape the cost of reproduction. We currently know very little about how costs of reproduction are actually incurred, since the majority of studies have focussed on the ultimate outcomes rather than the proximate mechanisms. It has been suggested that oxidative stress may be one key cellular mechanism underlying the costs of reproduction. This chapter examines how the need to manage oxidative stress has possibly influenced the evolution of reproductive strategies, from the tactics adopted to find a suitable mate to how much effort parents put in offspring rearing. This chapter also discusses the possible prominent role of hormesis in determining the extent to which (1) reproductive activity may be stressful or not and (2) mild stress may stimulate reproductive performance.

7.1 Reproduction is a Time of Trade-Offs

Reproduction is a demanding phase of animals' lives. In lactating laboratory mice *Mus musculus*, metabolic rate can increase to over 400 % of non-reproductive laboratory females (Hammond 1997), while metabolic rate of gravid eastern fence lizards *Sceloporus undulatus* increases to 122 % of that measured in non-gravid females (Angilletta and Sears 2000). Metabolic rate and energy expenditure can also dramatically increase in birds while chick rearing (Nilsson 2002). In sexually reproducing species, considerable resources can be invested in mate choice strategies, which also contribute to shape the cost of reproduction. Hence, individuals must optimise the many trade-offs they have to face; for example, it is thought that the parental effort is adjusted in order to achieve a close fit between

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parental capacity and environmental circumstances (the prudent parent paradigm, Drent and Daan 1980).

The cost of reproduction is a central paradigm of life history theory (Bell 1980; Stearns 1992; Roff 2002). It states that high investment in current reproduction reduces survival or future fecundity (Williams 1966; Stearns 1992). Moreover, pre-reproductive conditions that elicit stress or jeopardise future survival might constrain the next reproductive investment. Brood size manipulations cause parent kestrels (*Falco tinnunculus*) to alter investment in chick rearing, with an estimated mean increase or decrease of 12.3 kJ day^{-1} $young^{-1}$ (=2.9 % of mean daily energy expenditure of parents of control broods) for enlarged and reduced broods, respectively (Deerenberg et al. 1995). The manipulation of workload affected the survival perspectives of the kestrels: 60 % of parents raising two extra nestlings died before the end of the first winter compared to 29 % of those raising control or reduced broods (Daan et al. 1996).

Costs of reproduction also emerge during the next reproductive season. *Poa annua* plants flowering heavily in their first season produced fewer inflorescences in the next season compared with plants that flowered less (Law 1979). In the common guillemot (*Uria aalge*), the more chicks' parents raised at an early stage of their lives, the shorter their lives and the less likely it was that any chicks they produced later in life survived through the nestling period (Reed et al. 2008).

The strength of costs of reproduction can also be contingent on scheduling of reproductive effort. Rotifers that produced more offspring during their life lived longer than rotifers that produced fewer offspring (Snell and King 1977). However, long-lived rotifers produced fewer offspring per day but for a longer time interval than short-lived rotifers, meaning that offspring produced later in life impacted less on survival than those produced earlier in life (Snell and King 1977). In contrast, there are also studies where trade-offs between current and future reproduction or survival did not emerge (Stearns 1992). This apparent lack of trade-offs was contingent on the degree to which environmental conditions were stressful: trade-offs were more likely to occur when food was limited (Stearns 1992). Moreover, the nature of trade-offs also varies across environmental contexts: milkweed bugs (*Oncopeltus fasciatus*) males fed a poor-quality diet (sunflower) lived longer but invested less in reproduction, both in mating and fertility (Attisano et al. 2012). In contrast, bugs fed a high-quality diet (milkweed) invested in both mating and fertility at the expense of survival (Attisano et al. 2012).

The trade-off between parental effort and survival may, however, be more complex (Santos and Nakagawa 2012). A meta-analysis of experimental studies that manipulated parental effort in birds showed that (1) the effect of parental effort on survival was similar across species regardless of phylogeny; (2) individuals that experienced reduced parental effort had similar survival probabilities than control individuals, regardless of sex; and (3) males that experienced increased parental effort were less likely to survive than control males, whereas females that experienced increased effort were just as likely to survive as control females (Santos and Nakagawa 2012). Males and females may, therefore, differ in the life history consequences of parental effort.

There is striking among-species variation in the way they reproduce and so in how costs come about and are dealt with. For example, one aspect that has generated particular interest is the use of stored energy for reproduction. A distinction is generally recognised between income breeders, in which reproduction is financed using current energetic income, and capital breeders, in which compensatory feeding takes place in advance of breeding, so that reproduction may be fuelled from stored energetic capital (Stearns 1992; Stephens et al. 2009). Such a clear distinction between capital and income breeders is not biologically real. In fact, there is a continuum of species ranging from pure income breeding at one extreme to pure capital breeding at the other extreme, some species are versatile and may use one of the strategies depending on the circumstances under which they reproduce (Stearns 1992). We can further distinguish between those species that reproduce once during their lifetime (semelparous), putting all available energy into a single reproductive event with the trade-off being the mortality of the adults (e.g. mayflies, the Pacific salmon), and those species that reproduce multiple times throughout their lifetime (iteroparous) and are able to adjust their reproductive effort to suit current environmental conditions (e.g. most vertebrates; Roff 2002).

We currently know very little about how costs of reproduction are actually incurred, since the majority of studies have focussed on the ultimate outcomes rather than the proximate mechanisms. It has been suggested that oxidative stress may be one key cellular mechanism underlying the costs of reproduction (Costantini 2008; Monaghan et al. 2009; Metcalfe and Alonso-Alvarez 2010). This is because high investment in reproduction would result in faster somatic deterioration and reduced life expectancy since resources allocated to reproduction are no longer available for self-maintenance. There are many examples in the literature of how oxidative stress may influence individual fertility. However, there is almost a complete lack of clear experimental demonstrations of the link between oxidative stress and reproductive effort (Metcalfe and Monaghan 2013).

My aim in this chapter is to examine how the need to manage oxidative stress has possibly influenced the evolution of reproductive strategies, from the tactics adopted to find a mate to how much effort parents put in offspring rearing. To this end, I also rely on examples of species that may be interesting study models for the evaluation of the link between oxidative stress and reproductive activity. I finally discuss the links between hormesis and reproductive activity.

7.2 Mating Systems, Reproductive Tactics and Social Stress

7.2.1 Courtship Displays

Courtship display is a special set of behaviours, which some species evolved as part of mating phase. Courtship behaviours can include special calls, postures and

movements; may involve the development of extravagant ornaments, bright colours or other ornamentation (see Chap. 6); and may also include intense fighting to access mates. Hence, performance of courtship displays may impose costs for the male because the physical effort of courtship, injuries caused by among-male fights or development of ornaments might induce oxidative stress.

There are many relevant examples in nature, where the physical effort of courtship may be relevant. Males of bowerbirds are renowned for their capacity of building bower nests. This is a demanding task that only healthier males may afford. There is in fact variation among bowers that females may use to get information about the male's quality (Doucet and Montgomerie 2003). Building a bower may require an intense physical effort for the male. Moreover, the cognitive abilities of the male are important in determining how good it is in performing the many tasks needed to assemble a bower. There is clear room for oxidative stress to influence this courtship behaviour given its links to intense muscle activity and to cognitive abilities.

Manakins are another interesting example of birds that evolved an elaborate courtship. Manakins perform some of the most physically demanding courtship dances in the animal kingdom, including acrobatic routines and mechanically produced sounds (Fusani et al. 2007; Barske et al. 2011). Display performance might be an indicator of sensorimotor coordination, physiological condition and development history; hence, working as a clue females may use to evaluate the male's quality (Fusani et al. 2007; Barske et al. 2011). Manakins offer an exciting model to address questions related to the connections among oxidative stress, muscle and heart activity, courtship and evolutionary fitness. Oxidative stress might play a dual role: it may influence the muscle efficiency and so the motor skills of the male; dance-induced oxidative stress may carry over and influence sperm quality and ageing rate. The quality of the male and/or the environment may determine to what degree any oxidative costs are paid in terms of reduction in mating chances or fertilisation capacity.

There are many other examples of species that evolved courtship activities that require substantial energetic investments, such as the vocalisations in frogs or the leg-waving behaviour in spiders (Byers et al. 2010). Individual circumstances may also influence the oxidative damage level. For example, males of Carolina anole (*Anolis carolinensis*) appear to distinguish among individual females and use this ability to increase reproductive success by identifying and preferentially pursuing novel females that have come in their territories for the first time over previously inseminated resident females (Orrell and Jenssen 2002).

Costs of courtship can be exacerbated in those species, where males fight in order to increase their reproductive opportunities. This behaviour is very common in animals, from beetles and lizards to lekking birds or mammals. Oxidative costs may arise from the intense physical effort and the associated reduction in food intake and activation of immune and inflammatory processes induced by injuries. Another source of oxidative damage can be androgens. Testosterone secretion increases over the mating period. Testosterone increases aggressiveness and tolerance of injuries. It can also increase metabolic rate and activate physiological

pathways that result in production of reactive species. The quantification of oxidative stress in these species may inform behavioural ecologists about the proximate mechanisms underlying evolution of mating strategies.

7.2.2 The Waiting Male and the Fighting Female

It is not always only the male to pay costs for the competition with rivals (Alcock 2009). For example, at the start of the breeding season, males of the pipefish *Syngnathus typhle* wait for females, which compete among them in order to access the males with the larger ventral pouches (Berglund et al. 1986). This is because females lay eggs into the male ventral pouches; hence, a larger pouch allows the male to carry more eggs. This is important because males will deal with the incubation of eggs. Similarly, in the Mormon cricket (*Anabrus simplex*) competition for mates also takes place among females, which fight in order to obtain the spermatophore from the male (Gwynne 1981).

7.2.3 Cooperative Breeding

Cooperative breeding refers to a reproductive system in which one or more members of a social group care young that are not their own offspring (Stacey and Koenig 1990). Hence, a few individuals (dominants) frequently monopolise breeding opportunities. Caregivers may be non-breeding adults (so-called helpers or auxiliaries) or may be cobreeders (Stacey and Koenig 1990). It has been shown that helping behaviour can be costly in several ways, such as delayed maturation (Brown 1987), high-energy expenditure (Heinsohn and Cockburn 1994) or reduced direct reproduction. More recently, it has been proposed that oxidative stress might be a more proximate mechanism explaining the long-term costs of helping behaviour (van de Crommenacker et al. 2011). In the Seychelles warbler (*Acrocephalus sechellensis*), some subordinate individuals help dominant birds in chick rearing, while others do not so. Before breeding, female subordinates that did not subsequently help had higher oxidative damage (hydroperoxides) than helper or dominant females (van de Crommenacker et al. 2011). Female dominants and helpers showed higher non-enzymatic antioxidant capacity of plasma than female non-helpers during nest care. In males, oxidative damage was higher in dominants than helpers while nest guarding, while it was lower in dominants than non-helpers while chick feeding. Although the correlative nature of this study does not allow us to infer about causation, results of this study suggest that a link between social status and oxidative balance might occur in cooperatively breeding animals. However, social status does not appear to influence the deposition of carotenoids in eggs: sociable weaver (*Philetairus socius*) females assisted by helpers produced eggs with lower content of testosterone, androstenedione and corticosterone levels,

while deposition of carotenoids was not affected (Paquet et al. 2013). Importantly, the consequences of reproducing or helping may also depend on the size of the social group. For example, Cram (2013) found that any oxidative costs appear to be mitigated in large social groups, suggesting that the cooperative contributions of helpers may offset the costs of reproduction for all group members.

In animal societies, the competition for and acquisition of status may be very intense; hence, it may also carry significant fitness costs. In many cooperatively breeding mammals, a single female has a dominant status, and she prevents subordinate females from breeding (Solomon and French 1997). In wild meerkats (*Suricata suricatta*), it was found that dominant females that experienced a higher level of competition for the dominant position had also lower breeding success and higher rates of reproductive senescence (Fig. 7.1). Furthermore, females that were more frequently evicted as subordinates from the group had lower breeding success when dominant (Sharp and Clutton-Brock 2011). Although there has been no investigation as to whether oxidative stress is responsible for the higher reproductive senescence in contexts where social competition is intense, there is some indirect indication suggesting that it may be involved. Subordinate meerkat females were found to have double levels of faecal stress hormones ($\sim$200 ng g^{-1} faeces) when evicted than when within their group, and during this time, they either failed to conceive or aborted if pregnant (Young et al. 2006). Chronic elevation of stress hormones is known to increase oxidative damage (Costantini et al. 2011), and in turn, oxidative stress is known to promote cell senescence, as well as decreases in fertility or embryo viability. Hence, oxidative stress induced by stress hormones may be a plausible mechanism linking (1) social competition for dominance and female reproductive senescence and (2) stress level in evicted meerkats and failure to conceive or deliver. In many social mammals, competition among females leads to evictions and consequent increases in stress level of evicted females (Clutton-Brock and Huchard 2013). There is, therefore, considerable scope to test the role of oxidative stress in the reproductive senescence induced by social competition.

7.2.4 Hierarchical Societies

Up until the 1970s, there was general consensus that natural selection would favour males in every species that grow quickly and larger in order to get a dominant position. It was later realised that there are both costs and benefits of obtaining and maintaining a dominant social status in hierarchical societies. In this regard, it might be informative to look at those species where lower-ranking males may adopt alternative mating tactics (Alcock 2009). For example, subordinate baboons can form friendships with females and other males in order to have some chances of mating and to avoid fights with rivals, respectively (Palombit et al. 1997; Alberts et al. 2003). It would not pay for subordinate individuals to adopt other strategies because their physiological system might be unable to tolerate any

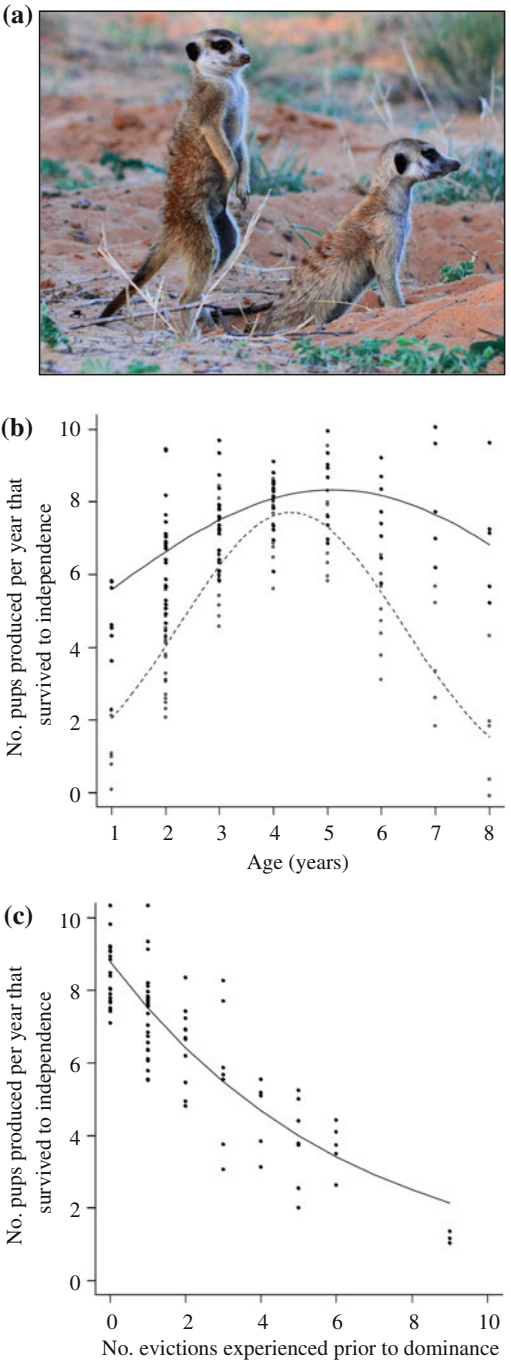

Fig. 7.1 **a** Family of meerkats *Suricata suricatta* in the South African Kalahari (Photograph by David Costantini); **b** effect of age and level of competition for dominance on the number of independent pups produced per year by dominant female meerkats; *the plot* shows generalised linear mixed effect model predictions with partial residuals for low competition (*solid line*, *solid circles*) and high competition (*dashed line*, *hollow circles*), plotted for the median weight (750 g) and median number of evictions experienced prior to dominance acquisition; **c** effect of the number of evictions experienced prior to dominance acquisition on the number of independent pups produced per year by dominant female meerkats; the plot shows generalised linear mixed effect model predictions and partial residuals for the median age (3 years), median weight (750 g) and low competition for dominance. Reproduced with minimal alterations from Sharp and Clutton-Brock (2011) with permission of John Wiley & Sons

costs these strategies may impose. Conversely, energetic or oxidative costs of maintenance of a dominant status may counterbalance the benefits of having more mating opportunities. Similarly, in many other animal species (e.g. insects, fish, frog, mammals), weaker males do better by switching to alternative mating tactics rather than trying to compete with rivals. The so-called satellite males or sneaker males patrol around until they come across females that are not being guarded by the dominant males. They take this opportunity to mate avoiding the attack of the dominant. At proximate level, this *carpe diem* strategy might pay in terms of reduced oxidative damage because a non-dominant male would not be faced with high testosterone levels, intense physical effort and/or high immune and inflammatory activity, which can be a consequence of among males competition for the maintenance of territory and control of females.

The position in the hierarchy may also impact on the physiological condition of partner over the breeding season. For example, early in the rearing period, jackdaw (*Corvus monedula*) females mated to subdominant males had higher oxidative damage than females mated to dominant males (Salomons 2009). This difference disappeared later in the chick-rearing period. Early in the breeding season, the female is largely dependent on her partner for food provisioning. This finding might, therefore, indicate that subdominant males do not provide enough food to their mates, which would make them be in poorer physiological condition (Salomons 2009).

Competition is not specific to males. Competition between females for reproductive resources is one of the cornerstones of the theory of natural selection, but it received comparatively less attention than competition between males (Rosvall 2011; Clutton-Brock and Huchard 2013). Studies on social mammals show, for example, that females can fight for access to breeding sites (Hoogland 1995) or to a dominant role (Jolly and Pride 1999). Females may, therefore, experience similar selective pressures as those of males. This suggests that oxidative stress might operate similarly in both sexes as a cost and constraint of reproductive competition in social species.

7.2.5 Two Sexes, But Many Morphs

In many animal species, males or females differ morphologically, and also by having different life histories and reproductive behaviours. For example, males of the marine isopod *Paracerceis sculpta* occur in three different sizes (large, medium and small), each with its own behavioural phenotype (Shuster 1989, 1992; Shuster and Wade 1991). There are three male morphs in ruffs (*Philomachus pugnax*), a medium-sized wading bird that breeds in marshes and wet meadows across northern Eurasia. Two of these morphs, independents and satellites, have elaborate courtship behaviour and plumage while a third one is a female mimic called faeder (Lozano et al. 2013). During the breeding season, independents establish lek mating courts and defend them against other independents. Satellites

do not fight but are pecked and chased by independents if they fail to behave submissively, while faeders, like females, do not incur the costs or benefits from the consequences of displaying and fighting in reproductive contexts (Lozano et al. 2013). Hence, independents might be more exposed to oxidative stress than both other morphs and females because of their higher energetic demands and potential costs associated with a high immune activity because of injuries due to territory establishment and defense (Lozano et al. 2013). Lozano et al. (2013) found that innate and cell-mediated immunity decreased from independents to females, supporting a risk-of-injury explanation. No such pattern was, however, evident for oxidative damage or antioxidant status of plasma (Lozano et al. 2013). Hence, in this particular case, oxidative stress did not appear to differ among morphs; however, studies using more specific parameters of oxidative damage or antioxidant protection would be needed in future studies.

7.2.6 Polyandry and Sperm Competition

Oxidative stress to sperm might be an important mechanism that influences post-copulatory sexual selection given its critical impact on male fertility. It is, therefore, possible that sperm oxidative stress may have influenced a number of evolutionary processes, including the evolution of polyandry, sexual conflict over mating and sperm competition dynamics (Siva-Jothy 2000; Arenas-Ríos et al. 2005; Dean et al. 2007; Pizzari et al. 2008). This may be the case for the jungle fowl (*Gallus gallus*), where females often mate multiply to force the sperms of different males into competition and the capacity of males to mate and fertilise successfully declines with age (Dean et al. 2010), possibly because of accumulation of oxidative damage (Noguera et al. 2012). This is also the case of houbara bustards (*Chlamydotis undulata*), where males investing most in sexual displays experience a rapid deterioration of spermatogenic function (Preston et al. 2011). Investment in sperm protection against oxidative damage is, therefore, essential for the male. The level of investment may vary among males, depending on various factors, such as individual condition, competition or reproductive strategy. For example, in the bluegill sunfish (*Lepomis macrochirus*), males may adopt three different reproductive tactics (see also Sects. 7.2.5 and 7.2.6). Guards make nests, court females and provide solitary parental care for the embryos; sneaks include small cuckolders, which are termed sneakers, that dart in and out of nests in order to ejaculate between the spawning pair and larger cuckolders, which are termed satellites, that mimic females in order to ejaculate between the spawning pair (Fu et al. 2001). Sneakers can fertilise more eggs than guards during sperm competition and are superior to satellites in sperm competition (Fu et al. 2001). It may be hypothesised that, compared to guards and satellites, sneakers prioritise protection of sperm against oxidative damage. Guards may, for example, be more constrained than sneakers in the allocation of resources to sperm because they spend lots of resources in mate guarding and nest construction. There are also other potential

routes through which differences in oxidative status between reproductive phenotypes may come up. For example, in the side-blotched lizard (*Uta stansburiana*), reproductive morphs differ in both hormonal and immune traits (Mills et al. 2008); both of these traits are known to influence the oxidative balance.

7.2.7 Socially Monogamous, but Genetically Polygamous

Many bird species are classically considered to be monogamous, but they routinely adopt extra-pair copulations, a quota of which leads to extra-pair offspring (Griffith et al. 2002; Westneat and Stewart 2003). Several hypotheses have been proposed to explain why females might benefit by mating voluntarily with more than one male. One of the most recent hypotheses stated that females may gain fitness benefits because their extra-pair offspring may be more resistant to oxidative stress (Losdat et al. 2011b). The authors found that while similar in body mass or size, extra-pair offspring of wild great tits (*Parus major*) had higher blood antioxidant protection than their within-pair half-siblings (Losdat et al. 2011b). Having offspring more resistant to oxidative stress might, therefore, pay in terms of evolutionary fitness. It is, however, not established yet whether the higher antioxidant protection is a direct benefit of multiple mating (i.e. higher genetic variation) or a consequence of higher maternal allocation of antioxidants into the eggs. More studies are also needed in order to quantify the short- and long-term effects on fledging success, survival until first reproduction and future reproductive performance of offspring.

Males of some species have evolved tactics (e.g. mate guarding, repeated copulation) to prevent their mates from mating with other males. For example, mate guarding provides clear fitness benefits compared to the opposite strategy. However, mate guarding may impose nutritional, energetic and other physiological costs for the male (Alberts et al. 1996; Komdeur 2001; Saeki et al. 2005) that would counterbalance the benefits. Mechanistic evidence of costs remains elusive, but the analyses of oxidative damage and antioxidant mechanisms may provide the currency to quantify and elucidate the nature of these costs.

On the other hand, males of some species tend to be monogamous, while in many other cases, males evolved a polygynous style. There is large among-species variation in the tactics employed by males to achieve polygyny: female defence polygyny, resource defence polygyny, lek polygyny, and scramble competition polygyny (Alcock 2009). Such variation offers many opportunities to test the role of oxidative stress as a cost of a particular mating tactic and how evolving a certain tactic rather than another one may pay in terms of reducing the threat of oxidative stress and its deleterious consequences.

7.3 Male Fertility

Sperm cells are particularly vulnerable to oxidative stress (Surai et al. 1997; Sikka 2001). In humans and a few domesticated animals, it was found that oxidative stress to sperm can lead to decreased motility and viability, an inability to fuse with the oocyte and decreased post-fertilisation embryo survival (Fujihara and Howarth 1978; Wishart 1984; Aitken et al. 1989; de Lamirande and Gagnon 1992, 1993; Baumber et al. 2000; Bilodeau et al. 2002). Moreover, oxidative stress may also reduce the capacity of testes to differentiate normal spermatozoa, which would ultimately result in a reduction in sperm fertilising ability (Wu et al. 1973; Aitken and Roman 2008). Studies on wild birds and mammals corroborate results from captive animals in that oxidative stress contributes to reduce the quality of sperm in animals living under various stressful conditions (e.g. heavy metal exposure, Reglero et al. 2009; workload in chick rearing, Helfenstein et al. 2010; radioactive contamination, Bonisoli-Alquati et al. 2011) and that oxidative damage in sperm increases in older males (Velando et al. 2011).

Both sperm and seminal plasma of invertebrates and vertebrates possess non-enzymatic and enzymatic antioxidants (Surai et al. 1997; Sikka 2001; Weirich et al. 2002). Much attention has been given to the protective role of dietary antioxidants on sperm, given its direct links with nutrition and food availability in the environment. For example, carotenoids are present in the ejaculates of birds (Rowe and McGraw 2008) and insects (Heller et al. 2000); hence, they might have a direct protective role on sperm. Carotenoid supplementation actually improved sperm quality in humans (Gupta and Kumar 2002) and fertilisation success in fish (Ahmadi et al. 2006; Pike et al. 2010). Dexamethasone treatment (to mimic a stress condition) significantly reduced ejaculate volume and increased oxidative damage in the seminal plasma in dogs (Hatamoto et al. 2006). Supplementation with vitamin E prevented some of the negative effects of stress: it increased sperm motility, vigour and concentration, and decreased the percentage of major sperm defects and the concentration of oxidative damage (Hatamoto et al. 2006). Importantly, it was also demonstrated that dietary antioxidants (carotenoids) may have a protective role on sperm in wild animals under stressful conditions. Helfenstein et al. (2010) found that the percentage of motile sperm of great tit (*P. major*) males caring for enlarged broods or having a paler breast colouration benefited from carotenoid supplementation. However, sperm swimming ability and the lipid peroxidation level in sperm were both unaffected by carotenoid supplementation, suggesting that (1) different aspects of sperm quality may be influenced by antioxidant availability; (2) dietary antioxidants may also improve sperm quality independently from their effects on oxidative damage levels. Moreover, Helfenstein et al. (2010) found that more colourful males produced sperm of better quality. Velando et al.(2011) found in blue-footed booby (*Sula nebouxii*) that old males with a duller foot colour (sexual signal) had higher DNA damage in sperm, while foot colour and damage were not associated in middle-age males. These results, together with previous findings in fish (Locatello et al. 2006;

Pitcher et al. 2007) or other bird (Peters et al. 2004) species, provided good evidence in favour of a link between body colourations and fertility in males.

The high sensitivity of sperm to oxidative stress may prevent eggs being fertilised by more than one sperm (polyspermy; Boldt et al. 1981; Coburn et al. 1981) and in those species with high levels of sperm competition (Costantini et al. 2010b). In these species, males with a greater capacity to defend sperm against oxidative stress might gain a greater share of paternity. Consequently, sexual selection may target cellular mechanisms underlying sperm function aimed at avoiding oxidative stress (Costantini et al. 2010b). Although these ideas remain largely unexplored, a comparative study of *Mus* spp. found evidence of positive selection in the protamine 2 gene in the species with the highest inferred levels of sperm competition (Martin-Coello et al. 2009). In addition, sperm competition levels across all species were strongly associated with high divergence in protamine 2 promoters that, in turn, were associated with sperm swimming speed, a major determinant of fertilisation success under sperm competition. Hence, it was concluded that in the incipient stages of species divergence, changes in behaviour (female sexual promiscuity) might have created new selective pressures (sperm competition), which favoured minor changes in gene sequence (molecular adaptive evolution) and major changes in its regulation (promoter genetic divergence; Martin-Coello et al. 2009). These changes were in turn associated with modifications in both sperm design and sperm performance (Martin-Coello et al. 2009).

Allocation of antioxidants to sperm might be vital in species, where females store sperm for prolonged periods (Costantini et al. 2010b). Stored sperm may in fact show reduced capacity to fertilise an egg because sperm quality degrades with time (Tarín et al. 2000; Wagner et al. 2004). This has led to the idea that in some species females may have evolved sperm storage as a post-copulatory mechanism of mate choice that is based on sperm quality. This is very relevant considering that a wide range of taxa show extended periods of sperm storage (e.g. birds up to 117 days; bats up to 198 days; reptiles up to 2,555 days; Birkhead and Møller 1993). Astonishing examples of species that do very prolonged sperm storage can be found among insects: in some species, females maintain viable populations of stored sperm for periods as long as thirty years for queens of the narrow-headed ant *Formica exsecta* (Pamilo 1991). Evolution of other strategies like sperm ejection or remating would have also been contingent on the necessity of retaining high-quality sperm soon before egg production (Siva-Jothy 2000; Collins et al. 2004; Wagner et al. 2004; Dean et al. 2007).

In summary, intense oxidative stress is clearly deleterious for sperm and fertility. Mild oxidative conditions are, however, required because they improve the sperm performance (see Sect. 7.7).

7.4 Egg Production

Egg production can be costly for females. Deposition in the egg of biomolecules like nutrients or antioxidants that naturally occur in limited supply can deprive females of important tools used for self-maintenance. Production of eggs is also energy demanding. Studies on birds, in particular, showed that tests of effects of reproductive effort, often limited to chick rearing, also need to encompass the full demands of producing and incubating eggs (Monaghan and Nager 1997). For example, female lesser black-backed gulls (*Larus fuscus*) stimulated to lay extra eggs by egg removal had a significant reduction in body energy reserves and capacity of chick rearing, laid lower-quality eggs, had lower return rates to the breeding site the next year and those that did return were less likely to produce a clutch compared to control birds (Monaghan et al. 1998; Nager et al. 2000, 2001).

Egg-removal experiments with food supplementation to avoid any resource limitation also showed that females laying many extra eggs suffered high oxidative damage and a reduction of plasma antioxidant defences, together with an increase in corticosterone and a reduction in immunoglobulins and fat reserves (Travers et al. 2010). Hence, the price of egg production is not only dependent on the metabolic requirements. Similarly, in reptiles, female Australian painted dragons (*Ctenophorus pictus*) that produced more clutches had higher DNA damage (Olsson et al. 2012).

Correlative evidence shows that female oxidative status may be associated with clutch size or egg volume. Results are, however, conflicting. In alpine swifts (*Apus melba*), females with higher blood antioxidant capacity laid larger clutches, and their eggs were more likely to hatch than those of females with lower blood antioxidants (Bize et al. 2008). In contrast, female great tits (*P. major*) with higher plasma non-enzymatic antioxidant capacity while incubating laid smaller eggs and clutch sizes (Costantini et al. 2010a). In wild starlings (*Sturnus vulgaris*), females with higher plasma non-enzymatic antioxidant capacity while incubating laid smaller eggs but larger clutch sizes and suffered less oxidative damage (Costantini et al. 2010a). Finally, in collared flycatchers (*Ficedula albicollis*), females that produced heavier eggs had a better plasma redox balance, with relatively lower levels of plasma hydroperoxides compared to plasma non-enzymatic antioxidant compounds (Markó et al. 2011).

Costs of egg production may also occur in other taxa. For example, Wang et al. (2001) stimulated egg production of female fruit flies (*Drosophila melanogaster*) by adding yeast to their standard diet. The authors then tested the resistance of fruit flies to oxidative stress and compared it to that of females on a non-enriched diet. Females on the enriched diet actually produced more eggs, but also suffered more mortality after exposure to a pro-oxidant agent (Wang et al. 2001). While diet may also influence the oxidative balance per se, added yeast to diet did not affect the resistance to oxidative stress in sterile females (Wang et al. 2001). In a study on a reptile species, egg production in Children's pythons (*Antaresia childreni*) was associated with a lower plasma non-enzymatic antioxidant capacity and a higher

concentration of thiobarbituric acid reactive substances (Stahlschmidt et al. 2013). Females with heavy clutches had low oxidative damage during early brooding. In addition, the percentage of muscle lost (a proxy for maternal protein allocation) from the onset of reproduction to early brooding was positively correlated with the oxidative damage level during early brooding. However, the level of oxidative stress did not influence reproductive perspectives. In fact, females with low antioxidants and high damage reproduced the next breeding season (Stahlschmidt et al. 2013).

The deposition in the egg of antioxidants (e.g. vitamin C, vitamin E, carotenoids) could be expected to reduce the capacity of the laying female to herself cope with reactive species. Female zebra finches (*Taeniopygia guttata*) that laid many eggs had a decrease in blood non-enzymatic antioxidants compared to females that also laid many eggs but were supplemented with carotenoids (Bertrand et al. 2006). The deposition of antioxidants into the eggs is not only influenced by their environmental availability, but also by the female's own antioxidant requirements. Supplementation of the maternal diet with antioxidants, such as vitamin E or carotenoids, can increase their concentration in the egg, as well as the antioxidant capacity of yolk (e.g. Blount et al. 2002a, b; Surai 2002; McGraw et al. 2005). These findings give support to the *resource limitation* paradigm, which states that the antioxidant content of the diet passively modulates the deposition of antioxidants by the female in the egg. However, some studies provided evidence in support of the *active regulation* paradigm, which states that females actively regulate the antioxidant deposition in the egg in an adaptive fashion, regardless of their availability in the diet (Royle et al. 1999, 2003; Blount et al. 2002a, b; Surai 2002). Therefore, it is very likely that both routes of deposition shape the antioxidant content of an egg, and their relative importance might be contingent on the circumstances under which egg formation takes place and on whether different classes of antioxidants are deposited in the yolk or albumen through different metabolic pathways. This might be one of the reasons why the correlation among antioxidants in egg, or between parameters of antioxidant capacity in a female and her eggs, while significant, are not strong or may be positive (mostly supportive of a diet effect) or negative (mostly supportive of an active physiological regulation by the female). In a study on captive pigeons (*Columba livia*) laying their first clutch in the season, females that laid eggs with higher yolk non-enzymatic hydrophilic antioxidant capacity tended to produce yolks with lower non-enzymatic lipophilic antioxidant capacity (Costantini 2010a). Moreover, females that laid eggs with higher yolk non-enzymatic hydrophilic antioxidant capacity had lower serum non-enzymatic antioxidant capacity, and led eggs with a lower content of antioxidants in albumen (Fig. 7.2). The complexity of these correlations suggests that trade-offs do not only occur between egg production and self-maintenance, but also between different classes of antioxidants (see also Blount et al. 2002a for a specific example on deposition in egg of various classes of carotenoids).

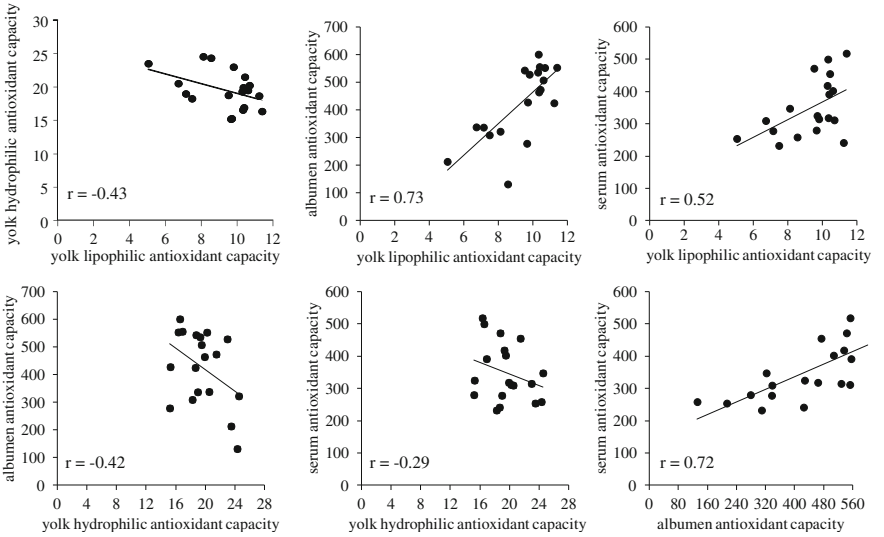

Fig. 7.2 Correlations between antioxidant status of egg and antioxidant status of captive pigeon (*Columba livia*) females laying their first clutch in the season. Females that laid eggs with higher yolk hydrophilic antioxidant capacity had higher levels of serum oxidative damage. The non-enzymatic antioxidant capacity of serum or albumen is expressed as mM HOCl neutralised, while that of yolk is expressed as μmol HOCl neutralised mg^{-1} yolk. See also Costantini (2010a)

7.5 Colostrum and Milk Production

Most of the ecological research has been focused on the strategies of maternal deposition of antioxidants in yolk. Comparatively, less attention has been given to antioxidant allocation through colostrum and milk. High investment in milk production (quality or quantity) is expected to come at a cost for the female. For example, Kumagai and Chaipan (2004) reported that colostrum α-tocopherol (a form of vitamin E) concentrations in the multiparous cows were significantly higher than those of the primiparous cows from 60 days before expected calving to 90 days of lactation. Consequently, plasma α-tocopherol concentrations of calves borne by the multiparous cows were significantly higher than those of the primiparous cows at 5 days of age (Kumagai and Chaipan 2004). Milk is also a source of other antioxidants (Hu et al. 2011; Abuelo et al. 2014), but it may also contain pro-oxidant molecules (Hu et al. 2011; Abuelo et al. 2014; Rizzo et al. 2013). For example, Rizzo et al. (2013) showed that lipid peroxides were constantly present in cow milk until 16 days after delivery. Moreover, the concentration of lipid peroxides in milk was positively correlated with both vitamins A and E. Clearly, domesticated animals have been selected for extreme reproductive output and so may not be considered representative. However, these studies suggest that it may be informative to look at how and why mothers regulate the

content of antioxidants and pro-oxidants in milk. The quality of milk may be dynamically adjusted, for example, depending on maternal condition or the reproductive value of pups.

7.6 Offspring-Rearing Effort

Several authors hypothesised that in iteroparous species (those with multiple reproductive cycles over the course of lifetime), a greater investment in reproduction might result in faster somatic deterioration since oxidative stress would increase consequently to a higher allocation of resources or time to reproduction (Costantini 2008; Monaghan et al. 2009; Metcalfe and Alonso-Alvarez 2010; Selman et al. 2012). However, there is almost a complete lack of clear experimental demonstrations of the link between oxidative stress and reproductive effort (Metcalfe and Monaghan 2013). Most studies did not manipulate reproductive effort, lacked suitable parameters of oxidative damage, did not manipulate the pre-reproductive oxidative stress or are of correlative nature, meaning that oxidative stress would be difficult to detect since individuals are probable to manage their reproductive effort so as to minimise damage (the prudent parent paradigm, Drent and Daan 1980). Increases in damage may also be a consequence of other physiological changes (e.g. sexual hormones) rather than the effects of the investment in offspring rearing. Studies have also rarely compared the oxidative balance between non-reproducing and reproducing individuals or were cross-sectional, so spurious associations may emerge because of selective disappearance of individuals having high oxidative stress, phenotypic plasticity or individual life history strategies.

Some studies on both captive and free-living birds have demonstrated that an increase in workload may decrease antioxidant defences. For example, in an experimental increase of brood size in zebra finches (*T. guttata*), males experiencing a higher chick-rearing effort did not change their daily energy expenditure, but had lower antioxidant enzyme activity, whereas females increased their energy expenditure but did not experience any changes in antioxidant enzyme activity (Wiersma et al. 2004). In a similar experiment on the same species, it was found that (1) males raising a six-chick brood had a reduction in blood non-enzymatic antioxidants compared to non-breeding or two-chick brood males; (2) breeding females had lower antioxidant defences than non-breeding females, although this pattern was mainly due to an increase in antioxidant defences in non-breeding females (Alonso-Alvarez et al. 2004). While in both studies, zebra finches might have experienced oxidative stress, the results are difficult to interpret, especially because those studies measured only components of antioxidant defences, while parameters of oxidative damage or, more specifically, of thiol oxidation were lacking. The same issues arise for studies on wild great tits (*P. major*), which showed that parents rearing experimentally enlarged broods had lower levels of blood non-enzymatic antioxidant defences (Losdat et al. 2011a; Christie et al. 2012).

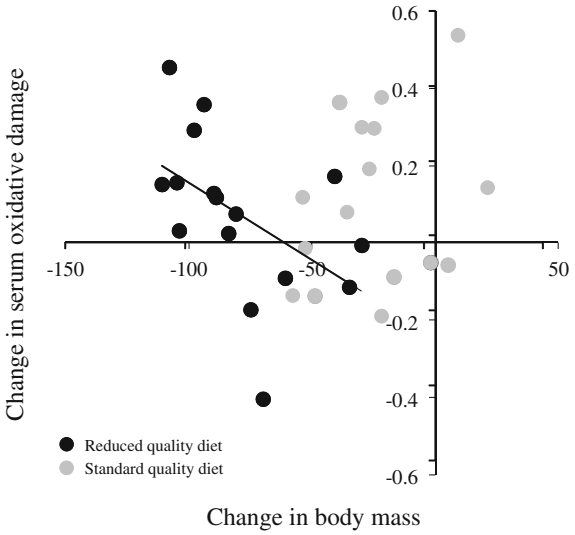

Fig. 7.3 The decrease in body mass of captive pigeons (*Columba livia*) recorded during the chick-rearing phase was accompanied by an increase in oxidative damage (serum hydroperoxides, mM H_2O_2 equivalents) in pigeons maintained on a decreased quality diet, but not on a standard quality diet. See also Costantini (2010b)

Circulating antioxidant defences do not necessarily decrease consequently to an increase in chick-rearing effort. Compared to broods whose size was reduced, jackdaw female parents (*C. monedula*) rearing enlarged broods had an increase in plasma non-enzymatic antioxidant capacity when it was expressed per volume of haematocrit but not when expressed per volume of plasma (Salomons 2009). Oxidative damage was not affected by the brood size manipulation, although there was a trend to increase in females rearing enlarged broods when it was expressed per volume of haematocrit (Salomons 2009). In male parents, there was no trend whatever parameters of oxidative status were expressed.

The cost of offspring-rearing activity can be influenced by the availability of resources, which may strengthen trade-offs when they occur in short supply. Captive pigeons (*C. livia*) lost more body mass and had a higher increase in oxidative damage while chick feeding on a low-quality diet than pigeons maintained on a higher-quality diet (Costantini 2010b). Moreover, female pigeons on the low-quality diet suffered more oxidative stress than all other experimental groups (Fig. 7.3). Although diet quality might have contributed per se to influence the parental oxidative status, these results suggested that the chick-rearing effort might increase oxidative stress when the environmental conditions (diet in this particular case) are poor.

Another study attempted to manipulate the reproductive effort by attaching rectangular cuboids to the middle-back feathers of Adélie penguins (*Pygoscelis adeliae*) in order to increase their foraging effort (Beaulieu et al. 2011). Birds carrying this extra weight lost more body mass and had higher plasma non-enzymatic antioxidant capacity than control birds, while the level of oxidative damage and telomere length (proxy of oxidative DNA damage) were both unaffected by the experimental treatment.

Overall, these studies highlight that males and females may differ in how their oxidative status is influenced by the chick-rearing effort. This might be because males and females strongly differ in how they contribute to offspring feeding. For example, a member of the pair may increase its effort in order to compensate for a lower breeding effort of its mate who had previously stressful experiences (Spencer et al. 2010), and this may reduce its life expectancy (Monaghan et al. 2012). Differential investment of males and females in chick rearing also differs among species. For example, in species where the male provides food to the female during incubation and to both the female and nestlings during the first 1–2 weeks of the nestling stage, the oxidative damage level of males was found to be higher than that of females while chick rearing independently from changes in sexual hormones (Eurasian kestrel *F. tinnunculus*, Casagrande et al. 2011) or after fledging young (Florida scrub jay *Aphelocoma coerulescens*, Heiss and Schoech 2012).

In mammals, females usually invest substantially more in parental care than males (Clutton-Brock 1991), and lactation is the most energetically demanding period for the female. As a consequence, it has been hypothesised that oxidative stress could increase during this reproductive period (Speakman 2008). As with birds, there is little direct experimental evidence that investment in reproduction elevates oxidative stress. In wild Soay sheep (*Ovis aries*), plasma lipid peroxidation level of females was not related to total reproductive effort nor to recent reproductive effort (Nussey et al. 2009). In captive house mice (*M. musculus*), lipid peroxidation level in liver was even lower in females that were allowed to reproduce or to reproduce and defend a territory than in non-reproducing females (Garratt et al. 2011). Oxidation of proteins was also lower in reproducing than non-reproducing females. Moreover, lipid peroxidation level in the livers of female mice that had not reproduced was higher than that of females that were in peak lactation or had just weaned their litter (Garratt et al. 2011). However, when looking at the individual variation within the reproducing females, protein thiols in liver and gastrocnemius muscle were lower in lactating females that had the heaviest litters. Similar to this last finding, Bergeron et al. (2011) and Stier et al. (2012) found, respectively, that wild eastern chipmunks (*Tamias striatus*) and captive mice (*M. musculus*) with larger litter sizes had higher levels of plasma oxidative damage. In a study on wild American red squirrels (*Tamiasciurus hudsonicus*), Fletcher et al. (2013) found that the highest levels of plasma protein oxidative damage occurred during the energetically demanding period of lactation. Moreover, plasma protein oxidative damage was elevated in squirrels that expended the most energy and had the lowest antioxidant protection. Finally, squirrels that were food-supplemented during lactation and winter had increased antioxidant protection and reduced plasma protein oxidative damage, which provided the first experimental evidence in the wild that access to abundant resources might reduce oxidative stress during reproduction (Fletcher et al. 2013). This finding is also very important because it shows how to test the oxidative cost of reproduction hypothesis in captive animals maintained on ad libitum food may not be appropriate.

In order to assess whether reproducing and non-reproducing individuals differ in oxidative balance, Yang et al. (2013) compared the oxidative status between lactating and non-reproducing female Mongolian gerbils (*Meriones unguiculatus*). The authors found that some parameters of oxidative protection (serum superoxide dismutase activity and glutathione peroxidase activity) were reduced, and a marker of oxidative damage (protein carbonyls in serum) was increased, in lactating compared with non-reproductive gerbils. However, other markers of oxidative damage (thiobarbituric acid reactive substances and protein carbonyls in the liver) were actually lower in lactating compared with non-reproductive gerbils, consistent with increased levels of superoxide dismutase activity and non-enzymatic antioxidant capacity in the liver (Yang et al. 2013). These findings showed that the effect of reproductive activity on oxidative balance may be tissue-dependent and biomarker-dependent. This might indicate that reproducing individuals selectively allocate protection to some key tissues, while sacrificing protection of others (Yang et al. 2013; Wilson et al. 2014).

All these studies did not manipulate the effort of females. In order to fill in this experimental gap, Garratt et al. (2013) manipulated female investment in reproduction by allowing some females to breed and keeping others as unmated controls. They then manipulated the reproductive females' investment in lactation by cross-fostering pups so that each female lactated for either two or eight pups, representing either a large or a small litter for wild-derived female mice. In addition to manipulating reproductive investment, females were fed a standard or antioxidant-enriched diet, respectively. The investigators found no differences between reproductive groups at peak lactation for several markers of oxidative stress in the heart and gastrocnemius muscle (Garratt et al. 2013). Surprisingly, oxidative damage to proteins was lower in the livers of females with a litter size of eight than in females with two pups or non-reproductive control females. While protein oxidation decreased, activity levels of the antioxidant enzyme superoxide dismutase increased in the liver, suggesting this may be one pathway used to protect against oxidative stress (Garratt et al. 2013). In a similar related study, Pichaud et al. (2013) found that liver mitochondria of reproductive females showed lower activity of the electron transport system and an increase in mitochondrial density when compared to the non-reproducing females. It was proposed that this mitochondrial adjustment in liver might help spare substrates and, therefore, energy for milk production in the mammary gland. Given that the activity of superoxide dismutase increased in reproducing females (Pichaud et al. 2013), the mitochondrial adjustment might have led to a higher production of superoxide anion (Pichaud et al. 2013). In a similar study, Xu et al. (2014) compared markers of oxidative stress between natural large ($n \geq 9$) and small litter sizes ($0 \leq 5$), and between manipulated large (11–13) and small litter sizes (2–3) of Brandt's voles (*Lasiopodomys brandtii*). They found that liver oxidative damage and superoxide dismutase activity were higher in voles with natural large compared to natural small litter sizes, but there were no differences in other markers. There was no effect of litter size on all measures when it was experimentally manipulated. The effects of reproductive status on oxidative stress were

critically dependent on the exact markers and tissues used. Moreover, the effects of natural variation in reproductive effort suggested there might be an oxidative stress cost associated with large litter sizes, but this effect was not replicated in the experimentally manipulated litters (Xu et al. 2014).

Overall, these studies provided evidence that oxidative damage does not necessarily increase with lactating effort and that any increases may be tissue-dependent and marker-dependent. However, mice and voles were maintained under optimal diet conditions. This diet regime might not be appropriate to reflect conditions that occur under natural conditions. Although dietary restriction may induce confounding complications (e.g. malnutrition), a solution might be to increase the amount of effort required to obtain food rather than limit its abundance (Metcalfe and Monaghan 2013). However, we should not lose sight of the fact that shortage of food or of specific nutrients does occur in the real world. It is, therefore, ecologically relevant to test the oxidative cost of reproduction hypothesis manipulating the diet in a way to reflect wild conditions.

The lack of a simple relationship between reproductive effort and oxidative stress can also be explained by females using strategies to offset particularly costly periods of reproduction. For example, King et al. (2013) found that captive mouse females with a greater reproductive load had no consistent increase in oxidative damage above females who had a smaller reproductive load. The experimental groups differed, however, in their food consumption, reproductive scheduling and mean offspring mass. Importantly, females with a very high reproductive load delayed blastocyst implantation of their second litter, potentially mitigating the costs of energetically demanding reproductive periods.

Oxidative cost induced by offspring rearing may also be tissue-dependent. For example, reproductive male house mice that defended a territory tended to have higher oxidative damage in the gastrocnemius muscle, but lower lipid peroxidation in serum and liver (Garratt et al. 2012). Mice that defended a territory might have experienced an increase in muscle effort needed to sustain an intense physical activity. This increase in physical effort may have induced localised oxidative damage in muscle (Garratt et al. 2012). Conversely, correlative evidence in snow buntings (*Plectrophenax nivalis*) shows that males that defended higher-quality territories had a greater increase in plasma oxidative stress from arrival to incubation (Guindre-Parker et al. 2013). We certainly need more studies in order to elucidate the mechanisms that make tissues to differ in their susceptibility to oxidative stress. It might be possible that individuals prioritise protection of tissues at a cost of others, depending on the current circumstances. However, we first need to clarify the origin of oxidative damage. For example, necrosis of cells of a tissue, say liver, may cause release of oxidised molecules into the bloodstream, causing their decrease and increase in liver and blood, respectively. If this were the case, hypothesis stating that protection of a tissue is prioritised at a cost of another one would be in trouble. We also need to know whether tissues are active to the same extent over the various phases of reproduction. It might be that an increase or decrease in oxidative damage in a certain tissue just reflects the degree to which that tissue is operating. For example, blood and muscle might be more active (as so

more susceptible to oxidative stress) in order to sustain foraging effort, while gonads might be more active when the production of eggs or sperm is required.

Although most of the work on the links between oxidative stress and parental care has been limited to birds and mammals, there are also examples of associations between those two traits in fish. For example, male smallmouth bass (*Micropterus dolomieu*) care for their offspring for around 4 weeks until offspring develop into juveniles and become independent (Ridgway 1988). A reduction in oxygen radical absorbance capacity and an increase in the concentration of both oxidised and total thiols were observed during the parental care period, while no change was observed in the ratio of reduced thiols over total thiols, 8-hydroxy-2-deoxyguanosine, protein carbonyls and thiobarbituric acid reactive substances (Wilson et al. 2012). However, no data for non-reproducing males to be used as controls were available; hence, it is impossible to ascertain whether any changes in oxidative balance were related to parental care or to changes in environmental conditions.

Other taxa offer notable examples of species suitable to test costs of parental care effort. For example, females of some Dendrobatidae frog species have evolved a fascinating tadpole-carrying behaviour (Eaton 1941; Crump 1974; Young 1979). When ponds are almost dry, females take a tadpole on their back and carry it up a tree to deposit it into a large bromeliad (where water is available), where the tadpole can continue to develop. Depending on the species, females do so for each single tadpole, separately, or carry several tadpoles at once. Once the tadpole is in its new home, the work for the mother is not yet finished. Given that food supply is limited within a bromeliad, the female visits repeatedly each offspring and lays an infertile egg that the tadpole will feed on. The workload for the female is clearly very intense.

The parental investment in reproduction can also be limited by physiological constraints. We lack studies looking at whether pre-reproductive oxidative stress acts as a constraint to subsequent reproductive investment. For example, female house mice with low pre-reproductive oxidative damage produced larger litters at birth compared to females with high pre-reproductive oxidative damage (Stier et al. 2012). Similarly, wild female starlings (*S. vulgaris*) with higher plasma oxidative damage during incubation had lower reproductive success (Fig. 7.4). Therefore, addressing how pre-reproductive oxidative stress influences subsequent reproduction is very important because (1) it can elucidate the mechanisms that link the conditions experienced before reproduction to the reproductive decisions and (2) it can explain why oxidative stress and reproduction are sometimes positively or negatively correlated (Stier et al. 2012). Experimental manipulations of pre-breeding oxidative stress level or breeding effort and a combination of multiple parameters of oxidative damage and antioxidant status will be needed to address the dual nature of oxidative stress as a constraint and cost of reproduction.

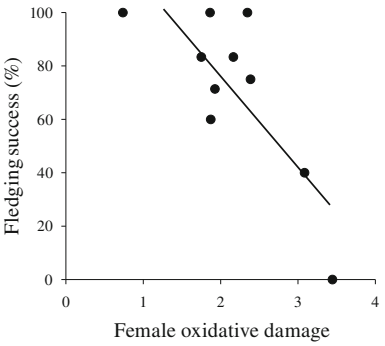

Fig. 7.4 Wild female starlings (*Sturnus vulgaris*) having higher plasma oxidative damage (hydroperoxides, mM H_2O_2 equivalents) while incubating had lower reproductive success (unpublished results of the author). See also Costantini et al. (2010a)

7.7 Hormesis and Reproduction

Whether oxidative stress may have a stimulatory effect on reproduction or whether reproduction may induce hormetic responses of other traits has almost never been directly explored. We have examples suggesting that this might be the case. The production of reactive species in semen may, for example, provide a range of beneficial effects for sperm function, including the ability to achieve and sustain hyperactivation and the promotion of capacitation (de Lamirande and Gagnon 1993; Zini et al. 1995; Godeas et al. 1997; de Lamirande et al. 1997). Mild oxidative conditions promote or are even required for binding of spermatozoa to the zona pellucida of the egg and acrosome reaction (de Lamirande et al. 1997). Although these initial studies were performed in vitro with human sperm and the mechanisms underlying these effects remain unclear, these reports demonstrate that reactive species are not always detrimental to the fertilisation process (Costantini et al. 2010b). Therefore, analysing the balance between the beneficial and harmful effects of reactive species and the balance between reactive species production and antioxidant defences under a hormetic framework is integral to understanding sperm function.

Sex steroids are the main endocrine regulators of reproductive activity. Hormonal organisation of reproductive behaviour involves coordinated changes in various traits, from production of gametes to parental care. They may, for example, determine the reproductive effort, such as that of lekking males (e.g. Siitari et al. 2007; Kervinen et al. 2012). Both oestrogens and androgens can induce or, in response to other molecules, show biphasic responses (Calabrese 2001a, b). It is, thus, reasonable to predict that endocrine hormetic responses may equate in hormetic responses of mechanisms that participate in the control of reproductive activity and that may determine the overall reproductive effort.

Reproductive effort per se may be considered as a stressful event for the individual. Discrepancies among studies on the effects of reproductive effort on oxidative stress might be explained to some degree by hormesis. For example,

increases in antioxidant defences following an increase in free radical production induced by reproductive effort suggest that the effort was mildly stressful for the animal and, therefore, was within the stimulatory range of the physiological performance. This implies that the link between oxidative stress and reproductive effort may follow a hormetic rather than a linear pattern. We need to take this into account when interpreting the effects and consequences of reproductive effort for individual evolutionary fitness. If we take the number of offspring generated across life as a proxy of reproductive effort, we have some evidence from various human populations that a mild reproductive effort may have a stimulatory effect on longevity of women. However, the mechanisms explaining this effect are likely not related only to oxidative stress resistance, but also include changes in hormonal status as those typical of reproductive status in humans (e.g. Brown-Borg 2007; Eskes and Haanen 2007).

Lund et al. (1990) found that nulliparous women had higher mortality than parous women. Parity showed a weak association with increasing mortality among high-parous women. Lowest mortality was found for parous women with 2–4 children. Similarly, Kuningas et al. (2011) found that women with two to three children had significantly lower mortality compared to women with no children. No such benefit was observed for women with four or more children, who had a similar mortality risk as women with no children. However, other studies found that the number of offspring at which detrimental effects on longevity become detectable may be much higher than that recorded in the above studies. For example, McArdle et al. (2006) analysed the genealogical data from an Amish community, a population that, conversely to others, is characterised by natural fertility patterns, large family sizes and little variation of possibly confounding variables like social status. The investigators found that lifespan of mothers increased linearly up to 14 children (0.32 years per additional child), but decreased with each additional child beyond 14. Such a detrimental effect of having beyond 14 children was still evident after adjusting for age at last birth, which was associated with longer lifespan of mothers.

Various studies found that hormetic stimulation of traits like longevity may come at a cost for reproduction (see Chap. 1). There are, however, various reports showing that reproductive performance may be increased through exposure to mild stress. López-Martínez and Hahn (2012) found that anoxia treatment improved mating success and decreased oxidative damage of Caribbean fruit fly males (*Anastrepha suspensa*) when males were also subsequently exposed to irradiation stress, but there was no effect of anoxia treatment on mating performance in unirradiated males. Despite improving performance and decreasing oxidative damage, anoxia-treated male flies were still sterile. This finding suggested the idea that damage to nuclear DNA responsible for sterility can be separated from somatic cellular damage that affects performance (López-Martínez and Hahn 2012). In another study, it was found that when fruit fly females (*D. melanogaster*) experience higher male harassment during the mating season, they have reduced fecundity, but also have offspring with a lower mutation rate (Maklakov et al. 2012). Harassment-induced stimulation of repair of mutagenised sperm indicates a

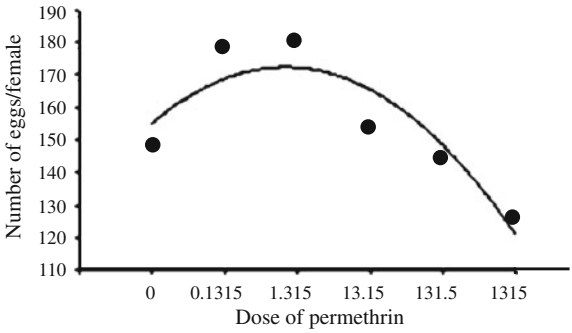

Fig. 7.5 Exposure of *Podisus distinctus* females to sublethal doses of permethrin increased egg production in comparison to controls or insects exposed to high doses. Doses of permethrin are expressed as ppb. The control group was acetone. For each treatment, tree replications were used with 30 nymphs each totalling 90 nymphs/treatment. Reprinted with minimal alterations from Zanuncio et al. (2011) with kind permission from Springer Science+Business Media

possible hormetic response, which may have counterbalanced the reduced fecundity of females. On the other hand, various studies found that exposure to mild stress also increased reproductive output of treated individuals or of their progeny (e.g. Parkhurst et al. 1981; Morse and Zareh 1991; Sclar et al. 1998; Cardoso et al. 2002; Calabrese and Baldwin 2003; Calabrese and Blain 2005; Wang et al. 2005; Cutler et al. 2009; Schreck 2010; Yu et al. 2010; Zanuncio et al. 2011). For example, compared to controls or high concentrations, it was found that the hatching success in many bird species was higher in eggs containing low doses of methylmercury that was artificially injected or naturally deposited by methylmercury-fed mothers (Heinz et al. 2010; 2012). Exposure to a low dose of 4-nonylphenol increased the number of eggs laid by fathead minnows (*Pimophales promelas*) through an indirect stimulation of oestrogen secretion compared to controls or to high doses (Giesy et al. 2000). Similarly, exposure of *Podisus distinctus* females to sublethal doses of permethrin increased both egg production (Fig. 7.5) and number of nymphs per female in comparison to controls or insects exposed to high doses (Zanuncio et al. 2011). It cannot be excluded that this increase in reproductive output might be a terminal investment response that only mild doses of stress may trigger.

Transgenerational hormesis was also observed in the green peach aphid, *Myzus persicae*, when exposed to sublethal concentrations of the insecticide imidacloprid (Ayyanath et al. 2013). Although fitness trade-offs occurred with hormetic responses, this appeared to not necessarily compromise overall biological fitness (Ayyanath et al. 2013). In fact, while reproduction was decreased in intermediate generations, it was observed a recovery to levels of reproduction equal to that of controls in subsequent generations and a significantly greater total reproduction after four generations (Ayyanath et al. 2013).

Hormesis of reproduction was also demonstrated by exposure of individuals to non-anthropogenic stressors. Increased partial pressure of CO_2 in seawater

dramatically stimulated breeding activity of cinnamon clownfishes (*Amphiprion melanopus*) rather than impaired it as expected (Miller et al. 2013). This positive hormetic effect on reproduction did not come through an effect on fish body mass, which did not differ among groups. There is, therefore, potential for hormesis to increase or to buffer against decreases in evolutionary fitness (see Chap. 1).

7.8 Conclusions

This chapter shows that there is strong potential for research linking the cost of reproduction and oxidative stress. The large taxonomic variation in reproductive strategies offers evolutionary ecologists and physiologists numerous systems where to address the role of oxidative stress as a cost and constraint of reproductive activity.

Certainly, we lack clear experimental demonstrations of the link between oxidative stress and reproductive effort, possibly because of inadequate experimental designs or use of available biomarkers of oxidative status (Metcalfe and Monaghan 2013). We have to consider, however, that (1) mild stress episodes may stimulate both the reproductive performance and resistance to oxidative stress and (2) reproductive investment per se may result in hormetic responses at physiological level. We have, therefore, to take into account hormesis when interpreting the effects and consequences of reproductive effort for individual fitness. If, for example, we aim to increase the chick-rearing effort of birds, we have to make sure that we do increase it to a degree that will exceed the hormetic zone, that is, that area in which the stressful event is stimulating for the organism (Chap. 1).

We also need to assess the cost of reproduction in individuals of different age. This is, for example, important to examine the consequences of maternal effects on offspring. Older females of the marine pelagic copepod *Acartia tonsa* produced offspring with higher protein oxidative damage than younger females (Rodríguez-Graña et al. 2010). This might indicate that reproductive strategies differ among individuals of different age and/or simply that ageing of the reproductive system constrains how many resources older individuals may put into reproduction. These results also suggest that if individuals born from older mothers have fewer chances to survive, the total reproductive output over lifetime might be a poor indicator of evolutionary fitness. That offspring born from older mothers suffer from a reduced lifespan was first demonstrated in 1947 with a study of *Rotifer vulgaris* (Lansing 1947). The so-called Lansing effect was later reported in various studies on offspring performance (e.g. growth, reproductive senescence, survival) in invertebrates and vertebrates, which showed that it decreases with increasing maternal age (Fox and Dingle 1994; Priest et al. 2002; Giron and Casas 2003; Jamieson and Santer 2003; Tarín et al. 2005; Bouwhuis et al. 2010). This suggests that at least for some species, it is beneficial to invest more in the first part of the reproductive life. Different life history trajectories may, however, result in a similar fitness

payoff, with young and old mothers having offspring of similar reproductive lifespan (Bouwhuis et al. 2010). There is, therefore, clear need that future experiments should not only measure fitness effects of reproductive or parental effort in the manipulated individuals (intragenerational trade-off), but should also measure the fitness consequences for progeny (intergenerational trade-off) and for males and females, separately (Lindén and Møller 1989; Martin 2004; Santos and Nakagawa 2012).

We should also not forget the importance of extrinsic mortality risk for the investment in reproduction and, consequently, for the magnitude of the proximate costs of reproductive activity. For example, guppies (*Poecilia reticulata*) from high-predation environments have a longer reproductive lifespan than guppies from environments with low predation risks (Reznick et al. 2006). In contrast, the post-reproductive lifespan was similar between the two populations, showing that differences in lifespan evolving in response to selection are confined to the reproductive lifespan, or those segments of the life history that make a direct contribution to fitness (Reznick et al. 2006). Investment in protection against oxidative stress may vary, depending on the risk of predation: there would not be any selective advantage in investing lots of resources in antioxidant protection whether chances of survival are low. In this particular case, it would be more convenient to invest in reproduction at a cost for self-maintenance.

The study of the links among oxidative stress, hormesis and reproduction might gain from the application of gene expression analyses and manipulations. For example, Béguel et al. (2013) manipulated the reproductive investment of Pacific oysters *Crassostrea gigas* by using an in vivo RNA interference technique to knockdown the *oyster vasa-like gene*, a gene implicated in germ cell proliferation in this particular oyster species. The highest reproductive investment was then found to be associated with upregulation of genes encoding enzymes involved in the first step of reactive species detoxification (Béguel et al. 2013).

There is, finally, important avenue for the study of oxidative stress as a proximate cause of evolution of sexual reproduction. While in most eukaryotes, sexual reproduction is an obligate part of the life cycle, it is facultative in prokaryotes and lower eukaryotes. In these groups, sex is an adaptive response to environmental conditions, with sexual reproduction being triggered under stressful conditions because being able of producing more resistant spores. In particular, studies on species like *Volvox carteri* and *Chlamydomonas reinhardtii* showed that antioxidants inhibit sexual induction, sex is triggered by an increase in reactive oxygen species production, and that there may be an evolutionary connection between sex and oxidative stress at the gene level (Nedelcu and Michod 2003; Nedelcu et al. 2004; Nedelcu 2005). These results suggest that sexual reproduction may have also been evolved as a response to oxidative stress threat.

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Chapter 8
Combating Parasites: Immune Response and Inflammation

Abstract An organism's physiological equilibrium is critically reliant on its immune system, which provides protection against parasites, pathogens, toxic substances, and cancerous cells and allows recovery from injuries. The immune response is, however, not cost free. It demands various kinds of resources, but also more subtle costs. In the last couple of decades, there has been growing interest by ecophysiologists and evolutionary ecologists in the investigation of oxidative stress as a potential cost of immune activation and induction of anti- and pro-inflammatory mechanisms. A core idea of the immuno-oxidative ecology is that oxidative stress may provide a currency to quantify physiological costs that impinge on growth, reproduction or senescence. This chapter deals with such research and also examines how exposure to mild stress may stimulate activity of immune cells through hormetic mechanisms.

8.1 Ecoimmunology and the Arms Race

An organism's physiological equilibrium is critically reliant on its immune system, which provides protection against parasites, pathogens, toxic substances, and cancerous cells and allows recovery from injuries, like wounds. Generally speaking, we can recognise the following: (1) an innate immunity (non-specific) that represents the first line of defence and that encompasses reactions like phagocytosis and inflammation; it relies on constitutively produced receptors that bind to distinct molecular structures (i.e. those of microbes) and activate the host's immune cells, and (2) an acquired immunity that develops with exposure to various antigens; it is characterised by a large variety of cells that recognise specific antigenic configurations of pathogens and respond by triggering cellular and humoral effectors (Sorci and Faivre 2009). Young individuals also have passive

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immunity because they are born with antibodies that are transferred through the egg or placenta from their mothers. Inflammation is part of the complex biological response that an organism mounts to cope with harmful stimuli, such as pathogens. It is considered to be a mechanism of innate immunity. The inflammatory response is regulated by many molecules (e.g., cytokines, glucocorticoids) and may result in various symptoms, such as fever, loss of body mass or lethargy (Dantzer et al. 2008).

The *arms race* paradigm (Red Queen hypothesis) appropriately reflects the need of every living organism's life to keep pace with the evolution of parasites and pathogens by evolving mechanisms to withstand their detrimental effects. An entire discipline (ecoimmunology) has been devoted to describing and explaining host–parasite coevolution and natural variation in immune responses (Sheldon and Verhulst 1996; Martin et al. 2011). It seeks to answer the questions of how and why biotic and abiotic factors contribute to variation in immunity in free-living organisms (Sheldon and Verhulst 1996; Martin et al. 2011). Life history theory provides an important framework to further our understanding of among-species variation in immunity. The organism invests energy and nutrients to sustain its immune response, but such resources are also required for other functions, such as reproduction, growth or self-maintenance. Given that these resources occur in limited supply, trade-offs arise between investment in other attributes that promote, say, reproductive success versus the immune system, leading to complex relationships between life history decisions and levels of infection and parasitism (Sheldon and Verhulst 1996; Norris and Evans 2000; Schulenburg et al. 2009). Hence, the immune response is clearly not cost free. It certainly demands various kinds of resources (Klasing 2004, 2007), but there are also more subtle costs. In the last couple of decades, there has been growing interest by ecophysiologists and evolutionary ecologists in the investigation of oxidative stress as a potential cost of immune activation and induction of anti- and pro-inflammatory mechanisms (Costantini and Dell'Omo 2006; Costantini and Møller 2009; Hasselquist and Nilsson 2012; Sorci and Faivre 2009). A core idea of immuno-oxidative ecology is that oxidative stress might provide a currency for quantifying physiological costs that impinge on life history strategies. For example, Krams et al. (2013) showed that prolonged immune activity can reduce lifespan in mealworm beetles *Tenebrio molitor*. Although this study does not prove that oxidative stress was involved, it provides valid support for investigation of the role of inflammation-related oxidative damage as promoter of senescence. Further studies showed that immune activation may cause apoptosis and cell necrosis in the testes and thus impairment of spermatogenesis and reduction in sperm quality with (Reddy et al. 2006; Brecchia et al. 2010) or without (Losdat et al. 2011) oxidative stress. In this framework, another important avenue of research is to assess the extent to which oxidative stress may constrain the immune response itself through the generation of damage to immune cells themselves.

8.2 Oxidative Stress and Immune Response

8.2.1 Immune Cells as Generators of Reactive Species

In 1961, Iyer et al. showed that the phagocyte respiratory burst results in the generation of hydrogen peroxide. In 1973, it was described for the first time that activated phagocytes produce the superoxide anion as initial product and not hydrogen peroxide (Babior et al. 1973). Further work showed that the production of reactive species is essential for various kinds of immune cells in order to degrade and kill parasites and/or pathogens (Babior 2004; Levy 2004; Segal 2005; Halliwell 2006; Üllen et al. 2013). Phagocytes and lymphocytes contain a multicomponent enzyme complex, the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Hampton et al. 1998; Babior 1999, 2004). This enzyme complex is responsible for the production of reactive oxygen species after immune stimulation. Phagocytes increase their oxygen uptake as much as 10–20 times the resting values (*respiratory burst*). The NADPH oxidase is oxidised into $NADP^+$, releasing two electrons, which are used to reduce oxygen to superoxide free radical:

$$NADPH + 2O_2 \rightarrow NADP^+ + H^+ + 2O_2^{\bullet -}$$

The superoxide anion generated by this enzyme does not seem to pass through the cell wall and membrane of the pathogen (Hassan and Fridovich 1979; Halliwell 2006). Hence, it contributes to killing microorganisms serving as the starting material for the production of various reactive species, such as hydrogen peroxide, hypochlorous acid, peroxynitrite and, possibly, hydroxyl and ozone (Halliwell 2006). For example, the superoxide radical undergoes either non-enzymatic dismutation or enzyme-catalysed dismutation by superoxide dismutase to hydrogen peroxide:

$$2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$$

This is important because hydrogen peroxide, being less reactive than the superoxide radical, can easily enter invading cells and kill them, often working as material for the formation of hydroxyl radical (Repine et al. 1981; Halliwell 2006).

Hydrogen peroxide can also be used to produce hypochlorus acid (HOCl), a powerful antibacterial and antifungal reactive species (Winterbourn 2002; Halliwell 2006):

$$Cl^- + H_2O_2 \rightarrow OCl^- + H_2O$$

$$OCl^- + H^+ \leftrightarrow HOCl$$

$$HOCl + H^+ + Cl^- \leftrightarrow H_2O + Cl_2$$

The above reaction is catalysed by the enzyme myeloperoxidase, which is absent in some animal groups, like certain bird species (Brune et al. 1972; Penniall and Spitznagel 1975; Stabler et al. 1994).

As with hydrogen peroxide, hypochlorous acid can enter pathogens, where it may trigger a damaging oxidative cascade. Downregulation of antioxidant enzymes (catalase and glutathione peroxidase) that reduce hydrogen peroxide to water can, therefore, be essential because the activity of those enzymes might limit the rate of production of hypochlorous acid and, consequently, the effectiveness of the immune response.

Hypochlorous acid can also generate the hydroxyl radical by reaction with either Fe^{2+} (Dukan and Touati 1996) or with superoxide (Shen et al. 2000):

$$Fe^{2+} + HOCl \rightarrow Fe^{3+} + OH^{\bullet} + Cl^{-}$$

$$HOCl + O_2^{\bullet -} \rightarrow O_2 + OH^{\bullet} + Cl^{-}$$

The production of reactive nitrogen species is another important route through which immune cells can kill pathogens (Halliwell 2006). They can do so, for example, by inhibiting respiration mechanisms (Shi et al. 2005) or by reacting with superoxide to generate peroxynitrite (Halliwell 2006):

$$O_2^{\bullet -} + NO^{\bullet} \rightarrow ONOO^{-}$$

Antimicrobial mechanisms do not only rely on the oxidative burst. For example, Vilchèze et al. (2013) found that vitamin C, a compound known to drive the Fenton reaction, sterilizes cultures of drug-susceptible and drug-resistant *Mycobacterium tuberculosis*, the causative agent of tuberculosis. The bactericidal activity of vitamin C against *M. tuberculosis* was dependent on high ferrous ion levels and reactive oxygen species production and caused a pleiotropic effect by affecting several biological processes, such as lipid biosynthesis (Vilchèze et al. 2013).

It is evident that various biochemical mechanisms can be responsible for the increase in oxidative stress during the organismal response to a parasite infection. Moreover, species are not all equipped with the same basic defence toolkit. The evolutionary reasons for this among-species variation in immune defences remain to be unravelled.

8.2.2 Immune Response and Oxidative Stress In Vivo

Independent evidence showed that pathogens with impaired antioxidant defences are more sensitive to phagocytic killing, indicating an in vivo role for reactive species as microbicidal agents (Halliwell and Gutteridge 2007). Further studies found that individuals deficient in oxidase are susceptible to severe bacterial and fungal infections (Nathan and Shiloh 2000). For example, wild-type mice had

100 % survival at 20 days post-infection with *Salmonella typhimurium*, while knockout mice that did not express *phox* (NADPH-based respiratory burst) or iNOS (nitric oxide production) died during the course of the experiment (Mastroeni et al. 2000). Moreover, *phox* mice died more quickly (100 % mortality at day 5 post-infection) than iNOS mice (100 % mortality at day 19 post-infection), suggesting that the respiratory burst precedes the nitric oxide response during the acute inflammatory response (Mastroeni et al. 2000). The role of nitric oxide as an anti-parasite molecule is not restricted to intracellular parasites, but can also kill extracellular organisms. For example, studies on mice infected with larvae of the nematode *Brugia malayi* were less or more resistant to infection when the production of nitric oxide was experimentally inhibited or stimulated, respectively (Rajan et al. 1996).

The activity of reactive species is not specific for pathogens because they can also oxidise host cell tissues, possibly generating detrimental effects (*immunopathology*). This is particularly evident when the oxidative stress level dramatically increases during chronic inflammation (Sorci and Faivre 2009). Immune cells can also be damaged. However, immune cells have their own antioxidant defences, such as glutathione, superoxide dismutase, glutathione peroxidase or ascorbate. Neutrophils deficient in glutathione reductase activity are, for example, more rapidly inactivated during phagocytosis than normal neutrophils (Halliwell and Gutteridge 2007). There is also among-species variation in the antioxidant content of immune cells, which calls for caution in generalising findings from a single species to others (Halliwell and Gutteridge 2007). The increase in oxidative stress during an inflammatory event may also be furthered by systemic increases in metabolic activity, such as those associated with fever (Demas et al. 1997; Riedel and Maulik 1999; Ots et al. 2001).

In a meta-analysis of 16 studies of ten species of birds including 49 estimates of effect size from experimental studies, Costantini and Møller (2009) found that induction of an immune response may determine oxidative stress (variance explained: 4.1 %), but, most notably, may determine changes in parameters of oxidative damage or antioxidant status (variance explained: 15.0 %). There was, however, significant heterogeneity among studies, part of which could be accounted for by whether the study was based on birds studied in captivity or in the wild. Effect size was actually always high in free-living birds, while it was more variable in captive birds (Costantini and Møller 2009). On the one hand, this may be explained by either the quality of the diet or the fact that free-living birds cannot readily recover from the costs of an immune response because trade-offs are more stringent. On the other, this may mirror the fact that selection lines of captive birds may not be subject to a full complement of natural and sexual selection, emphasising the need of many replicates (Costantini and Møller 2009). Significant changes in oxidative status induced by immune response were found in both the short (e.g., 1 day after immunostimulation, Costantini and Dell'Omo 2006; Fig. 8.1) and long terms (e.g., 6 days after immunostimulation, Casagrande et al. 2012). Moreover, such changes emerged in response to artificial immune challenges (e.g., phytohaemagglutinin in Costantini and Dell'Omo 2006) or to real

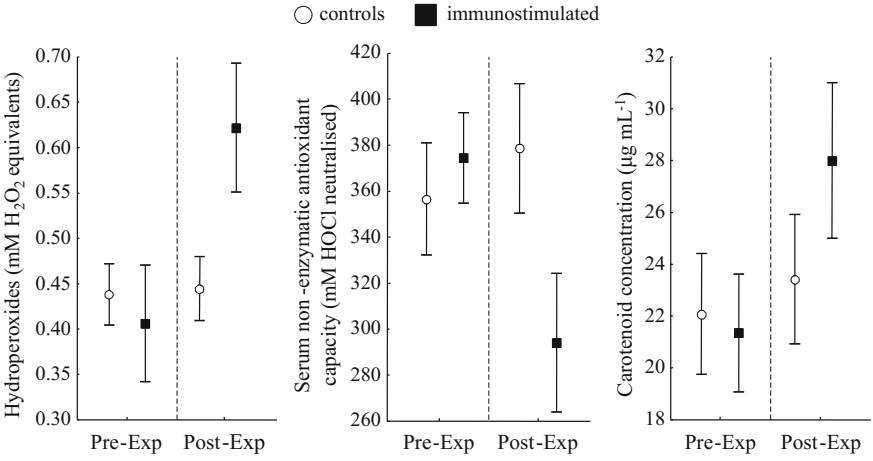

Fig. 8.1 Effects of immune response stimulated by an intradermal phytohaemagglutinin injection on serum oxidative damage (hydroperoxides), serum non-enzymatic antioxidant capacity and serum carotenoid concentration in wild kestrel nestlings (*Falco tinnunculus*). *Pre* = value before immunostimulation; *post* = value 24 h after immunostimulation. Data are shown as mean ± standard error. Reprinted with minimal alterations from Costantini and Dell'Omo (2006) with permission from Elsevier

pathogens (e.g., *Trichostrongylus tenuis* larvae in Mougeot et al. 2009; *Isospora* coccidians in Sepp et al. 2012). The increase in oxidative stress during an immune response may also be strengthened by a pre-treatment with molecules involved in immunity like lysozyme (van de Crommenacker et al. 2010).

Mounting an immune response had also a significant impact on antioxidant defences, which were possibly important for mitigating oxidative stress to host tissues. Studies on birds found that dietary antioxidants like vitamin E and carotenoids may stimulate the immune response. In ovo administration of vitamin E improved the phagocytic potential and production of nitric oxide by macrophages isolated at 4 weeks of age (Gore and Qureshi 1997). Vitamin E may have preserved macrophage membrane integrity through antioxidant protection or downregulation of synthesis of the immunosuppressive prostaglandins (Qureshi et al. 2000). As regards other dietary antioxidants, male zebra finches (*Taeniopygia guttata*) supplemented with carotenoids produced significantly stronger immune responses than control birds to a phytohemagglutinin injection, which induces a non-specific response of T lymphocytes and macrophages (Blount et al. 2003; McGraw and Ardia 2003), as well as to injection of sheep red blood cells, which induces a humoral response to T-dependent antigens (McGraw and Ardia 2003). However, it has been shown that carotenoids have low to negligible antioxidant properties in birds (Costantini and Møller 2008; Simons et al. 2012) and do not influence the oxidative burst of phagocytes (Sild et al. 2011). These results might be reconciled with each other if carotenoids mainly act as immuno-stimulating and immuno-modulating biochemicals (e.g., Bendich 1989; Chew 1993;

Kim et al. 2000), while vitamins A and E and other fat-soluble antioxidants or vitamin C mainly act as free radical scavengers (e.g., Cadenas et al. 1998; Surai 2002; Hartley and Kennedy 2004). Such dual roles for these two groups of biochemicals could help explain their positive covariation within and among species, because the consequences of high levels of immuno-stimulation or immunomodulation by carotenoids for immune response would later be partly neutralised by the effects of antioxidants, thereby preventing immuno-pathological damage to host tissues (Costantini and Møller 2009). In fact, enhanced immune cell activity can overproduce free radicals, and antioxidant protection in this case may be vital. However, it cannot be entirely ruled out that carotenoids may offer some antioxidant protection to immune cells that studies so far failed to detect because of an inappropriate methodological or experimental setting. For example, carotenoids, but not vitamin E supplementation reduced oxidative damage in greenfinches (*Carduelis chloris*), although it did not fully compensate for the effects of immune activation (Hõrak et al. 2007). The immune response may be constrained by free radicals when immune cells are being damaged by the free radicals themselves or negative feedback mechanisms related to reactive species levels are activated. For example, Tobler et al. (2011) found that pre-immune challenge anion superoxide levels were negatively correlated with the strength of the subsequent immune response towards phytohaemagglutinin in male, but not in female painted dragon lizards (*Ctenophorus pictus*), and that the immune response was stronger in females than in males. These results also suggested that there might be sex-specific selective pressures on immune investment (Tobler et al. 2011). Similarly, Ridgway et al. (2014) found that exposure to a pro-oxidant molecule (tert-butyl hydroperoxide) reduced haemocyte counts in four bivalve species.

The change in redox balance following an immune response is not specific to birds, but is highly conserved across animals. Studies on mammals found that stimulation of immune cells enhanced the blood or liver levels of oxidative damage (Sirak et al. 1991; Cadenas et al. 1998; Schneeberger et al. 2013). However, among free-living adult female sheep (*Ovis aries*), there was no evidence that current parasite burden (strongyle faecal egg count) significantly predicted oxidative damage levels (Nussey et al. 2009). In contrast, it has been found that blood oxidative stress may be higher in dogs with *Leishmaniasis* infection (Paltrinieri et al. 2010; Almeida et al. 2013) or dogs affected by lymphoma (Winter et al. 2009) or mast cell (cells involved in wound healing and defence against pathogens) tumour (Finotello et al. 2014).

In fish, three-spined sticklebacks (*Gasterosteus aculeatus*) with high levels of expression at major histocompatibility complex class IIB loci had elevated levels of acrolein, a marker of oxidative protein damage (Kurtz et al. 2006). At spawning, prioritisation of investment in egg production may make female brown trout (*Salmo trutta*) especially vulnerable to ulcerative dermal necrosis and fungal infection (*Saprolegnia* spp.) with associated increases in oxidative damage and decreases in antioxidant protection (Kurhalyuk et al. 2009, 2010; Hoogenboom et al. 2012; Zahran and Risha 2013). Correlative data also suggest that when the fungal infection becomes very intense, immune activity may be suppressed through the action of

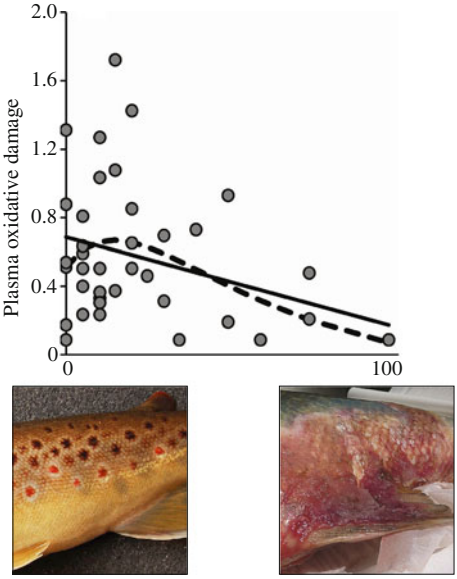

Fig. 8.2 Relationship between intensity of *Saprolegnia* fungal infection (% cover of fins) and plasma content of oxidative damage (hydroperoxides). Data points represent individual trout females sampled at the time of spawning. *Solid line* indicates linear regression depicting the statistically significant relationship identified using analysis of variance. *Dashed line* represents fit of the equation $y = a + (b - e^{-\alpha x})e^{-\beta x}$ with parameters estimated as $a = 0.2$, $b = 1.7$, $\alpha = 0.04$ and $\beta = 0.02$. However, while a nonlinear regression explained an additional 5 % of the variation in the data, model selection techniques supported the retention of the simpler (linear) model (Akaike weight 81 % for the linear regression and 19 % for the nonlinear regression). Reprinted with minimal alterations from Hoogenboom et al. (2012) with permission from Elsevier. Photograph of healthy fish courtesy of Mia Hoogenboom; photograph of fish infected with *Saprolegnia* fungus courtesy of David Bruno

stress hormones, and the production of early derivatives of oxidative damage consequently goes down (Fig. 8.2; Hoogenboom et al. 2012). High levels of glucocorticoids can, for example, suppress activity of antioxidant enzymes and production of hydrogen peroxide in macrophages (Pereira et al. 1995). Plants also generate reactive species as signalling molecules to control various processes including pathogen defence (Apel and Hirt 2004), and this may result in oxidative stress whether the infection is long lasting (Hernández et al. 2004).

Although immune cells of invertebrates are different from those of vertebrates, they also rely on production of reactive oxygen and nitrogen species to degrade pathogens (Philipp et al. 2012). Immune cells in bivalves increase their production of reactive species 15–70 min after infection, although at much lower concentrations than those produced in vertebrates (Donaghy et al. 2009; Philipp et al. 2012). However, it was found that immune cells differ in the quantity of reactive

species that they can produce (Pipe et al. 1997; Hégaret et al. 2003), suggesting that bivalves might up- or downregulate specific immune cells, depending on current needs and costs-benefits associated with the activity of a certain group of immune cells. Antioxidant defences of bivalves may also be upregulated while being infected. For example, *Crassostrea gigas* larvae subjected to experimental infection with the bacterium *Vibrio coralliilyticus* upregulated various antioxidant defences, as revealed by the higher transcript abundance of antioxidant enzymes measured after 24 h of exposure for glutathione peroxidase 5 and after 48 h for glutathione reductase, superoxide dismutase, catalase and peroxiredoxine 4 and 5 (Genard et al. 2013). This overexpression of catalase and superoxide dismutase in challenged larvae was also reflected at the physiological level by the higher activities of the corresponding enzymes.

Several invertebrates may also rely on other mechanisms beyond immune cells to protect themselves against pathogens. For example, the enzyme phenoloxidase is used by many invertebrate species to synthesise melanin, which binds to the surface of bacteria and increases the adhesion of haemocytes to them, thus accelerating their removal (da Silva 2002; Bogdan 2007; Cerenius et al. 2008). While free radicals are produced during the synthesis of melanin, relying on this mechanism might allow the organism to avoid unnecessary production of highly toxic and reactive compounds that might damage host tissues.

The activation of an immune response is not always accompanied by a change in oxidative damage level or in antioxidant status (Costantini and Møller 2009). There are certainly various technical reasons, from methodological approaches used to elicit the immune response, the temporal window (e.g., time lag) when the parameter is measured, to which branches of the immune system are activated, and which components of oxidative balance are measured. For example, some inflammatory cells may produce little quantities of reactive species (Conlon et al. 1991). Therefore, it would be very important to quantify the total and relative numbers of different leucocytes that are activated following experimental stimulation. Intraindividual repeated measures would also help overcome any issue related to time lag in response.

Heterogeneity in the effects of an immune response on oxidative balance may also mirror different life histories, phases of the life cycle, intensities of sexual selection, histories of parasite-mediated selection, and/or feeding habits, and, consequently, different mechanisms that have been evolved to cope with immune response-induced oxidative stress threats. Avian malaria (*Plasmodium* sp.) is an interesting example of a globally widespread disease that may show considerable variation in both prevalence and parasitaemia at small spatial scales (Wood et al. 2007). This pathogen is transmitted by insect vectors like mosquitoes (*Culex*, *Aedes* and *Culiseta*), and chronic infection may have detrimental effects on reproductive activity and survival of avian hosts (Knowles et al. 2010). An increase in oxidative damage may be implicated in the reduction in evolutionary fitness, especially when the infection is chronic. Van de Crommenacker et al. (2012) found that malaria was associated with increased susceptibility to oxidative stress in Seychelles warbler (*Acrocephalus sechellensis*), but this depended on the

breeding stage: only during the energetically demanding provisioning stage did infected birds have higher oxidative stress susceptibility than non-infected birds. The imbalance in oxidative status was caused by a marked increase in oxidant levels observed only in infected birds during provisioning and by an overall reduction in non-enzymatic antioxidant capacity observed in all birds across the breeding cycle. These findings implied that higher workload while dealing with an infection could aggravate oxidative repercussions (van de Crommenacker et al. 2012), likely because individuals are more constrained in the optimisation of trade-offs. In a study of great tits (*Parus major*), while the reproductive effort decreased blood antioxidants and increased malarial parasitaemia, respectively, antioxidants and parasite load were not related to each other (Christie et al. 2012). In contrast, Isaksson et al. (2013) found that host oxidative balance shows species-specific patterns in relation to infection and that oxidative damage increases with the level of *Plasmodium* parasitaemia in wild breeding great tits.

Circumstances under which the immune response is activated are also important for other taxa, like fish. For example, it was found that the immune response and so the capacity to degrade pathogens may be suppressed by a combination of decreased oxygen and pH (Boleza et al. 2001) and increased carbon dioxide or may be lower at certain water temperatures than at others (Ndong et al. 2006).

Reasons for why the oxidative status parameters do not change while mounting an immune response may also be found in the strategies that parasites adopt to invade the host. For example, various strains or species of bacteria, protozoa and worms evolved mechanisms to suppress generation of reactive species of their hosts (see Sect. 8.2.3). It is clear that we should bear in mind the biology of the parasite when interpreting the effects of that parasite on the oxidative balance of its host.

The effect of parasites on oxidative stress may also be mediated by experiences of the mother, which in turn shape strategies of maternal investment in offspring. De Coster et al. (2012) assessed how mothers mediate the link between parasite infection and oxidative stress in nestling great tits (*P. major*). They carried out an ectoparasite treatment before egg laying combined with a partial cross-fostering experiment between broods of infested and uninfested nests. The ectoparasite treatment consisted of a first phase in which nest parasites were killed and a second phase in which half of nests were inoculated with hen fleas (*Ceratophyllus gallinae*), which are blood-sucking ectoparasites of great tits. Hen fleas induce immune responses (De Coster et al. 2010) and various deleterious effects, especially in males (Heeb et al. 1998; Tschirren et al. 2003). Then, half broods were reciprocally swapped between pairs of infested and uninfested nests. De Coster and collaborators found that when pre- and post-hatching environments were similar (control–control, infested–infested), (1) daughters had significantly lower plasma oxidative damage levels than sons in infested nests, but not in uninfested ones, and (2) daughters had significantly higher and lower plasma non-enzymatic antioxidant capacity than sons, respectively, in infested and uninfested nests.

Increases in oxidative damage observed during infection may not be entirely due to the physiological response of the host, but also to the release of reactive species from the invading microorganism. For example, *Mycoplasma* species are highly

contagious bacteria that cause severe and chronic respiratory diseases. It was reported that various *Mycoplasma* species can release hydrogen peroxide, superoxide anions and hydroxyl radicals that are used as a primary virulence mechanism, inflicting damage to host tissue (Lynch and Cole 1980; Baumann 1989).

What is evident from these studies is that quantifying the costs of an immune response is very complex. For example, measuring one class of antioxidants or a measure of in vitro antioxidant capacity of a biological matrix may give us a different picture from that obtained while measuring another. For example, Pašková et al. (2008) clearly showed that levels of two antioxidants, glutathione and glutathione peroxidase, may covary negatively after immunostimulation. Similar results emerged from other studies (Koinarski et al. 2005; Georgieva et al. 2006) that found levels of catalase and superoxide dismutase to covary negatively after an immune challenge. In a similar way, antioxidants may change, while oxidative damage markers may remain stable during an immune response (Meitern et al. 2013). These results may depend on the fact that antioxidants can detoxify the biological system in very different ways, for example, acting at different stages in the oxidative sequence (Chap. 1). However, some antioxidants may be downregulated in order to make the immune response more efficient. For example, catalase and glutathione peroxidase may be downregulated in order to keep the production of hypochlorous acid up (see Sect. 8.2.1).

Animals may adopt various strategies to sustain the immune response, while mitigating any costs of immune response. Selection of food rich in antioxidants and immunostimulant compounds help individuals self-medicate (see Chap. 4). Hosts can also show several physiological, anatomical and behavioural compensatory responses. Although it is hard to distinguish between direct parasite impact and host compensation, some alterations in hosts might represent examples of phenotypic plasticity that minimise the fitness costs of infection. For example, infection of deer mice (*Peromyscus maniculatus*) with a trematode parasite (*Schistosomatium douthitti*) caused an increase in gastrointestinal, liver, and spleen masses and a decrease in liver protein synthesis, while it did not affect food consumption, activity, basal metabolic rate and cold-induced maximal metabolic rate (Schwanz 2006). These results were also interpreted as an example of a plastic response of mice to mitigate any costs associated with infection. Moreover, it was suggested that mice might prioritise maintenance of some tissues over others whether any damages to them were more detrimental for evolutionary fitness (Schwanz 2006). Plastic responses to pathogens can also be determined by early life experiences. Exposure to pathogens early in life had a hormetic effect on adult nematodes, as it resulted in increased resistance to different pathogens and to heat shock (Leroy et al. 2012).

8.2.3 Inflammation from the Parasite's Viewpoint

Parasites have evolved a number of strategies to evade the host's inflammatory response (Hornef et al. 2002; Sacks and Sher 2002; Sorci and Faivre 2009).

Parasites may use mechanisms to avoid recognition; to avoid phagocytosis; or to interfere with the secretion of inflammatory cytokines that the host uses for transfer of information among immune cells.

Parasites may also rely on antioxidant defences to resist the host's free radical attack. They may rapidly raise antioxidant levels in response to the oxidative burst; may maintain high constitutive levels of antioxidant enzymes; or may express various proteins that repair oxidative damage (Halliwell and Gutteridge 2007). That antioxidant defences are important for parasites is well demonstrated by those studies showing that parasites with impaired antioxidants are more sensitive to phagocytic killing. For example, *S. typhimurium* strains deficient in superoxide dismutase were less resistant to reactive species produced by the host (De Groote et al. 1997). Similarly, strains of *Staphylococcus aureus* unable to synthesise carotenoids were less resistant to neutrophil oxidative burst and were less pathogenic compared to wild strains (Liu et al. 2005).

Some parasite species can also directly interfere with the production of reactive species of the host (Lambert and Nicolas 1998; Broeg and Steinhagen 2012). For example, *Helicobacter pylori* reduces the availability of substrates that the host uses for the production of nitric oxide (Gobert et al. 2001).

Similarly, *Leishmania* spp. inhibits the production of pro-oxidants by phagocytes. This ability allows parasites to survive and infect macrophages within which they replicate (Panaro et al. 1998). As a response, the organism recruits immune cells into inflammatory sites, where they can release pro-oxidants more intensely than in other inflammatory responses (Paltrinieri et al. 2010; Almeida et al. 2013).

8.3 Environmental Stress, Viruses Outbreaks and Oxidative Stress

Host exposure to environmental stressors can facilitate the spread, persistence or emergence of diseases or parasites (Martin et al. 2010). When exposed to a stressor, organism resistance to infection may actually be reduced through suppression of the immune system, which in turn may reduce survival perspectives (Apanius 1998). The likelihood to be infected and the morbidity of the infected animal consequently increase. Although it is now well established that a stress status is associated with an increase in susceptibility to diseases, the physiological mechanisms underlying the links among environmental stressors, resistance to pathogens and survival are not well understood, especially in free-living animals. Understanding such physiological mechanisms is of central importance to evolutionary ecology and conservation biology because spread of zoonotic infectious may cause impressive mortality outbreaks in natural animal populations, so working as a relevant selective agent.

Herpes viruses outbreaks are, for example, one of the most common viral agents in wild animals because their spread is facilitated by suppression of the

immune system consequently to exposure to stressful conditions. Many virus strains appear to be species specific or can only infect a limited range of species; in contrast, others can infect a wide species range (Kaleta 1990). The general signs of disease include depression of normal activity, respiratory distress, extremity paralysis, head-shaking, haemorrhagic lesions, twisting of the neck and sudden mortality (Kaleta 1990).

The appearance of a herpes virus outbreak in a population likely indicates that animals have been exposed to a severe environmental stressor. Vertebrates translate environmentally stressful stimuli into secretions of hormones (glucocorticoids) that through a cascade mechanism activate the physiological stress response (Sapolsky et al. 2000; Romero 2004). The action of glucocorticoids involves a high diversity of metabolic effects, such as the stimulation of hepatic gluconeogenesis, inhibition of glucose uptake by peripheral tissues and suppression of insulin (e.g., Munck et al. 1984; Romero et al. 2009), causing the mobilisation of stored energy and their redirection to muscles. Changes in physiological status are also coupled with behavioural responses, such as inhibition of reproductive activity, increase in anxiety, and changes in foraging and feeding rate (Sapolsky et al. 2000). This set of physiological and behavioural changes characterises the so-called emergency life history stage (Wingfield et al. 1998). When an individual enters this stage, resources are mostly used to sustain mechanisms activated to protect it from allostatic failure and to promote life-saving strategies essential to self-maintenance and survival (McEwen and Stellar 1993; Wingfield et al. 1998). For these reasons, circulating levels of glucocorticoids have increasingly been employed as physiological indices of stress condition and health of individuals and animal populations (e.g., Romero 2004). Prolonged high secretion of glucocorticoids normally reflects a state of chronic stress, which has been linked with an increase in susceptibility to cardiovascular and metabolic diseases (Sapolsky et al. 2000; Bjelakovic et al. 2010), and, possibly, to a decrease in survival probabilities (Romero and Wikelski 2001; Goutte et al. 2010) and evolutionary fitness (CORT-fitness hypothesis, Bonier et al. 2009; Angelier et al. 2010) in natural vertebrate populations. In fact, although brief exposure to stress (measured in minutes to a few hours) can stimulate immune responses, it has long been recognised that prolonged systemic elevation of stress-induced glucocorticoids is immunosuppressive and can lead to deterioration of health, including heart disease, cancer, susceptibility to infections and poor responses to vaccines (Webster et al. 2002; Bailey et al. 2003; Faivre and Sorci 2009). Antiviral T-cell immune responses are, for example, compromised by glucocorticoids that are either stress induced or pharmacologically administered (Elftman et al. 2010; Hunzeker et al. 2011). Glucocorticoids can also suppress $CD8^+$ T-cell activation, proliferation, cytokine production, and trafficking and impairs viral clearance (Elftman et al. 2010; Hunzeker et al. 2011). Exposure to stress at the earliest stages of herpes virus infection can also be important for the progression of disease because stress hormones suppress subsequent antiviral immunity (Elftman et al. 2010). Therefore, exposure to stress during an infection can have dire consequences for the survival of the host, because stress-induced changes in T-cell

responses result in profound increases in mortality rates from several kinds of viruses. Chronic exposure of vertebrates to glucocorticoids is also known to induce oxidative stress through the increase in production of free radicals and oxidative damage to biomolecules (lipids, proteins and nucleic acids), as well as the depletion of antioxidant defences (Costantini et al. 2011). This may be relevant because herpes virus infection can be facilitated by a cell state of oxidative stress, while antioxidants, like vitamin E or resveratrol, can inhibit virus replication and accelerate lesion healing and recovery (Faith et al. 2006; Kavouras et al. 2007; Sheridan and Beck 2008; Mathew et al. 2010; De Luca et al. 2012). For example, the duck viral enteritis is an acute, contagious herpes virus infection of ducks, geese and swans. Resveratrol (a dietary antioxidant) was found to inhibit in vitro duck enteritis virus replication in a dose-dependent manner (Xu et al. 2013). The inhibition of virus replication in the presence of resveratrol was not attributed to direct inactivation or inhibition of virus attachment to the host cells, but to the inhibition of viral multiplication in host cells (Xu et al. 2013).

Other kinds of viruses can also exploit a state of slight oxidative stress of the cell to increase their replication. For example, deficiency of dietary antioxidants (vitamin E) and cofactors of glutathione peroxidase (selenium) can cause transition of avirulent to virulent and virulent to extremely virulent mutation of coxsackievirus in mice (Beck 1997).

Environmental stressors that are able of soliciting an increase in secretion of glucocorticoids can take many forms, like harsh weather conditions, pollution, predation risk or food shortage. For example, in birds, exposure to food shortage is particularly relevant during the chick rearing period because probability of survival declines in undernourished nestlings. Although chicks of precocial species or some altricial ones can flexibly adjust their growth rate to current food availability with no detrimental effects, many altricial birds lack such developmental plasticity. Therefore, even a short-term food shortage can cause increased nestling mortality, permanent stunting or other detrimental effects. Seabirds, for example, may be in crisis during food shortage periods, experiencing poor breeding numbers and survival rates (e.g., Kitaysky et al. 2007, 2010). It has also been shown that poor nutritional status is related to levels of infection and stress hormones in wild mammals (e.g., Chapman et al. 2006). However, relationships between food availability and stress have been quantified only rarely in free-living animals, and the direct effects of food-related physiological and oxidative stress on survival of individuals and the links with outbreaks of herpes virus or of other viral strains are yet to be investigated.

8.4 Hormesis and Immunology

Reactive species released during the immune response may also stimulate the immune response itself. For example, reactive species may facilitate phagocyte adherence to endothelium or stimulate synthesis of cytokines (Halliwell and

Gutteridge 2007; Sorci and Faivre 2009). The effects of reactive species are likely to be hormetic: at low dose, they modulate cellular communication, while at high dose, they damage host tissues, as well as immune cells. The application of a hormetic framework to ecological immunology might prove fruitful, since it could prompt investigation of the conditions under which immune responses are costly rather than beneficial, or the range of pathogen exposure that could have stimulatory and, possibly, beneficial effects on the organism through hormesis. Immune-system-related hormetic-like biphasic dose–response relationships have been widely documented in the immunological literature. There is now evidence of over 90 different immune-related hormetic responses, induced by over 70 endogenous agonists, over 100 drugs and over 40 environmental contaminants (Calabrese 2005). These hormetic responses have been reported in a wide range of animal species, including strains of laboratory rodents, primates, cattle, rabbits, chickens, frogs, marine bivalves and protozoa. It is worth noting that many of these immunological hormetic responses were detected using methodological approaches commonly used by ecologists for other reasons, such as the activating agents phytohemagglutinin and lipopolysaccharide, and the antibody-forming response to sheep red blood cells (Calabrese 2005). The extent to which a hormetic response is triggered may help explain variation in effects of these activating agents on oxidative damage and antioxidant status that has been noted in ecological studies (Costantini and Dell'Omo 2006; Costantini and Møller 2009).

Lymphocyte activation has been widely studied due to its central role in overall immune responsiveness and its association with various disease processes. Experimental activation/stimulation of lymphocytes represents an in vitro correlate of an in vivo process that typically occurs once an antigen interacts with specifically sensitised lymphocytes in the host (Calabrese 2005). It provides a quantitative estimate of the functional ability of lymphocytes to proliferate following exposure to an antigenic challenge. An evaluation of the dose response for lymphocyte activation carried out by Calabrese (2005) has revealed that hormetic-like biphasic dose responses are prominent for this particular feature of the immune system. Such hormetic responses have been reported in various biological models following exposure to a number of endogenous molecules, such as estradiol, testosterone, adrenocorticotropic hormone, corticosterone, serotonin, histamine, interleukin-1, reactive oxygen species, melatonin or phospholipase A2 (Calabrese 2005). For example, sex steroid hormones can enhance immunity in both male and female Siberian hamsters *Phodopus sungorus* (Bilbo and Nelson 2001). It has also been found that exposure to some contaminants can induce hormetic responses of the immune system (paraquat, Seve et al. 1999; heavy metals, Brousseau et al. 2000). The stimulatory range was highly variable. Moreover, most (ca. 80 %) maximum stimulatory responses were less than twofold greater than the control response (Calabrese 2005). Hormetic responses are not limited to lymphocyte activation. They have also been described for other immune endpoints, such as platelet activity factor, interferon production, rosette formation or oxidative burst (Calabrese 2005), and for the time needed for tissue regeneration, such as wound healing (Calabrese 2013). Importantly, the low-dose stimulation was demonstrated

to occur within physiological ranges of various molecules, like estradiol (Kenny et al. 1976) or hydrogen peroxide (Los et al. 1995), and in both in vitro and in vivo systems (Colic et al. 2000; Cupic et al. 2001).

Numerous mechanisms have been proposed to explain the link between hormesis and immune activity. There is also a range of potential ecologically relevant factors that have been shown to underlie hormetic immune responses. Physical activity may, for example, induce immunological hormetic responses, hence determining the individual capacity to deal with a pathogen. Why physical effort should affect either individual susceptibility to catching a disease or how badly a particular parasite affects the organism is still unclear. But it does appear that intense workouts suppress the body's immune response. On the other hand, mild physical effort may stimulate the activity of immune cells, making the individual more resistant to infection. To test whether different doses of exercise, performed in the initial days after infection when the host is mounting an immune response, influenced survival and morbidity, Lowder et al. (2005) first infected laboratory mice with flu. One group then rested, a second group ran for 20 or 30 min at 8–12 m min^{-1}, while a third group ran for a strenuous two and a half hours at 8–12 m min^{-1}. Each group repeated this routine for 3 days, until they began to show flu symptoms. Mice that ran mildly after being infected with flu had a significantly higher survival rate (82 %) when compared to controls (43 %) or those mice that ran at a very demanding rate (30 %). Morbidity did not differ between controls and mice from the mild physical effort group, while it was significantly higher in mice from the high physical effort group. In another experiment, Murphy et al. (2008) allocated laboratory mice to one of the two groups: control and exercise, which consisted of a treadmill run to fatigue performed on three consecutive days. Fifteen minutes after the last bout of exercise or rest, mice were intranasally inoculated with a standardised dose of influenza virus. Mice were then monitored daily for morbidity (time to sickness), and symptom severity and mortality (time to death) for 21 days (Murphy et al. 2008). Exercise stress was associated with an increase in susceptibility to infection (morbidity, mortality and symptom severity on days 6 and 7). Hence, this experiment demonstrated that severe exercise can increase susceptibility to infections.

Results of these studies together with many other new lines of research supported the so-called *inverted J hypothesis* that physiologists proposed some years ago (Woods et al. 1999), which links exercise to immunity (Fig. 8.3). In this model, the risk of having a particularly severe form of infection drops if the physical effort is moderate, while goes up dramatically if the physical effort becomes intense. In reality, the so-called J-shaped model represents a case of hormetic response, where the physical effort is the stressor and the immune activity is the response variable, respectively.

The so-called dietary antioxidants like carotenoids and polyphenols also show variable effects on the infection levels and immune response. For example, carotenoid supplementation increased immune responsiveness of captive zebra finches (Blount et al. 2003; McGraw and Ardia 2003). A more recent meta-analysis of bird studies found that the intensity of the response to a phytohemagglutinin injection

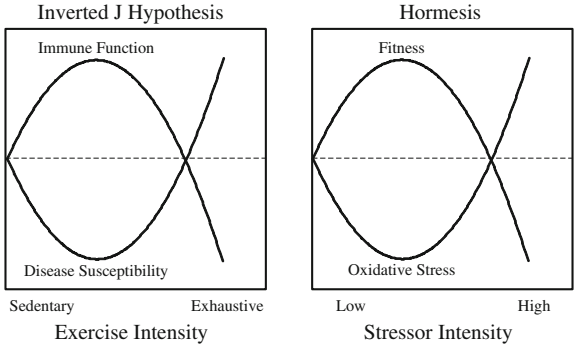

Fig. 8.3 The inverted J hypothesis states that the risk of having a particularly severe form of infection drops if the physical effort is moderate, while goes up dramatically if the physical effort becomes intense. The *J-shaped* model represents a case of hormetic response, where the physical effort is the stressor and the immune activity is the response variable. Evolutionary fitness would follow a same trend as that of the immune function, while oxidative stress would follow an opposite pattern, with higher evolutionary fitness at low oxidative stress. The graph of the inverted J hypothesis was redrawn from Woods and collaborators (1999) with minimal alterations

was significantly and positively associated with concentration of circulating carotenoids (Simons et al. 2012). Other studies, however, found that carotenoids may also have toxic effects (Costantini et al. 2007; Huggins et al. 2010). Similarly, it was found that polyphenols had hormetic effects on human immune cell function (Fig. 8.4; Falchetti et al. 2001), ameliorated neuronal markers of inflammation, and oxidative stress in rats (Shukitt-Hale et al. 2005) and increased the likelihood of blackcaps (*Sylvia atricapilla*) to mount a humoral immune response after being injected with sheep red blood cells compared to control blackcaps (54 vs. 30 % of birds; Catoni et al. 2008). Carotenoids may also play further roles in the interplay between parasites and host response. For example, Heath et al. (2013) found that the carotenoid content of galls of *Asteromyia carbonifera* was positively associated with the probability of parasitism by the egg parasitoid *Platygaster solidaginis*. The authors suggested that carotenoids could directly influence parasitoid behaviour as precursors to attractive volatile apocarotenoids.

Environmental challenges like cold stress can exert hormetic responses of the inflammatory system. Carter et al. (2013) provided compelling evidence for a hormetic effect of cold stress on wound healing in wild wood duck (*Aix sponsa*) ducklings. On both 1 and 2 days after hatching, ducklings were exposed for 1 h to either 5, 10 or 20 °C thermal challenges or served as a control (held at thermo-neutral temperature of 36 °C), thus creating four experimental groups. On 3-day post-hatch, all ducklings were exposed to an immune challenge. At 3 days post-hatch, a circular, superficial wound was administered on both the thermally challenged and control ducklings' left thigh using a 3-mm sterile punch. The procedure was minimally invasive and only removed the epithelium; no muscle

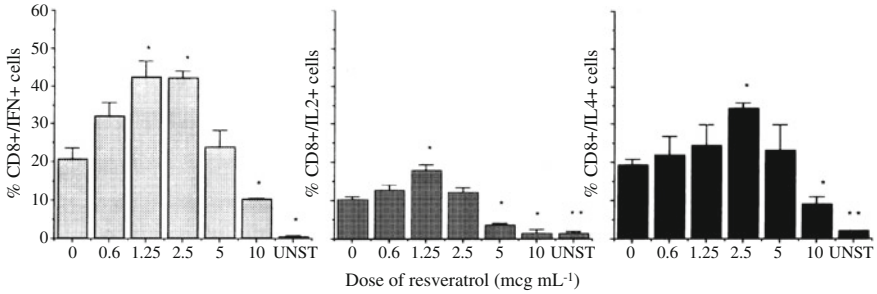

Fig. 8.4 Effects of resveratrol on the frequency of CD81 T cells expressing IFN-g, IL2 or IL4 in anti-CD3/anti-CD28-stimulated peripheral human blood mononuclear cells. Results are expressed as mean + standard error. UNST = unstimulated controls. * = $P < 0.05$ versus resveratrol-untreated controls; ** = $P < 0.01$ versus resveratrol-untreated controls. Reprinted with minimal alterations from Falchetti et al. (2001) with permission from Elsevier

was damaged or removed. The healing process was monitored daily for full recovery. The response of ducklings followed a hormetic pattern: the 20 °C group (mild cold stress) healed wounds 11 % faster than controls and the 5 °C group (high cold stress) healed wounds 11 % slower than controls (Carter et al. 2013). Results of this study have important implications for the capacity of young with a still immature immune system to withstand parasites and injuries. The extent to which early-life conditions are stressful may significantly impact on the future capacity of mounting a successful and, possibly, low-cost immune response.

8.5 Glucocorticoids and Inflammation

Glucocorticoids have a regulatory role of immune activity. They can, for example, regulate the activities of various antioxidant enzymes (e.g., superoxide dismutase, glutathione peroxidase) and the production of hydrogen peroxide of macrophages (Pereira et al. 1995). Although glucocorticoids can activate anti-inflammatory pathways and are often presumed to suppress the immune system, evidence from laboratory models suggests that their effect depends on the dose and on the type of immune cell. For example, cyclic adenosine monophosphate-mediated stimulation of thymocyte proliferation can be induced by low concentrations of cortisol (Whitfield et al. 1970). Administration of corticosterone induced a biphasic effect on cytokine production (IL-4 and IL-10) in T helper 2 cells of adult female mice, with low doses inducing cytokine production, whereas higher doses suppressed it (Fig. 8.5; Stanulis et al. 1997a, b), fitting the biphasic dose–response relationship of hormesis. In contrast, low doses of corticosterone had no effect on T helper 1 cells, while suppression occurred at higher doses, but lower than the threshold doses for suppression in TH2 cells (Stanulis et al. 1997a, b). Such findings suggest that direct exposure to corticosterone at low doses can selectively enhance TH2

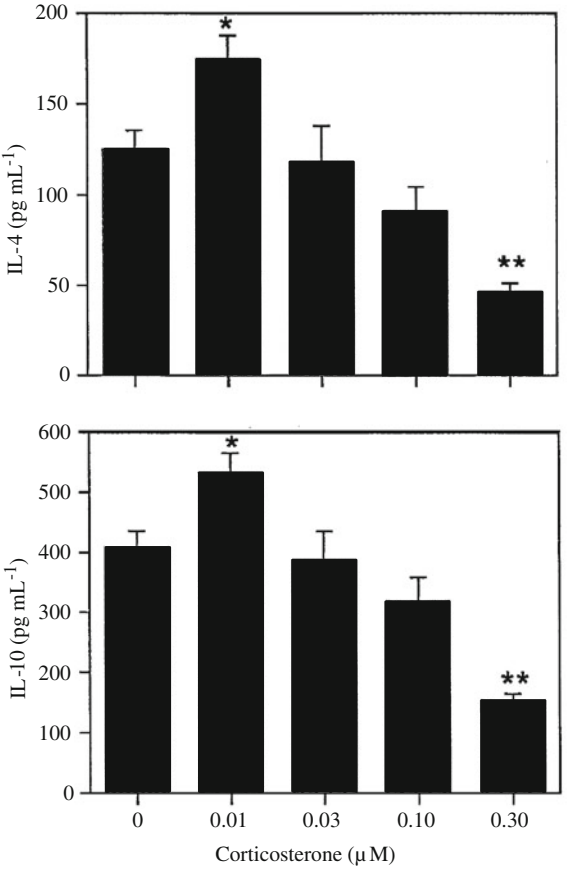

Fig. 8.5 Effects of corticosterone on Th2 cytokine IL-4 and IL-10 production in mice. Naive spleen cells were exposed to corticosterone in culture and were stimulated with anti-CD3 in a 24-well plate. Supernatants were collected 24 h later and analysed via ELISA for the respective cytokines. Results are shown as mean + standard error. Significant differences from respective vehicle controls are indicated: * = $P < 0.05$; ** = $P < 0.01$. Reprinted with minimal alterations from Stanulis et al. (1997a) with permission from Elsevier

cytokine production. Importantly, corticosterone concentrations employed in these studies (0.01–0.30 μM) were within the physiologic range of a number of stress models (Pruett et al. 1993; Calabrese 2005).

Hormetic effects induced by glucocorticoids were also demonstrated in relation to organism resistance to infections. While some studies showed that glucocorticoids enhanced the susceptibility to a wide variety of infectious agents, other studies found that they can increase the resistance to infections (see review in Calabrese 2005).

Effects of glucocorticoids also depend on the duration of the inflammatory status. One of the consequences of a prolonged secretion of glucocorticoids may be oxidative stress. Costantini et al. (2011) reviewed studies where glucocorticoids were administered to mimic a condition of physiological stress and performed a meta-analysis to evaluate the magnitude of the effects on the redox balance. Oxidative stress was weakly increased in studies where physiological responses to glucocorticoid administration were measured in the short term (after a few hours to

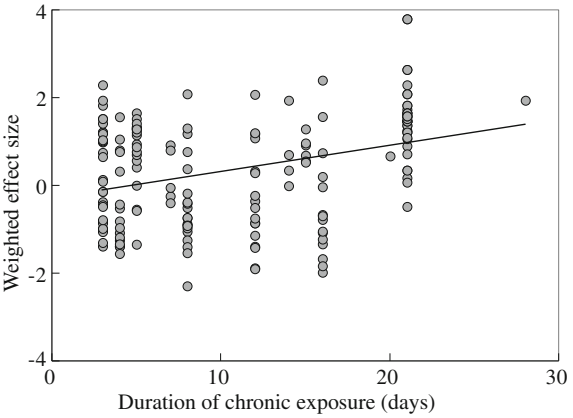

Fig. 8.6 Effect size of glucocorticoids on oxidative stress increased with treatment duration. Oxidative stress appears most notably increased after 3 weeks of administration of glucocorticoids. Reprinted with minimal alterations from Costantini et al. (2011) with kind permission from Springer Science + Business Media

4–5 days) because antioxidant defences were upregulated. In contrast, short-term stimulatory effects of glucocorticoids on the antioxidant machinery appeared dramatically reversed in the long term, and oxidative stress strongly increased with treatment duration, most notably after 3 weeks of administration of glucocorticoids (Fig. 8.6). The acute response is therefore important because glucocorticoids turn off the activity of various pro-inflammatory molecules and favour upregulation of antioxidant mechanisms. However, chronic organism response turns glucocorticoids in pro-oxidant agents. Under these conditions, glucocorticoids exacerbate the inflammatory status, leading to cell necrosis and death.

8.6 Conclusions

That the production of reactive species is important in the battle against parasites is well established. But that immune activation is associated with increases in oxidative damage is less straightforward. There is large among-species variation in anti-parasite mechanisms and the intensity of the oxidative burst. There is also among-individual variation that may explain whether an inflammatory response may result in oxidative damage. For example, there is individual flexibility in the relationships between hormones, immune response and life history traits. The individual physiological and social context may be important to explain such flexibility. Archie et al. (2012) found no evidence that high testosterone and intense reproductive effort associated with high rank suppress immune responses in wild baboons (*Papio cynocephalus*). In contrast, high-ranking males were less likely to become ill, and they recovered more quickly than low-ranking males. High-rank males, who also experience high stress hormones, healed significantly faster than other males (Archie et al. 2012).

There are certainly methodological limitations with both characterisation of immune activity and oxidative balance, in particular for field studies. Immune

functions and mechanisms remain challenging to characterise meaningfully in natural populations because (1) the individual parasite exposure history is rarely known; (2) it is difficult to take repeated sampling on the same individual; (3) there may be coinfection with pathogens that largely differ in their action; and (4) the complexity of the immune system calls into question extrapolating from simple assays or approaches aimed at stimulating the immune response (Keil et al. 2001; Adamo 2004; Martin et al. 2011; Pedersen and Babayan 2011). Lack of effects of inflammation on oxidative balance may, for example, depend on which immune cells are mostly activated. Avian heterophils are activated in acute inflammatory responses. Their antimicrobial activity depends primarily on non-oxidative mechanisms, and, conversely to mammalian neutrophils, avian heterophils lack the enzymes myeloperoxidase and catalase (Harmon 1998). For example, when challenged with phagocytic stimuli, there is activation of oxidative burst in chicken heterophils, but this does not result in increased production of hydrogen peroxide or superoxide anion (Penniall and Spitznagel 1975; Stabler et al. 1994). This emphasises the importance of determining the total and relative numbers of leucocytes activated during an inflammatory process (Conlon et al. 1991).

Evidence of delayed occurrence of costs of inflammation emphasises that time is a crucial dimension that should be considered when weighing the benefits and costs associated with this component of the immune response (Sorci and Faivre 2009). The time elapsed between the initiation of inflammatory damage and the occurrence of detectable costs may allow organisms to achieve crucial fitness-related functions, such as reproduction (Sorci and Faivre 2009). These costs may, however, increase the rate of senescence.

The threat of oxidative stress has likely constrained evolution of inflammatory response, making it impossible for natural selection to maximise. Sorci and Faivre (2009) suggested that, in order to optimise the immune response, natural selection might have favoured a rapid activation of the costly inflammatory response and also a rapid switch off of the response itself to avoid the deleterious consequences of chronic inflammation. Regulation of inflammatory response is therefore vital.

Interpretation of the link between immune response and oxidative damage also needs a life history framework. Long-lived species might, for example, favour investment in immunocompetence over other traits. Hence, for these species and conversely for short-lived species, oxidative stress induced by an immune response may contribute to shape the senescence rate. Life history strategies also vary between the sexes. Hence, we also need to explore sexual dimorphism in immune response, which appears to be based partly on differences in sex steroids (Grossman 1985). The interplay among immune response, oxidative stress and mate choice strategies appears a fruitful area of investigation (Kurtz et al. 2007; Torres and Velando 2007).

To further our understanding of the oxidative costs of immune response in wild animals, it will also be important to keep in mind that parasite infection may interact with environmental stressors, complicating interpretation (Hall et al. 2013). We also need to bear in mind that individual age is important because

immunocompetence and resistance to oxidative stress decrease with time (Cichoń et al. 2003; Gruver et al. 2007; Torres and Velando 2007).

Immuno-oxidative ecology is also interesting from the parasite's viewpoint. Beyond the various mechanisms parasites use to evade the host's response, some parasite species can divert the host inflammatory response against competitive microorganisms. Moreover, the host inflammatory response operates as an important selective pressure for pathogens (Sorci and Faivre 2009). We need to know more about which mechanisms are being used by parasites for self-defence.

Another important avenue of research is to assess the extent to which oxidative stress may constrain the immune response itself through the generation of damage to immune cells themselves. This research has also a strong link to nutritional ecology because various dietary antioxidants might play an important role in the protection of membranes of immune cells.

The last but likely more important question we need to elucidate is how the oxidative damage induced by an immune response affects evolutionary fitness. Fitness costs may manifest immediately, like in the form of reduced current reproduction, or in the long term, such as in terms or reduced future reproductive output. As usual for biology, there are likely more than a single answer.

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Chapter 9
Within- and Among-Species Variation in Resistance to Oxidative Stress and Hormetic Responses

Abstract Residual variation unexplained by life histories occurs in the comparisons among individuals or closely related species. There are many factors that contribute to generate this variation. Individual genetic quality, gene flow, genetic drift, presence of predators, synergistic or antagonistic stressors, early-life experiences, diet, rhythms, plasticity, coping style, metabolic rate, body size, quality of the wintering or breeding habitat, and further local selective pressures have been shown to contribute in some way to build up variation among individuals, populations or species. This chapter examines some of these factors. It briefly reviews the long-term effects of early-life experiences on individual variation in adult oxidant phenotype because this topic has been fully discussed in Chap. 2. It then explores how variation in individual stress responsiveness or in environmental circumstances under which an individual lives contribute to build up variation in oxidative status; research that quantified the relative contributions of environment, maternal effects and genetic background to variation in oxidative status and hormesis; comparative studies that assessed the links between life history traits and parameters of oxidative status or analysed among-species variation in oxidative stress resistance or hormesis.

9.1 The Essence of Biology: Variation

The previous eight chapters have examined the numerous ways through which oxidative stress and hormesis may influence evolution of life history strategies and adaptations to environmental pressures. If we take a closer look at variation within species, resistance to oxidative stress is highly variable among individuals and such variation may be associated to individual life history strategies. Comparisons among species also provide support for a link between life history variation and oxidative damage or antioxidant protection. However, residual variation unexplained by life histories still occurs in the comparisons among individuals or closely related species. There are many factors that contribute to generate this variation. Individual genetic

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quality, gene flow, genetic drift, presence of predators, synergistic or antagonistic stressors, early-life experiences, diet, rhythms, plasticity, coping style, metabolic rate, body size, quality of the wintering or breeding habitat, and further local selective pressures contribute in some way to build up variation among individuals, populations or species (e.g., Hardeland et al. 2003; Pigliucci et al. 2006; Badyaev 2009, 2011; Cohen et al. 2009; Gilbert and Epel 2009; Wilson and Nussey 2010; Carere and Maestripieri 2013; Grether 2014; Konarzewski and Książek 2013; White and Kearney 2013). Although it is common practice in biological research to focus on means, it is the variation among and within individuals that provide the raw material natural selection works on. Hence, in this chapter, I examine how some of these factors generate variation in oxidative stress and hormesis.

9.2 Early-Life Experiences

That early-life experiences influence future basal oxidative balance and resistance to oxidative stress has been established by many experimental investigations in various animal species. Moreover, it is increasingly recognised that early-life exposure to mild stress primes the system to better withstand stressful episodes experienced in adulthood. Hence, early-life experiences significantly contribute to build up among-individual variation in phenotype. Here, I present a few examples because the topic was fully examined in Chap. 2.

Early nutrition may have long-lasting effects on basal levels of circulating antioxidants. For example, studies on zebra finches (*Taeniopygia guttata*) found that nestlings fed on a poor-quality diet had later in life significant lower concentrations of circulating vitamins A and E (Blount et al. 2003) and carotenoids (Alonso-Alvarez et al. 2006). Whether this reflects poorer capacity of assimilating antioxidants from diet, higher storage of dietary antioxidants in other tissues, or higher oxidation of antioxidants, remains to be tested.

Early life may also influence adult resistance to an oxidative insult through hormetic responses. For example, early-life exposure of nematodes (*Caenorhabditis elegans*) to heat stress (35 °C for 2 h), hyperbaric oxygen or juglone (a chemical that generates reactive species) significantly increased subsequent resistance to the same challenge, resulting in a longer lifespan (Cypser and Johnson 2002). Similarly, mild heat stress experienced in juvenile life primed zebra finches (*T. guttata*) to better withstand oxidative stress induced by exposure to high ambient temperature in adulthood (Costantini et al. 2012a). Such early-life conditioning resulted in the birds showing no increase in plasma oxidative stress on exposure to high temperatures in adulthood. In contrast, birds that had either no previous experience of heat stress or only experience of high temperatures showed a significant increase in plasma oxidative damage; birds also exhibited a bigger decrease in red blood cell thiol antioxidants than in the mild conditioning group.

9.3 Styles of Coping with Stressful Situations

Conspecific individuals of the same sex or age often differ from each other in clusters of behaviour and underlying physiology, even under standardised conditions. Most of this variation is non-random, and such individual differences are consistent across situations or contexts and across time (Carere and Eens 2005). Individual differences in behaviour and physiology in non-human animals have often been neglected as biologically meaningful variation. In contrast, the study of individual variation in humans has a crucial role in biomedical research because of its links with individual disease vulnerability and response to medication (Carere and Eens 2005). In the last decade, such an old-fashion scenario has changed, and individual differences in non-human animals are now recognised as a real biological phenomenon (Wolf and Weissing 2012; Carere and Maestripieri 2013).

One of the fundamental features that characterises behavioural types is the responsiveness to environmental stimuli. Whereas some individuals tend to be highly responsive to such stimuli, others are unresponsive and show routine-like behaviours (Wolf et al. 2008). Variation in the way individuals behaviourally cope with stressful episodes can be modelled along an axis polarised at the two extremes by proactive and reactive responses (Koolhaas et al. 1999, 2010). Proactive individuals are generally bold, superficial explorers, active and aggressive. Reactive individuals are lowly active and aggressive and explore a novel environment thoroughly. Differences in coping styles have been considered to reflect consistent behavioural differences among individuals and may be refereed as personalities (Sih et al. 2004; Groothuis and Carere 2005; Réale et al. 2007; Carere and Maestripieri 2013). There is increasing evidence that such individual variation in personalities has important ecological and evolutionary consequences (Carere and Eens 2005; Sih et al. 2004; Réale et al. 2007; Wolf and Weissing 2012). It is generally thought that variation among individuals is maintained because, while certain behavioural phenotypes do better than others under some environmental conditions, an opposite pattern can emerge under different conditions (e.g., Sih et al. 2004; Carere et al. 2010a). Furthermore, recent studies have suggested that selection should have favoured integrated sets of behavioural, physiological and morphological traits along with variation in life history strategies (Sih et al. 2004; Wolf et al. 2008; Biro and Stamps 2008; Careau et al. 2008b; Réale et al. 2010). In other words, these integrated neuro-physiological and behavioural sets of traits may be part of more general life history strategies with proactive individuals showing high growth rate, early sexual maturity and short lifespan, and reactive individuals being characterised by a slower pace of life (Biro and Stamps 2008; Careau et al. 2008b; Wolf et al. 2008; Réale et al. 2009, 2010). Thus, in this context, we should expect that natural selection has also led to some potential coevolution between coping styles and mechanisms involved in life history trade-offs. Some of these mechanisms involve stress responsiveness modulated by glucocorticoids. When confronted to a challenging situation (e.g., predator attack or social conflict), proactive individuals tend to respond with a strong sympathetic

activation and increase in noradrenergic stimulation, resulting in a general fight-or-flight behavioural response (e.g., Koolhaas et al. 2010; Carere et al. 2010a; Coppens et al. 2010). On the other hand, reactive individuals respond to a challenge with a strong hypothalamic–pituitary–adrenocortical (HPA) reactivity (Koolhaas et al. 2010; Carere et al. 2010a; Coppens et al. 2010), resulting in a freezing response to stimulus and an increase in circulating glucocorticoids. However, the link between behavioural and physiological response is not so straightforward. In an updated version of the reactive-proactive model, Koolhaas et al. (2010; see also Steimer et al. 1997) have proposed the existence of two independent axes: coping style and stress reactivity. This new model seems to be supported by a recent study on coping styles in wild marmots *Marmota marmota* (Ferrari et al. 2013). Thus, the behavioural reaction to an environmental stimulus may reflect a certain coping style, but it does not need to be necessarily related to the glucocorticoid response to a stressor (stress reactivity, Koolhaas et al. 2010). Many studies on artificially selected or inbred lines in laboratory settings (Carere and Maestripieri 2013) and, more recently, in wild animals (Ferrari et al. 2013) suggest that an association between behavioural and glucocorticoid response may generally occur, but that its strength may be weaker in a natural context.

The link between coping style and reactivity of the HPA axis is very important in terms of consequences for the maintenance of oxidative balance and downstream effects related to any change in oxidative stress levels. In fact, stress responsiveness may influence the generation of oxidative damage and/or the antioxidant response (Costantini et al. 2011). Importantly, the pro-oxidant effect of glucocorticoids appears to be consistent across multiple tissues (Fig. 9.1; Costantini et al. 2011). In addition, reproductive and physical activities in sociosexual contexts, which are known to differ among coping styles (e.g., mice *Mus musculus*, Koolhaas et al. 1999; great tits *Parus major*, Groothuis and Carere 2005), could also contribute to significantly affect the oxidative status and thus expose individuals with differing coping styles to oxidative challenges of different magnitudes (e.g., Alessio 1993; Costantini et al. 2008). They might do so through changes in metabolic activity or energy expenditure, which are both correlated with some traits of personalities (Ros et al. 2006; Careau et al. 2008a; Biro and Stamps 2010). This route appears particularly relevant because acute stress-induced corticosterone levels, and to some extent baseline corticosterone levels, were found to be repeatable within individuals in a free-living population of Florida scrub-jays (*Aphelocoma coerulescens*) sampled across ages (from the age of 11 days post-hatch up to 4 years of age) and including multiple cohorts (Rensel and Schoech 2011). Individual variation in feeding styles might be another route that links personality to oxidative stress resistance, if feeding styles differ in the intake of antioxidants and nutrients or cost of foraging (Costantini et al. 2005a).

Importantly, there is good evidence in laboratory models and humans that individual differences in behaviour and physiology may influence longevity and susceptibility to certain diseases and resilience to others (Cavigelli and McClintock 2003; Cavigelli 2005; Korte et al. 2005). For example, laboratory male rats identified as neophobic during infancy were 60 % more likely to die at any point in time

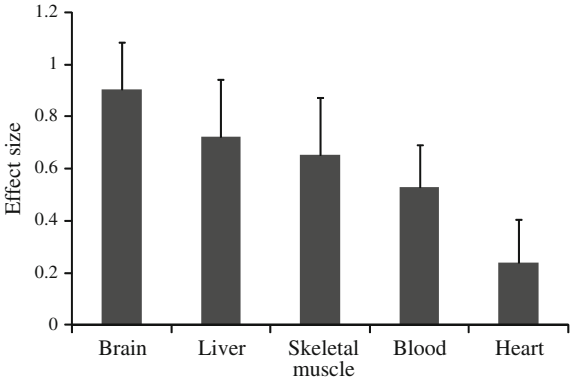

Fig. 9.1 Mean effect size (plus standard error) of glucocorticoid administration on oxidative stress in various tissues most commonly analysed in studies included in the meta-analysis from Costantini et al. (2011). The analysis included 191 effect size estimates from 19 studies on seven vertebrate species

than their less fearful brothers, although they died of similar pathologies (Cavigelli and McClintock 2003). It seems therefore reasonable to expect that, if a link between personality and oxidative stress exists, oxidative stress may be a mechanism underlying the link between personality and longevity perspectives or health span.

In the last years, research on various vertebrate species showed that personality types could indeed differ in oxidative profile, although results do not appear to be consistent across species, different personality traits or parameters of oxidative balance. Mice selected for aggression and coping (long attack latency (LAL), reactive coping strategy; short attack latency (SAL), pro-active coping strategy) are a useful model for studying the physiological background of animal personalities. These mice also show differential stress responsiveness, especially in terms of HPA axis reactivity, to various challenges. LAL mice were shown to have higher serum non-enzymatic antioxidant capacity than SAL mice, while no differences emerged for serum hydroperoxides (Costantini et al. 2008). Moreover, the two mouse lines showed inverse relationships between hydroperoxides or non-enzymatic antioxidant capacity and body mass corrected for age, respectively. Oxidative damage level decreased with body mass in SAL mice, while the antioxidant capacity increased with body mass in LAL mice. Overall, results of this study indicated that variation in oxidative status may be linked to personality. Results of this study also suggested that oxidative state may have a genetic basis because all subjects were adolescent and experimentally naive concerning social challenges. However, this difference might arise because of artificial selection of the two mouse lines. Results further suggested that LAL mice may invest more than SAL mice in long-term physiological homeostasis by maintaining high constitutive antioxidant levels in blood (1) to cope with higher free radical production or (2) to prepare the organism for the repeated stressful events they will experience throughout adulthood because of their higher stress responsiveness.

Another study on laboratory mice also found a correlation between aggressiveness, one of the most reliable proxies of personality type, and a parameter of oxidative balance. Mice with a higher basal production of reactive species in blood

granulocytes were those having a shorter latency time ($r = -0.87$) and a higher number of attacks ($r = 0.88$; Rammal et al. 2010). This study also suggested that more aggressive mice have a higher basal activity of immune cells. This might be important if aggressive mice are more exposed to injuries and inflammation consequently to fights among males. Both of the above studies have been done using artificial selected lines of mice. In natural conditions, however, an individual's social experience may affect its behavioural profile and stress responses to subsequent challenges (Jansen et al. 2010; Sachser et al. 2010) and there is not the potential confounding of artificial genetic selection. In a study on wild Alpine marmots (*M. marmota*), it was showed for the first time that different individual coping styles may also be associated with differences in pre-restraint or acute stress-induced blood oxidative statuses in a natural setting (Costantini et al. 2012b). Marmots with a more proactive coping style had higher baseline levels of plasma non-enzymatic antioxidant capacity, through a positive action of plasma cortisol on circulating antioxidants (Fig. 9.2a). Coping style was, however, not associated with two parameters of oxidative damage in plasma (hydroperoxides and 4-hydroxynonenal-histidine protein adducts). A second path model including changes in blood oxidative statuses induced by an acute stressor (i.e. a 30-min restraint and an open-field test) further showed that more proactive marmots experienced a higher increase in hydroperoxides, but also an unexpected higher increase in non-enzymatic antioxidant capacity (Fig. 9.2b). In contrast to pre-restraint statuses, changes in cortisol under restraint were not associated with coping style nor with parameters of blood oxidative status. Correlative analyses also showed that 4-hydroxynonenal-histidine protein adducts were not associated with coping style.

Links between personality and oxidative profile were also found in birds and reptiles using wild-caught animals tested under captive conditions. A report on greenfinches (*Carduelis chloris*) showed that neophobic individuals had higher plasma hydroperoxides and lower plasma non-enzymatic antioxidant capacity than neophiliac ones; that fast exploring individuals had higher plasma non-enzymatic antioxidant capacity and lower plasma malondialdehyde than slow exploring ones; and that greenfinches with extremely high or low neophobia had lower malondialdehyde than intermediate responders (Herborn et al. 2011). In a study on the White's skink (*Egernia whitii*), Isaksson et al. (2011) found that more aggressive males had higher plasma non-enzymatic antioxidant capacity but not higher oxidative damage (hydroperoxides) than less aggressive males. Isaksson et al. (2011) also showed that the link between aggressiveness and antioxidants was independent from testosterone, suggesting that other behavioural patterns associated with an aggressive phenotype (e.g., territory defence, mate acquisition) might influence the individual oxidative status.

Overall, these studies showed both similarities and discrepancies. Aggressive individuals from wild species tended to have higher circulating antioxidant capacity. In contrast, the association between personality and oxidative damage levels was not consistent across studies. Once again discrepancies among studies may be caused by differences in treatments among them. LAL and SAL mice come from artificial selection lines, which represent the two most extreme phenotypes/genotypes of the

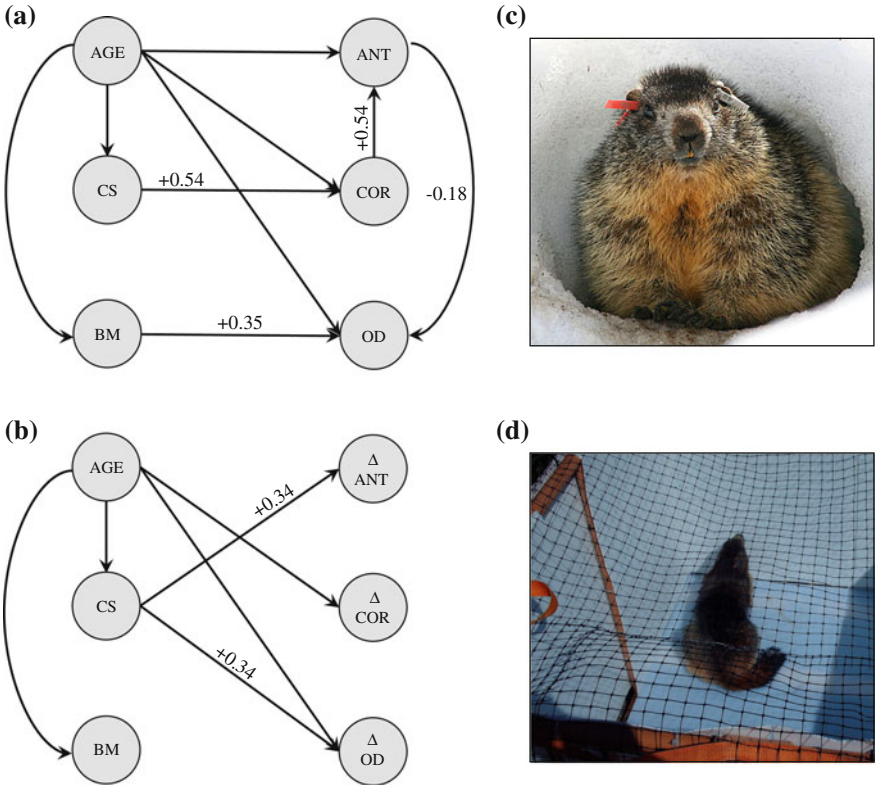

Fig. 9.2 Best-fitting path models of relationships among **a** coping style (*CS*), pre-restraint values of cortisol (*COR*), plasma oxidative damage (*OD*) and plasma non-enzymatic antioxidant capacity (*ANT*), and among **b** coping style (*CS*), restraint-induced changes in cortisol (*ΔCOR*), plasma oxidative damage (*ΔOD*) and plasma non-enzymatic antioxidant capacity (*ΔANT*) in Alpine marmots (*Marmota marmota*) while controlling for age and body mass (*BM*). **c** The picture (*Photograph* courtesy of Caterina Ferrari) shows an individual from a marmot population in Orvielles (Valsavarenche, Aosta, Gran Paradiso National Park, North-Western Italian Alps, 45°34′ N/7°11′ E) that has been tagged for individual recognition. **d** The picture (*Photograph* by David Costantini) shows a marmot within an open-field arena, where the individual behavioural response to a novel environment was tested. Reproduced from Costantini et al. (2012b) with permission

range existing in the original population, and they are maintained under optimal and standard husbandry conditions in the laboratory. In contrast, the marmots studied in Costantini et al. (2012b) represent some variation existing in a natural population. For example, LAL and SAL mice included in the study by Costantini et al. (2008) were naïve concerning resident-intruder tasks (age: 38–64 days old), whereas marmots tested had totally different social experiences prior to the analyses. These multiple effects of social experiences and life history trajectories may make difficult to find any pattern in wild populations consistent with the ones found in controlled

laboratory experiments. However, studies in the wild are essential to understand the link between oxidative status and evolutionary fitness. It will be very important to test in future studies whether personality types differ in oxidative balance across different stages of life cycle and, if so, how and why they differ and if such variation translates in differences in evolutionary fitness. The existence of personality-associated oxidative costs might also explain why the behaviour shows various degrees of flexibility. Individuals would switch from a behavioural response to another only if it pays to do so under the environmental conditions they live. The existence of such costs would explain the coexistence in a same population of more responsive and flexible and of unresponsive rigid individuals.

9.4 Population Differentiation in Oxidative Stress Physiology

The extent to which conspecific populations differ in oxidative profile is not well known, nor have the causes of such variation been clearly elucidated yet.

A 3-year study on Galápagos land iguanas (*Conolophus subcristatus*) from various islands of the archipelago found significant differences among populations in plasma oxidative damage, plasma non-enzymatic antioxidant capacity or plasma carotenoids (Fig. 9.3; Costantini et al. 2005b, 2009). Results of these studies suggested that reproductive activity, food availability and local pressures related to human activity might have contributed to explain variation in oxidative profile because mating seasons, food abundance or human impact differed among islands (Snell et al. 1984). That oxidative profiles may differ among populations in relation to context-dependent life history strategies has been later demonstrated in a study on western terrestrial garter snakes *Thamnophis elegans* (Robert and Bronikowski 2010). Populations of this snake species may be differentiated in a lakeshore fast (fast growth, earlier sexual maturation, intense reproduction, no trematode infection and lower longevity) and a meadow slow ecotype (slow growth, later sexual maturation, moderate reproduction, high trematode infection, higher longevity). Ecotype differentiation has been likely driven by differing predation pressures (higher for the fast ecotype) and food availability (fluctuating for the slow ecotype). Robert and Bronikowski (2010) found that life history differences between ecotypes are also translated at physiological level. Compared to the short-lived phenotype, offspring snakes from the long-lived ecotype consumed equal amounts of oxygen, produced more energy for a given amount of consumed oxygen, had lower mitochondrial production of reactive species, had DNA in red blood cells that was damaged more readily but was also repaired more efficiently (Fig. 9.4). Differences between ecotypes were enhanced in offspring from mothers that were given corticosterone before parturition, which supported the role of non-genetic maternal effects in shaping oxidative stress resistance of offspring (Robert and Bronikowski 2010). Results of this study also supported the idea that physiological

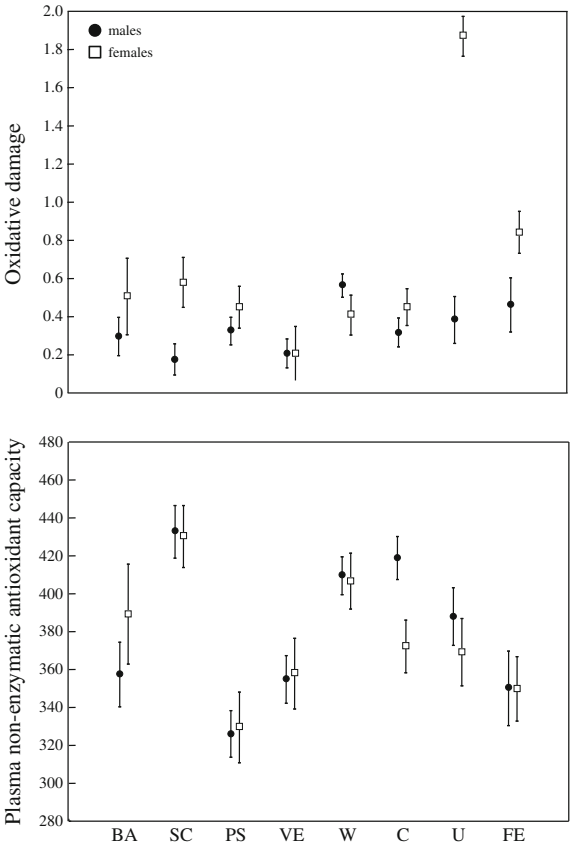

Fig. 9.3 Oxidative damage (plasma hydroperoxides, mM H_2O_2 equivalents) and plasma non-enzymatic antioxidant capacity (mM HOCl neutralised) significantly varied across sexes and populations of Galápagos land iguana, *Conolophus subcristatus*. Samples are from the June–July period. Hence, differences likely reflect the among-population variation in reproductive period and local pressures (e.g., food availability, competition and human activities). Data are shown as mean ± standard error

mechanisms may be evolved in order to respond to the demands of life history strategies that are more advantageous to adopt under certain circumstances. In another study, Schwartz and Bronikowski (2013) sought to identify which nodes of the molecular stress network were more divergent between the two populations of western terrestrial garter snakes under both unstressful and stressful conditions. They found that two common parameters of stress (plasma corticosterone and liver gene expression of heat shock proteins) increased under stress in both life history phenotypes (Schwartz and Bronikowski 2013). In contrast, the two phenotypes diverged at four nodes both under unstressful conditions and in response to stress: circulating levels of superoxide and hydrogen peroxide (under unstressful conditions and in response to heat stress); DNA damage within erythrocytes (under unstressful conditions and in response to heat stress); liver gene expression of glutathione peroxidase 1 in response to heat stress; allele frequencies at superoxide dismutase 2. These results supported the hypothesis that life history phenotypes of western terrestrial garter snakes have diverged at the molecular level in how they respond to stress, particularly in nodes regulating oxidative stress (Schwartz and

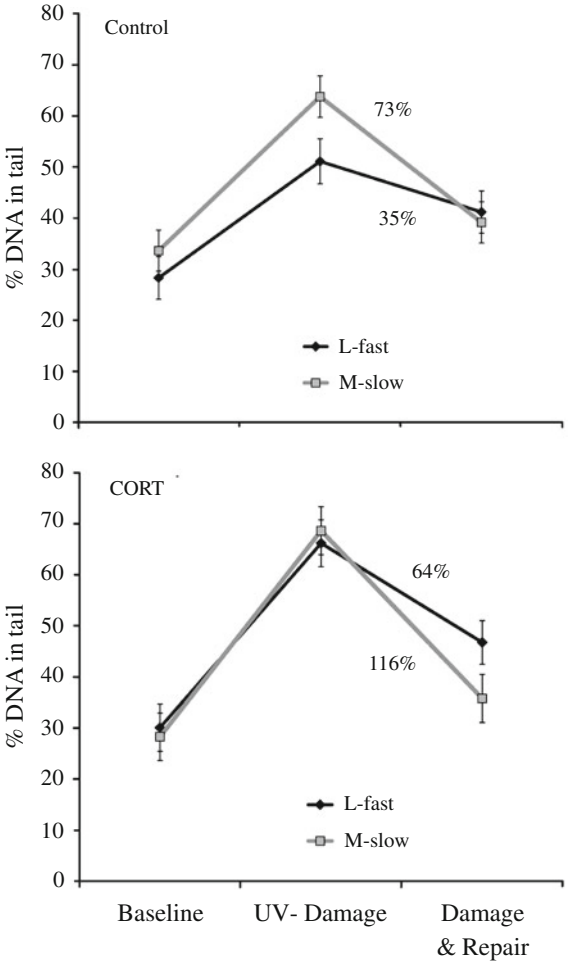

Fig. 9.4 Least square means (± standard error) of percent DNA in comet tails of erythrocytes in offspring from control mothers (*above*) or mothers treated with stress hormones during pregnancy (*below*) originating from lakeshore-short-lived (*black diamonds*) or meadow-long-lived (*grey squares*) ecotype of western terrestrial garter snakes (*Thamnophis elegans*). Efficiency of repair expressed as per cent baseline damage ([UV damage—damage and repair]/baseline) computed with least-square means from analysis of variance. Reproduced from Robert and Bronikowski (2010) with permission

Bronikowski 2013). Hence, oxidative stress has probably represented an important agent of microevolutionary differentiation in garter snakes. Another finding of the study was that differences appeared to be stronger in females than males. It was suggested that this might be due to females likely investing more into reproduction than do males due to their viviparity. Thus, the fitness costs of mismatched regulation of their stress responses might be predicted to be higher if stress networks have pleiotropic effects on longevity and reproduction (Schwartz and Bronikowski 2013). This hypothesis remains to be tested. Finally, results of both studies on garter snakes (Robert and Bronikowski 2010; Schwartz and Bronikowski 2013) supported the idea that the long-lived ecotype invested more in protective mechanisms to promote longevity rather than in keeping low the basal production of reactive species.

Divergence between ecotypes in mechanisms regulating the oxidative status appears to be a public rather than a private phenomenon. Philipp et al. (2012) found that changes in expression of genes involved in the response to oxidative insults differed between short- and long-lived populations (living less than 50 and until 410 years, respectively, as determined by shell ring counts) of the bivalve *Arctica islandica*. Compared to the long-lived populations, the short-lived populations are exposed to frequent and prolonged environmental hypoxia and anoxia episodes. Most of the hypoxia/anoxia-related genes (including antioxidant ones) investigated in the long-lived population were downregulated under experimentally forced anoxia and hypoxia and in animals with anoxia self-induced by shell closure, whereas the opposite happened in individuals from the short-lived populations. Such changes were transient and disappeared within six hours of reoxygenation, suggesting a high flexibility in transcriptional regulation (Philipp et al. 2012). The increase in expression level of genes involved in antioxidant defence was not always accompanied by a change in protein level in tissues, especially for bivalves from the short-lived populations (Philipp et al. 2012). Results of this study open various scenarios for further investigation: enzymes become less functional with time and are replaced by new synthesised molecules; availability of amino acids or energy might have constrained the synthesis of enzymes, because bivalves also needed to use them to sustain other physiological functions; constraints are stronger for short-lived populations, which might explain their minor capacity to cope with stress.

Population differentiation in oxidative status suggests that processes regulating the redox balance may be important targets of selection underlying adaptive divergence. For example, Nikinmaa et al. (2013) found that there was divergence among three populations of three-spined sticklebacks (*Gasterosteus aculeatus*) in both genetic and protein markers linked to antioxidant response. Physiological performance might be under disruptive selection due to local environmental adaptation. Processes regulating oxidative balance in turn can exert or influence selection on other fitness-related traits and vice versa. Hence, diversification in redox mechanisms might contribute to evolutionary diversification and, possibly, to speciation. For example, outcrossing between populations with divergent mtDNA of the copepod *Tigriopus californicus* can exacerbate cellular oxidative stress in hybrid offspring, resulting in low fecundity (Barreto and Burton 2013).

Cichlid fish living in the East African great lakes offer another notable example of divergence in physiological performance among closely related species. For example, when body colourations and aggression are experimentally maximised, red males of *Pundamilia nyererei* experienced more oxidative stress than blue males of *Pundamilia pundamilia* (Dijkstra et al. 2011). It was hypothesised that a higher oxidative stress in red males has negative evolutionary fitness consequences, and as a result, this fitness cost may counterbalance the advantage of red males in aggressive interactions, stabilising the coexistence of red and blue fish in the population (Dijkstra et al. 2011). Direct fitness estimates were lacking, but data on the distribution of the two fish species gave some indirect support for the hypothesis (Dijkstra et al. 2011).

Differences in behaviour and stress physiology were also found between urban and rural populations, suggesting the importance of human activities as a new selective agent (Partecke et al. 2006; Herrera-Dueñas et al. 2014; Miranda et al. 2013; Sol et al. 2013). A comparative study on birds found that rural avian populations had higher concentrations of vitamin E and carotenoids in liver than conspecific urban populations (Møller et al. 2010). Differences between conspecific populations were accounted for by diet (Møller et al. 2010). Compared to rural populations, urban populations of common side-blotched lizards (*Uta stansburiana*) showed higher levels of stress hormones, which were associated with higher oxidative damage levels and suppressed immunity. Moreover, urban populations had higher reproductive output and decreased survival (Lucas and French 2012). As with studies on garter snakes, selection on certain components of the stress network might contribute to drive the evolutionary differences between populations.

It should not be overlooked the importance of differing local human pressures (e.g., contaminants, tourism) when comparing the oxidative stress level of different populations. Local exposure to high pollutant levels may in fact hidden biological patterns or biases our conclusions on the ecological meaning of the observed variation (Carere et al. 2010b; Isaksson 2010). For example, the different antioxidant profiles observed in populations of East Pacific green turtles (*Chelonia mydas*) appear to be explained by differences in abundance and class of contaminants between coastal lagoons (Labrada-Martagón et al. 2011). A similar thinking also applies to comparisons among conspecific populations exposed to differing levels of tourist-induced disturbance. This is, for example, the case of southern stingrays (*Dasyatis americana*), which suffered higher oxidative stress levels in tourist than in non-tourist sites (Semeniuk et al. 2009). It is therefore evident that interpretations about the ecological or evolutionary meaning of population differentiation in oxidative status or hormetic responses need to keep into account the potential bias related to human influences.

9.5 Oxidative Profiles in Specific Ecological Circumstances

9.5.1 Predation Risk

Individuals may live under very different circumstances and may be consequently exposed to differing oxidative threats. The risk of predation may be one of these threats. Predation has a strong impact on life histories of prey since it may demand increases in hormonal stress responsiveness and metabolic rate and allocation of resources to support the emergency (Hawlena and Schmitz 2010; Clinchy et al. 2013). Moreover, exposure to predation may generate correlations between behavioural traits, hence, acting as a selective force on personalities (Bell and Sih 2007). Changes in oxidative balance have been described in relation to many of the

above-listed aspects. Hence, we might expect that oxidative stress can work as a mechanism underlying behavioural and life history adjustments to the risk of being predated. For example, if growth is slowed down in environments with high presence of predators, this strategy may carry benefits in terms of reduced generation of molecular damage (see Chap. 2). If growing up quickly is a best solution because the individual attains sexual maturity earlier, this might carry costs in terms of accelerated rate of ageing. Under predation risk, larvae of the common blue damselfly (*Enallagma cyathigerum*) reduced their growth while increased their consumption of oxygen compared to larvae not exposed to predators (Slos and Stoks 2008). In the presence of predators, larvae had also higher levels of heat shock proteins 70 and lower levels of catalase, while heat shock proteins 60 and superoxide dismutase were not affected (Slos and Stoks 2008). In another experiment on the same damselfly species, larvae exposed to predation risk had lower activity of superoxide dismutase, higher production of superoxide anion and of lipid oxidative damage (Janssens and Stoks 2013a). Exposure to additional stressors can exacerbate the pro-oxidant effect of predation risk. Exposure of larvae of the common blue damselfly to predator cues reduced activity of superoxide dismutase and increased oxidative damage, but only in the presence of an additional stressor—the pesticide glyphosate (Janssens and Stoks 2013b). These findings indicated an additive effect of the two stressors on the oxidative balance of larvae. However, the emergence of these hidden physiological synergisms did not occur for the growth rate and food intake (Janssens and Stoks 2013b). In contrast, while exposure to chemical cues from predators decreased activity of superoxide dismutase and of glutathione reductase in tadpoles of the Western spadefoot (*Pelobates cultripes*), the activity of both enzymes remained unchanged when tadpoles were exposed simultaneously to both predator clues and to a low concentration of the herbicide glyphosate (Burraco et al. 2013). Catalase, glutathione peroxidase and oxidative damage were not influenced by predation risk (Burraco et al. 2013).

Metabolic demands of escapes from predators may also influence the oxidative balance. Philipp et al. (2008) simulated an escape from a predator by exposing queen scallops *Aequipecten opercularis* to the common sea star *Asterias rubens*, which is a natural predator for scallops. Exposure to the risk of predation increased the swimming activity of scallops. This resulted in an increase in reactive species generation in muscle, but there was no increase in oxidative damage. However, effects of predator-induced stress may vary across tissues. Guerra et al. (2013) found that Catarina scallops (*Argopecten ventricosus*) experimentally exposed to natural predators (blue crab *Callinectes sapidus*) had not only lower antioxidant capacities (superoxide dismutase and catalase), but also lower oxidative damage (protein carbonyls and thiobarbituric acid-reactive substances) in gills and mantle compared to individuals not exposed to predators. In contrast, oxidative damage and catalase in the swimming muscle were higher in predator-exposed scallops than in scallops not exposed to predators.

A study on pied flycatchers (*Ficedula hypoleuca*) found that individuals breeding in closer proximity to predators' nests (sparrowhawk *Accipiter nisus*) had

higher levels of heat shock proteins 60 and 70 (Thomson et al. 2010). These studies suggested that there might be an indirect link between predation risk and oxidative stress, highlighting the importance of habitat selection decisions. Increased secretion of stress hormones in the presence of predators might be a mechanism linking the perceived predation risk to changes in oxidative profile. Exposure of passerine birds to predator decoys can, for example, increase levels of stress hormones in the plasma (Canoine et al. 2002), and in turn, stress hormones might induce increases in oxidative stress levels (Costantini et al. 2011) and changes in life history decisions (e.g., reduction in growth; Wingfield et al. 1998). Importantly, the increase in oxidative damage level caused by stress hormones becomes stronger with duration of the physiological stress and appears to be consistent among different tissues (Fig. 9.1; Costantini et al. 2011). This suggests that having high chronic secretion of stress hormones may impinge on the oxidative balance at systemic rather than local level.

Overall, these studies suggest that changes in oxidative damage and antioxidant protection may be part of the response to predation risk. Hence, oxidative stress might work as a selective constraint of prey response to predators. It remains to be tested if predator-induced oxidative stress influences evolutionary fitness.

9.5.2 Habitat Quality

The quality of the habitat in which an animal lives can have major consequences for its physiology through different routes. For example, the availability of food is very important because it may influence the individual oxidative status through the intake of nutrients and antioxidants, as well as through the effort needed for foraging activity. Seychelles warblers (*Acrocephalus sechellensis*) inhabit a fixed feeding territory year round and experience profound and local fluctuations in food availability. van de Crommenacker et al. (2011) found that spatio-temporal variation in territory quality can be associated with differing levels of oxidative damage being higher when the quality of territory is low. This study has been carried out in such a way that it is hard to disentangle the effects of territory quality per se from those of low-quality individuals in case they are forced to occupy lower-quality territories by high-quality competitors. Nevertheless, such study provided the first evidence that the individual oxidative damage level can actually track the quality of its territory and it is therefore not a fixed trait, rather it may reflect individual responses to environmental changes. Consequences for fitness might differ if costs of sustaining high levels of oxidative damage differ among individuals of different quality.

Breeding in a high-quality habitat does not necessarily pay in terms of better oxidative balance whether, for example, individuals are pushed to overinvest in reproduction or to make other costly decisions. For example, great tits (*P. major*) breeding in deciduous-dominated habitats (i.e. higher abundance of caterpillars) had higher baseline levels of oxidative damage than birds that bred in evergreen

habitats (Isaksson 2013). Moreover, environmental quality may impinge on senescence trough mechanisms sensitive to oxidative stress, such as the telomere dynamics (Haussmann and Marchetto 2010). Angelier et al. (2013) reported that telomeres of male American redstarts (*Setophaga ruticilla*) wintering in a low-quality non-breeding habitat shorten faster than those of individuals wintering in a high-quality non-breeding habitat. As with the study on Seychelles warblers, this study is of correlative nature; hence, it does not allow to disentangle the effect of non-breeding habitat from a potential effect of individual quality on the rate of telomere shortening. This would be the case if low-quality American redstarts winter in low-quality habitats. Previous experimental work has, however, shown that the quality of the non-breeding habitat may actually determine individual performance in the American redstart: wintering birds that were upgraded from a low-quality habitat to a high-quality habitat had higher breeding success and return rates in the next year than control birds in the low-quality habitat (Studds and Marra 2005, 2007, 2011; Reudink et al. 2009). Similarly, Mizutani et al. (2013) found that telomere dynamics in black-tailed gulls (*Larus crassirostris*) were influenced by dramatic changes in environmental conditions, highlighting the importance of environmental fluctuations in affecting stress and lifespan.

Research on plants provided strong evidence for an important role of habitat in determining resistance to oxidative stress. Studies on three closely related oak species (*Quercus petraea*, *Quercus pubescens* and *Quercus robur*) transplanted randomly to different soil typologies showed that changes in reactive species production and foliar antioxidants in response to stressful events differed between calcareous and acidic soil: reactive species and antioxidants were lower and higher on calcareous than on acidic soil, respectively (Contran et al. 2013; Hu et al. 2013). These results indicated that the capacities of antioxidant defence and osmotic stress adjustment developed better on calcareous compared with acidic soil; however, this effect was metabolite- as well as species-specific (Hu et al. 2013). Hu et al. (2013) suggested that one of the reasons for such soil-dependent differences in resistance to oxidative stress is the lower water and nutrient availability plants have to cope with on calcareous compared with acidic soil (Marion et al. 1993; Xie et al. 1998). Regardless of mechanisms, these studies clearly showed a strong link between habitat characteristics and organism oxidative balance.

9.5.3 Daily and Seasonal Variation

Individual oxidative profile is not a fixed trait. It may show daily rhythmicity (Fig. 9.5). Diurnal fluctuations in free radical production, oxidative damage, antioxidant enzyme activity, synthesis of low-molecular weight antioxidants or dietary antioxidants were described in various species, including both invertebrates and vertebrates (van den Driessche et al. 2000; Hardeland et al. 2003; Costantini and Bonadonna 2010; van de Crommenacker et al. 2011; Beaver et al. 2012; Giannetto et al. 2012; Peek et al. 2013). For example, rhythms of nitric oxide

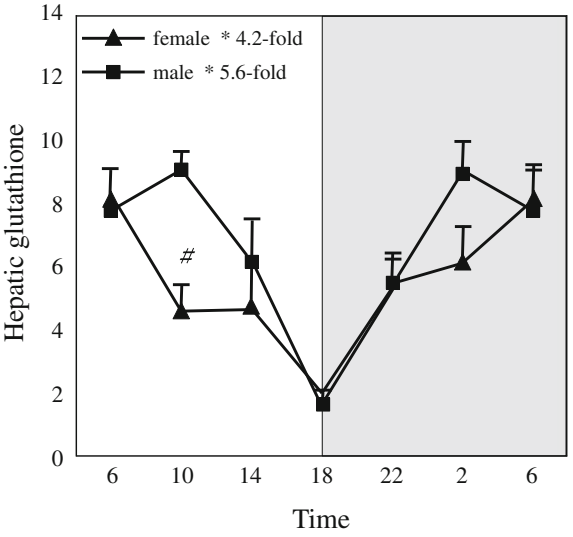

Fig. 9.5 Circadian variations of hepatic glutathione in adult female and male mice. *Asterisk* (*) indicates a significant circadian rhythm at $P < 0.05$; *hash* (#) indicates significant sex differences $P < 0.05$. Reproduced with minimal alterations from Xu et al. (2012)

synthase activity were recorded in various tissues of mice (Tunçtan et al. 2002). Generation of oxidative protein damage (protein carbonyls) was found to be rhythmic in various taxa, like in the dinoflagellate *Lingulodinium polyedrum* (Burkhardt et al. 1999), *Drosophila melanogaster* (Coto-Montes and Hardeland 1997) and Syrian hamsters *Mesocricetus auratus* (Coto-Montes et al. 2001). Extracellular ascorbate and glutathione peroxidase were observed to peak at night in rat brain (O'Neill et al. 1982; Barlow-Walden et al. 1995). There may also be variation among tissues, sexes or species in the rhythmicity of some antioxidants (Fig. 9.5; Hardeland et al. 2003; Xu et al. 2012). Daily changes in retinol, α-tocopherol and β-carotene appear to be of low amplitude in human blood (Nierenberg and Stukel 1987). In contrast, β-carotene strongly oscillates over the day in the dinoflagellate *Lingulodium polyedrum*, a species capable of synthesising this particular carotenoid (Di Mascio et al. 1995; Hollnagel et al. 1996). Melatonin and glucocorticoids appear to be important regulators of daily changes in oxidative balance (Goncharova et al. 2006; Ashkenazi and Haim 2012, 2013).

The degree to which rhythms of antioxidants and oxidation production reflect a direct genetic control through differential expression of genes, a post-translational regulation by means of other mechanisms (e.g., daily fluctuations in secretion of stress hormones or melatonin, mitochondrial activity), variation in food intake and/or a response to environmental stimuli remains to be tested in natural animal populations. Regardless of mechanisms, the occurrence of daily changes in antioxidant protection suggests that there might be time-dependent selection on behavioural schedules, i.e. a certain activity would be better done at sometimes of the day than others because physiological costs vary. Changes in some antioxidants might also reflect an anticipatory response to prepare for regular tasks. Biological clocks exert a feed-forward control over effectors: it initiates changes in

physiological systems, rather than correcting for changes after they happen (Hill et al. 2008). The extent to which a feed-forward control over redox system is important would depend on whether an individual or a species is strictly dependent or is not on external cues. Selective advantages of antioxidant rhythmicity are still far from being understood and so are the perturbations of such rhythmicity induced by oxidative stress itself. Research on mutants suggests that antioxidant rhythmicity may be important for evolutionary fitness. For example, loss of rhythmicity was found to reduce lifespan (Klarsfeld and Rouyer 1998; Krishnan et al. 2012) and reproductive performance (number of eggs laid, quantity of sperm, success of egg fertilisation; Beaver et al. 2002) in *D. melanogaster*. The extent to which oxidative stress contributes to the decreases in lifespan and reproduction induced by an impaired rhythmicity remains to be tested.

Oxidative status may also vary seasonally. Female great tits (*P. major*) and starlings (*Sturnus vulgaris*) breeding later in the season had higher plasma non-enzymatic antioxidant capacity and, but for starlings only, lower oxidative damage (Costantini et al. 2010). Activity of blood glutathione peroxidase was found to be lower in late broods of great tits (Norte et al. 2009). Similarly, the resistance of red blood cells to haemolysis induced by a free radical in vitro exposure was lower in late broods of yellow-legged gull *Larus cachinnans* (Kim et al. 2010). Second-brood chicks of starlings were also found to have lower plasma non-enzymatic antioxidant capacity than first-brood chicks within a same breeding season (Serra et al. 2012). Seasonal variation was also found for the antioxidant content of eggs: yellow-legged gulls breeding later in the season laid eggs with a lower content of antioxidants (Rubolini et al. 2006). This seasonal variation in oxidative damage or antioxidant status may reflect changes in ambient temperature and humidity, food quality and quantity, differential investment of females between first and second broods and/or different qualities of early and late breeders. For birds, as well as for other animals that go through a moulting phase, some seasonal variation in oxidative damage and antioxidant status might be induced by moult, which is a high-energy-demanding process. Correlative evidence in barn swallows (*Hirundo rustica*) suggests that this might be the case: birds with nearly completed moult had lower lipid hydroperoxides and activity of glutathione S-transferase than those in the initial stages of moult (Raja-aho et al. 2012).

Seasonal changes in antioxidant status were also recorded in other taxa, such as trouts. Aras et al. (2009) found that higher activities of various antioxidant enzymes (but not oxidative damage) in liver of female trouts (*Salmo trutta*) were found in autumn, and this coincided with an increase in the gonado-somatic index, which indicates the proportion of gonad mass of the total body mass of the animal. Hence, seasonal fluctuations in sexual hormones might underlie some variation in individual antioxidant protection (Hoogenboom et al. 2012).

9.6 Environmental, Maternal and Genetic Contributions to Oxidative Balance

That some components of the oxidative balance have a genetic basis is well established, antioxidant enzymes being a renowned example (e.g., Larsen 1993; Ito et al. 2004; Martin et al. 1996). The perspective that variation in production of reactive species and resistance to oxidative stress in natural animal populations may reflect the expression of different genetic polymorphisms is supported by various studies, such as those reporting allelic variants in cytochrome P450 enzymes in fish and mammals (Daly et al. 1993; Roy and Wirgin 1997). However, very limited information is available on how resistance to oxidative stress is determined by genetic, maternal or environmental effects and by the interaction between genotype and environment. This limits our capacity to infer the potential for evolutionary change in individual ability to resist oxidative stress within a population. To the end of defining whether selective processes directly shape resistance to oxidative stress, it is essential to assess the magnitude of additive genetic variance and genetic heritability (proportion of phenotypic variance explained by additive genetic variance; Falconer and Mackay 1996). It is also very important to assess the interaction between environment and genetics in order to evaluate the degree to which heritability and reaction norms vary across environments (Lynch and Walsh 1998).

Establishing the extent to which a marker of oxidative damage or antioxidant status is a stable trait across time within an individual may, at least partially, provide some clue about the control of its genotype and thus if it is subject to the forces of natural selection. Studies so far found that some parameters of oxidative status may be significantly repeatable across time, ranging from a few weeks to years, while others are more sensitive to short-term changes (e.g., Costantini et al. 2007; Galván and Alonso-Alvarez 2009; Saino et al. 2011; Sepp et al. 2012).

Studies of reptiles and birds indicated that some components of oxidative balance may have an inherited component, while others are mostly influenced by the environment. However, the data were collected in such a manner that these heritability estimates are sometimes based on a combination of additive genetic effects and maternal/paternal effects (i.e. indirect genetic or environmental effects on the phenotype of the offspring, caused by the mother or father). This makes difficult to say how much variation in oxidative balance of offspring was due to female allocation strategies of nutrients or genetics.

In reptiles, Olsson et al. (2008) found significant heritability in production of reactive species in painted dragon lizards (*Ctenophorus pictus*). In birds, various techniques of cross-fostering (e.g., partial, total) have been used by some authors to estimate the amount of heritable phenotypic variation in a certain measure of oxidative balance. Costantini and Dell'Omo (2006) found that the nest of origin (reflecting the genetic but also the early maternal effects) significantly contributed for a 23.5 % to the variance in serum oxidative damage as measured by hydroperoxides, while the nest of rearing (reflecting the environmental contribution)

significantly contributed for a 52.8 % to the variance in serum non-enzymatic antioxidant capacity in nestling kestrels (*Falco tinnunculus*). Rubolini et al. (2006) showed that the non-enzymatic antioxidant capacity of yolk (expressed as per unit or total) in yellow-legged gulls had a significant maternal contribution (nest of origin explained more than 48 % of variance). Among-chicks variation in plasma non-enzymatic antioxidant capacity was mostly explained by the rearing environment at both four (18.7 %) and eight (56.5 %) days of age. Norte et al. (2009) found a 17.7 % contribution of rearing environment to the among-chicks variation in blood glutathione peroxidase in great tits (*P. major*). Using a cross-fostering design that limited the influence of maternal effects, Kim et al. (2010) found that heritability and common environmental effects were low and non-significant for resistance of red blood cells to haemolysis induced by a free radical in vitro exposure on the day of hatching in nestling yellow-legged gulls. However, resistance of red blood cells at day 8 showed significant heritability, but the rearing environment of the chicks did not influence the variation in resistance of red blood cells. Hence, results suggested that genetic effects on variation in resistance of red blood cells increase as gull chicks grow. Expression of genetic variance for resistance to oxidative stress may be influenced by the environmental conditions. This is, for example, the case for yellow-legged gulls, where the phenotypic variance in non-enzymatic antioxidants in plasma increased in the chicks supplemented with vitamins mainly due to increased additive genetic variance (Kim et al. 2013). Supplementation of dietary antioxidants may induce downregulation of antioxidant enzymes (Chap. 1); hence, some, but not all, chicks might have reduced the expression of endogenous antioxidants in response to an increased intake of dietary antioxidants. On the other hand, the change in genetic variance for plasma non-enzymatic antioxidant capacity suggested that individuals differed in the ability to capture, absorb and transport dietary antioxidants (Kim et al. 2013). Although circulating non-enzymatic antioxidants showed some genetic variance, two parameters of oxidative damage (hydroperoxides and malondialdehyde) in plasma did not show any heritable variance. In another study on wild great tits, it was found that the common environment plays a major role in determining two parameters of blood oxidative status (in vitro resistance of red blood cells to haemolyse; plasma concentration of malondialdehyde) early in life, suggesting a low potential for evolution of individual ability to resist to oxidative stress (Losdat et al., in preparation).

Studies on invertebrates provided strong evidence for a high heritability of resistance to oxidative stress. Reynolds and Phillips (2013) quantified the natural genetic variation for lifespan and stress response using the *Caenorhabditis remanei* rather than the *C. elegans* because it possesses very high levels of molecular genetic variation and low levels of linkage disequilibrium. Heritability (and the coefficient of additive genetic variation) was high for resistance to oxidative stress (exposure to hydrogen peroxide) and heat stress, low (but significant) for

longevity, and essentially zero for stress response to ultraviolet radiation. The resistance to oxidative stress is also a determinant of longevity. However, heritability estimates differed between these two traits. This was possibly a consequence of measuring longevity under non-challenging conditions. Reynolds and Phillips (2013) suggested that, at least for this population of *C. remanei*, lifespan under relatively benign conditions was not determined by the same suite of genes as was resistance to oxidative stress in the environmental context of this study.

Indirect evidence highlighting the contribution of genetics to oxidative stress resistance comes from studies about inbreeding (increase in homozigosity). Inbreeding depression results in reduced evolutionary fitness in a given population because of breeding of related individuals (e.g., Lynch and Walsh 1998). Inbreeding makes the organism less resistant to environmental stress, but also and more specifically to oxidative stress (e.g., Pedersen et al. 2008; Okada et al. 2011). For example, levels of testicular oxidative stress were significantly elevated in inbred fruit fly males, although sperm viability did not differ between inbred and outbred males (Okada et al. 2011).

Overall, these results suggest that genetics significantly contributes to shape the oxidative balance in wild animals. However, the components of the oxidative balance analysed so far did not appear to be influenced to the same extent by the genetic background. The concentration of a molecule in a biological matrix is the result of a complex interplay of multiple components, from gene expression, post-translational regulation to interaction with other molecules and response to stressful stimuli. For example, we might expect a different impact of genetic background on oxidative damage parameters in case these molecules are produced early (e.g., hydroperoxides) or late (e.g., end products of lipid peroxidation) in the oxidative cascade.

A low heritability of a trait is usually related to its positive contribution to fitness, since strong directional and constant selection is supposed to decrease the genetic variation because the alleles conferring the highest fitness are expected to be driven quickly to fixation by natural selection (Fisher 1930; Kimura 1958; Gustafsson 1986; Mousseau and Roff 1987; Kruuk et al. 2000). However, many examples in the literature showed that fitness-related traits may actually have high additive genetic variation, whose expression is influenced by environmental conditions (Hoffman and Parsons 1991; Price and Schluter 1991; Merilä and Sheldon 1999; Charmantier and Garant 2005). Environmental stimuli may make the genetic variation cryptic to be expressed, generating novel developmental trajectories; this will depend on how plastic the genotype is, in other words on the ability of a genotype to respond to different environments by expressing differing phenotypes (Pigliucci 2001; West-Eberhard 2003; Badyaev 2009). Also in the case that additive genetic variance and heritability would be low, oxidative stress resistance might still show potential for evolution whether it were influenced by non-genetic inheritance (e.g., parental effects, epigenetics: Bonduriansky and Day 2009; Danchin et al. 2011; see also Chap. 2).

9.7 Among-Species Variation in Oxidative Damage and Antioxidant Defences

Intraspecific studies showed that oxidative damage or antioxidant mechanisms may be associated with individual life history strategies. Comparative studies also found connections between oxidative balance and life history, suggesting that oxidative stress may have played an important role in determining patterns of variation in animal life histories. However, comparative studies were so far mostly limited to antioxidants in birds or, when having a broader taxonomic approach, mostly focussed on the link between oxidative stress and longevity (Chap. 10).

Cohen et al. (2008) analysed various serum non-enzymatic antioxidants (carotenoids, vitamin E, uric acid, non-enzymatic antioxidant capacity) from a sample of 95 bird species (mostly passerines). They found that higher antioxidant levels were generally characteristic of more rapid development, lower survival rate, smaller body size, larger clutch size and higher mass-adjusted metabolic rate. However, there was high variation among antioxidants in the link with life history traits, with vitamin E showing the smaller number of significant connections. That the complexity of the relationship between life histories and antioxidants is remarkable is also supported by the fact that the relationship was also dependent on which species were included in the analyses (Cohen et al. 2008). Phylogenetic subsampling and division into tropical and temperate subsets both produced changes in the antioxidant-life history correlation structure (Cohen et al. 2008). Moreover, results of the study suggested that, while larger species may have low levels of circulating antioxidants because they would increase them only in response to a specific stressor, smaller species may have evolved high constitutive levels in order to anticipate any stressors they will encounter in life. Hence, smaller and short-lived species would invest in long-term physiological homeostasis because they experience repeated stressors on a daily basis if compared with larger ones (Cohen et al. 2008).

Results from Cohen et al. (2008) were in disagreement with those of Galván et al. (2012), who analysed the connections between life history traits and concentrations of carotenoids or vitamin E in the liver of 125 bird species. Galván et al. (2012) found that high carotenoid concentrations were present in species with large body, brain and egg sizes, high absolute metabolic rates and resident lifestyles, while high vitamin E concentrations occurred in species with large brain sizes and long lifespans and incubation periods.

Discrepancies between studies were probably dependent on differences in activity of vitamin E while circulating in the blood or stored in liver (Galván et al. 2012). Plasma vitamin E would be ready to protect from reactive species, while vitamin E in liver would reflect a strategy of storing an important antioxidant that can be then used in time of need.

As regards carotenoids, Tella et al. (2004) also found that diet significantly explains some among-species variation in plasma carotenoids. Dietary influences were, however, less important than phylogeny, which explained 65 % of variation

in a sample of 80 bird species. Although the phylogenetic effect could be due partly to its covariation with diet, there may also be the contribution of differences among species in the mechanisms that regulate the absorption of carotenoids from food (Trams 1969; Yang and Tume 1993; Rock et al. 1996; Surai et al. 1998; Tella et al. 2004).

Connections between lifestyles and oxidative balance were also found in taxa beyond birds. For example, López-Cruz et al. (2010) found that two shark species (*Isurus oxyrinchus* and *Carcharhinus falciformis*) with intense swimming habits and a diet based on highly active prey had higher production of superoxide and activities of glutathione peroxidase and glutathione S-transferase in skeletal muscle than in a shark species (*Sphyrna zygaena*) with less active swimming. However, the oxidative damage level was lower in *I. oxyrinchus* than in the other two species. Hence, sharks with high demanding lifestyles may need to maintain high constitutive antioxidant protection in order to mitigate cellular damage.

Parasite helminths express a comprehensive repertoire of both cytoplasmic and secreted antioxidant enzymes, but there are significant differences between species and life-cycle stages. For example, adult individuals of *Brugia malayi*, *Dirofilaria immitis* and *Onchocerca volvulus* secrete copper–zinc superoxide dismutase and the former two species also secrete a selenocysteine-independent glutathione peroxidase (Selkirk et al. 1998). Adult *O. volvulus* may compensate for an apparent deficiency in expression of this enzyme by means of a variant of glutathione *S*-transferase (Liebau et al. 1994; Selkirk et al. 1998). Further variation was observed in genes coding for glutathione peroxidases and peroxiredoxins, with different helminth phylogenetic lineages coding for different isoforms (Kim et al. 2009; Bae et al. 2011).

Closely related algal species can rely on different strategies to withstand oxidative stress. For example, differently from the green alga *Ulva procera*, the cohabiting green alga *Cladophora glomerata* responds to the increase in photosynthetic active radiation by increasing the synthesis of carotenoids and activating the violaxanthin xantophill cycle (Choo et al. 2005). Moreover, *C. glomerata* has higher baseline activities of catalase and ascorbate peroxidase than *U. procera* (Choo et al. 2004). These differences in antioxidant mechanisms make *U. procera* more sensitive to oxidative stress than *C. glomerata* (Choo et al. 2005). *U. procera* algae produce high amounts of hydrogen peroxide while exposed to light. Given their low activities of hydrogen peroxide removing antioxidant enzymes, *U. procera* algae remove it through diffusion out of the cells into the seawater (Choo et al. 2005). These differences in antioxidant strategies reflect ecological differentiation of the two algal species. Although both species have opportunistic habits, *C. glomerata* is a persistent annual alga, while *U. procera* has ephemeral habits, appearing and disappearing in a same site depending on the current local conditions (Choo et al. 2005). It may therefore be hypothesised that *C. glomerata* invests more than *U. procera* in self-maintenance mechanisms to support their higher persistency. In contrast, *U. procera* algae invest more in growth and reproduction over the short periods

over which they are active. *U. procera* algae have in fact very high photosynthetic activity compared to other algal species, including *C. glomerata* (Johansson and Snoeijs 2002).

Variation in oxidative stress resistance was also reported for closely related plant species. Hu et al. (2013) showed that foliar antioxidant and free amino acid levels were significantly affected by drought plus air warming; however, this effect was species-dependent with the drought-tolerant species *Q. pubescens* having a higher reactive oxygen species scavenging capacity than the less drought-tolerant species *Q. robur* and *Q. petraea*.

Being more resistant to oxidative stress may, for example, be vital in a competitive context. Li et al. (2013) investigated the effects of ozone (a pro-oxidant agent) on winter wheat (*T. aestivum*) and on its natural competitor, the flixweed (*Descurainia sophia*). The production of hydrogen peroxide and oxidative damage to lipids was increased in both plant species, but the effect was stronger when plants were grown in mixture than singly. Importantly, the winter wheat was less resistant to the pro-oxidant agent than the flixweed. The higher susceptibility to oxidative stress of winter wheat resulted in lower growth and grain yield than those of the flixweed. Competition has therefore exacerbated the pro-oxidant effect of an environmental stressor, but the different resistance to oxidative stress of the two plants determined the success in the competition.

9.8 Among-Species Variation in Hormetic Responses

There has not been so far almost any attempt to study among-species variation in hormetic responses within a comparative framework. Theoretical considerations and a limited number of experimental investigations suggest that we cannot assume a hormetic response we describe in a species to be comparable to that of another one. The hormetic zone, i.e. the stimulatory area under the curve (Chap. 1), may strongly differ among species, possibly reflecting specific adaptations to differing habitats (Parsons 2001) or differences in developmental rates (e.g., heterochrony). This is well exemplified by an experiment on five species of *Drosophila* fruit flies (Holmes et al. 1980). When adult fruitflies are exposed to differing concentrations of ethanol, only four out of the five species showed a hormetic response and these four species showed different threshold concentrations above which ethanol became a metabolic stressor, inducing a decrease in longevity. Consequently, the width of hormetic zone for the response of longevity to ethanol differed among species and was smaller for *Drosophila* species that normally feed on food poor in ethanol content. These findings indicated that it is very important to consider the hormetic zone when predicting the responses of an individual, population or species to an environmental perturbation.

9.9 Is Hormesis a Target of Natural Selection?

It has been debated for a long time whether we might expect hormesis to be under natural selection. Recent evidence shows that natural variation in hormetic effects on lifespan for heat shock response may be genetically determined (Rodriguez et al. 2012), suggesting that hormetic responses, at least those to heat stress, may be a target of natural selection.

Rodriguez et al. (2012) investigated the genetic variation of heat-shock-dependent hormetic effects on lifespan and the associated quantitative trait loci in various strains of *C. elegans*. Wild-type CB4856 worms exposed to heat stress survived 18 % longer than controls of the same strain, while there was not hormetic effect on lifespan in wild-type Bristol N2 worms. Using recombinant inbred lines (RILs) derived from a cross between wild types N2 and CB4856, Rodriguez et al. (2012) also found natural variation in stress-response hormesis in lifespan. Some 28 % of the RILs displayed a hormetic effect in lifespan. Importantly, the ability to recover from heat shock mapped to a significant quantitative trait locus on chromosome II. The QTL was confirmed by introgressing relatively small CB4856 regions into chromosome II of N2.

Hormetic responses might also be under selection whether they were influenced by non-genetic inheritance, such as epigenetic modifications. Many hormetic responses induced by a variable range of agents have been characterised by large-scale changes in gene expression (Vaiserman 2008, 2010, 2011; Sarup et al. 2013). For example, hormetic responses to heat stress have been routinely found in various animal species, and in 1998, it was suggested to be a plausible molecular mechanism underlying the emergence of epigenetic effects in natural animal populations (Rutherford and Lindquist 1998).

Although the importance of inheritance of epigenetic modifications and their impact on micro-evolutionary patters are still under intense debate, some studies indicated that hormesis may have trans-generational effects, although effects persisted only until the second or third generation (Chap. 2). It should be recognised, however, that these conclusions were drawn from studies mostly limited to laboratory animals. There is therefore the need to assess the genetic and epigenetic bases of hormesis in natural animal populations in order to definitely corroborate the hypothesis that hormesis is under selection.

9.10 Conclusions

Studies show that there are multiple sources of variation in oxidative stress resistance and hormetic responses. We need to know more about the causes and consequences of such variation and to better appreciate how they contribute to life history variation. For example, exposure to predators may increase the oxidative damage level in individuals highly responsive to stressors (e.g., through an

increase in secretion of stress hormones). However, adjustments of life history traits (e.g., speeding up or slowing down growth, investing more or less into reproduction) in relation to predation risk can also influence the oxidative balance. Another important source of variation is the daily rhythmicity in reactive species generation and antioxidant levels. Although it was described in various species, including both invertebrates and vertebrates, we are still far from appreciating which selective advantages it may bring to the individual.

We should not forget that sex differences are an important source of biological variation. Comparatively to others features, we know very little about how sexual differences in resistance to oxidative stress or hormesis (see Chap. 7 for examples on reproductive strategies) come out. Males and females have distinct life styles and experience different selection pressures. These differences in selection have likely given rise to some of the sexual dimorphisms in life history, physiology, morphology and behaviour observed across animal species. There are therefore good reasons to expect that males and females differ in the way they invest in protection against oxidative stress and how this investment strategy impinges on the resolution of life history trade-offs.

Finally, in order to understand the evolution of oxidative stress and hormetic responses, we need to fully understand how they are inherited. This is clearly an essential step to ascertain whether both oxidative stress and hormesis are a target of natural selection. Heredity does not necessarily need to be genetic in origin. Oxidative stress resistance and hormesis responses might also be under selection whether they are influenced by non-genetic inheritance, such as epigenetic modifications that are passed from the mothers to their offspring. So far, data were collected in such a manner that heritability estimates are sometimes based on a combination of additive genetic effects and maternal/paternal effects. We need a combination of different approaches (e.g., common garden experiments to reduce phenotypic plasticity; analysis of gene expression; and detection of quantitative trait loci) to overcome these issues.

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Chapter 10
Integrating Oxidative Stress and Hormesis into Research on Senescence and Survival Perspectives

Abstract Progressive deterioration in performance with age is what we refer to as senescence or ageing. It varies greatly among species, but also among conspecific individuals because of the influence of genetic, environmental and stochastic factors. The recent emergence of long-term field studies has provided widespread evidence that animals in nature do senesce, a finding that has opened new horizons for research on causes and consequences of senescence. Empirical evidence is often supportive of the prediction that long-lived species produce less reactive species, have lower oxidative damage levels and antioxidants and have molecules more resistant to oxidative damage. However, full understanding of mechanisms underlying ageing is still elusive. This chapter reviews briefly the main mechanistic and evolutionary theories of senescence that may be linked to oxidative stress, and discusses comparative studies that tested the link between oxidative status parameters and senescence. The chapter ends with an examination of how mild doses of oxidative stress or other forms of stress experienced during sensitive windows of life may increase longevity through hormetic mechanisms.

10.1 The Secret Nature of Longevity

Individuals change over their lifetime in how they look like or how they behave, sometimes irreversibly, but sometimes temporarily. Changes that occur in old age are often irreversible and mostly are due to a loss of body functionality, as with reduced immunocompetence, increased susceptibility to oxidative damage or reduced skeletal muscle strength. Such a progressive deterioration in performance is what we refer to as senescence or ageing. It varies greatly among species, but also among conspecific individuals because of the influence of genetic, environmental and stochastic factors (Kirkwood et al. 2005; Jones et al. 2014).

Various questions have permeated the research on senescence since the very beginning: Why does an individual senesce? When does an individual start ageing? How might senescence have evolved? Why do species differ so greatly in longevity? The classic approach to studying senescence has been the use of experimental

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approaches on a few model laboratory species, or analysis of observational data on humans. Although this research has provided very important insights into the mechanisms of senescence, our knowledge is still very limited and many secrets underpinning the mechanisms controlling lifespan and the impressive biological variation in longevity among individuals and species are yet to be revealed. A major limit to our understanding of senescence may be attributed to the erroneous assumption that senescence does not occur in natural animal populations. This has deprived us of a unique natural laboratory, where to challenge the generality of laboratory findings in species differing not only in lifespan, but also in many other life history traits and the harshness of the environment. The recent emergence of long-term field studies has provided widespread evidence that animals in nature do indeed senesce (e.g. Møller et al. 2005; Monaghan et al. 2008; Nussey et al. 2008, 2013; Bouwhuis et al. 2010; Martin and Festa-Bianchet 2011). This finding has opened new horizons for research on the causes and consequences of senescence.

In this chapter, I present briefly the main mechanistic and evolutionary theories of senescence that may be linked to oxidative stress; I discuss comparative studies that seek to explain why species differ in lifespan; and I examine how mild doses of oxidative stress or other forms of stress may increase longevity through hormetic mechanisms.

10.2 Mechanistic Hypotheses of Ageing

10.2.1 From the Rate of Living to the Oxidative Stress Hypothesis of Ageing

More than a century ago, Rubner (1908) noted that while the lifespan in a sample of five mammalian species varied approximately fivefold, the amount of energy consumed per gram of body mass was much less variable. Moreover, he observed that the maximum lifespan of mammals increased with body size and that the mass-specific rate of metabolism of mammals decreased with body size. Rubner's observations were later further elaborated by Pearl (1928) in the *rate of living hypothesis of ageing*, which states that organisms with a high metabolic rate have shorter lives. The rate of living theory of ageing lacked any mechanistic support until the 1950s, when Gershman et al. (1954) and Harman (1956) suggested that the production of free radicals might be the mechanism linking metabolic rate to longevity (Harman 1956): high metabolic rate would result in high production of free radicals, which would cause damage to cells and, consequently, senescence. However, in the 50 years following this proposal, the rate of living theory lost credibility in favour of the Harman's hypothesis that we now known as the *free radical hypothesis of ageing* (Beckman and Ames 1998; Weinert and Timiras 2003). The decline of the rate of living hypothesis of ageing has largely been because of the following findings: increases in metabolic rate do not necessarily reduce lifespan;

the production of reactive species or oxidative damage is not one-to-one proportional to metabolic rate; studies on various animal species have not found any correlation between lifespan and mass-specific metabolic rates at the individual level or found that high metabolic rate was linked to greater longevity; caloric restriction can extend lifespan independently of changes in metabolic rate; birds and mammals with similar paces of life differ in longevity; birds generally have higher metabolic rates and body temperatures than mammals of similar body size, but live substantially longer; supposed correlations between longevity and metabolic rate were an artefact of not correcting for the confounding effects of body mass and phylogeny (Storer 1967; Speakman et al. 2004; Speakman 2005a, b; Hulbert et al. 2007; Furness and Speakman 2008). For example, studies on microchiropteran bats (Filho et al. 2007), colubrid snakes (Robert et al. 2007) or zebra finches *Taeniopygia guttata* (Moe et al. 2009) did not find any relationship between mortality and basal metabolic rate. Experimental manipulation of energy expenditure via cold exposure did not shorten lifespan in field voles *Microtus agrestis* (Selman et al. 2008). Moreover, in mice, lifespan was not affected by an increase in energy expenditure induced by either cold exposure (Vaanholt et al. 2009) or aerobic exercise (Vaanholt et al. 2010). Møller (2008) found that maximum lifespan was positively correlated with the mass-specific field metabolic rate (i.e. that of a free-living animal), while controlling for annual adult survival rate and sampling effort, in 35 species of birds.

Comparative studies also failed to find an association between basal metabolic rate and lifespan, although this result was not consistent across taxa (Speakman 2005a; de Magalhães et al. 2007). For example, de Magalhães et al. (2007) found that, while basal metabolic rate did not correlate with longevity in eutherians or birds, it negatively correlated with longevity in marsupials. Longevity records were, however, obtained from captive animals; hence, caution is required when extending these results to wild animals.

Despite this evidence against the role played by metabolism in ageing process, the importance of metabolic rate cannot be disregarded. Ruggiero et al. (2008) found that, independent of age, basal metabolic rate of humans who died was higher than in those who survived. Basal metabolic rate was a significant risk factor for mortality independent of secular trends in mortality and other well-recognised risk factors for mortality, such as age, body mass index, smoking, white blood cell count and diabetes. In a comparative study of wild birds, Cohen et al. (2008) found that species with higher body mass-adjusted metabolic rates tended to live shorter while controlling for phylogeny. Recent studies on tropical birds, which generally live longer than their temperate counterparts, suggested that differences in metabolic rate linked to differences in organ size might be important (Wiersma et al. 2012).

Since this time, the free radical theory has been refined and a number of hypothetical iterations have been presented, including the *mitochondrial theory of ageing* (Harman 1972; Miquel et al. 1980) and the *oxidative stress theory of ageing* (Yu and Yang 1996; Sohal et al. 2002), which have highlighted the importance of free radical-induced oxidative damage, especially to mitochondria, rather than free radical production *per se*, to drive the cell senescence rate (Beckman and Ames 1998; Finkel and Holbrook 2000).

That protection against oxidative damage is important is well exemplified in studies where transgenic animals lacking specific antioxidant enzymes are used. For example, mice lacking superoxide dismutase 2 did not survive more than a few weeks and had increased susceptibility to oxidative mitochondrial injury in the central nervous system neurons, cardiac myocytes and other metabolically active tissues after post-natal exposure to ambient oxygen concentrations (Lebovitz et al. 1996). Similarly, mice lacking mitochondrial manganese-dependent superoxide dismutase survive for only 30–40 days before they die because of high levels of accumulated oxidative damage (Melov et al. 1998, 1999). Administration of catalytic exogenous antioxidants has been shown to attenuate the negative effects of not having mitochondrial manganese-dependent superoxide dismutase, further demonstrating the importance of oxidative damage as a promoter of senescence in these mice (Melov et al. 1998). Transgenic mice that overexpressed an antioxidant enzyme (catalase) lived longer, had a delayed development of cardiac pathology and cataract development, a reduction in oxidative damage, an attenuation of hydrogen peroxide production and hydrogen peroxide-induced aconitase inactivation, and a reduction in the development of mitochondrial deletions (Schriner et al. 2005). Treatment with a pro-oxidant molecule (diquat) resulted in a significant increase in oxidative damage within 3–6 h in wild-type mice without any lethality (Han et al. 2008). In contrast, treatment of $Sod1^{-/-}$ or $Gpx1^{-/-}$ mice with a similar concentration of diquat resulted in a significant increase in oxidative damage within an hour of treatment and was lethal (Han et al. 2008). The expression response to elevated oxidative stress in vivo did not involve an upregulation of classic antioxidant genes, although long-term oxidative stress in $Sod1^{-/-}$ mice led to a significant upregulation of thiol antioxidants (Han et al. 2008). A critical evaluation of studies where animals lack certain antioxidant enzymes, or have it downregulated or overexpressed, however, suggested that oxidative damage had more significant impacts on the probability of becoming ill rather than on longevity (Salmon et al. 2010).

The rate at which oxidative damage is generated is, however, just one piece of the puzzle. Animals are in fact equipped with numerous mechanisms that may make them more or less resistant to oxidative damage (Chap. 1). Hence, other mechanisms have been proposed to explain among-species variation in longevity, and this led to the definition of various derivatives of the oxidative stress theory.

10.2.2 The Homeoviscous-Longevity Adaptation and the Membrane-Pacemaker Hypotheses of Ageing

The *homeoviscous-longevity adaptation* (Pamplona et al. 2002) and the *membrane-pacemaker* (Hulbert 2005) are two such hypotheses of ageing that have been derived and integrated into the oxidative stress theory. They state that long-lived species have fatty acid profiles that make their cell membranes more resistant to oxidative damage than short-lived species (Pamplona et al. 2002; Hulbert 2005).

It has long been known that fatty acids differ substantially in their vulnerability to peroxidation, with polyunsatured fatty acids being generally the most vulnerable (Chap. 1). Therefore, membrane susceptibility to peroxidation can be determined from its fatty acid composition and the relative susceptibility of each fatty acid to peroxidation damage (Holman 1954). On the basis of these cell characteristics, a peroxidation index can be calculated, with higher values indicating a higher presence of fatty acids most susceptible to damage. Peroxidation index was negatively correlated with maximum lifespan in both birds and mammals (Fig. 10.1) (Hulbert et al. 2007; Hulbert 2008). Interestingly, avian cell membranes are richer than mammalian ones in fatty acids less susceptible to peroxidation (Hulbert et al. 2002a, b). The naked mole rat (*Heterocephalus glaber*) lives about 5 times longer than predicted from its body mass (Buffenstein 2005), and although its basal metabolic rate is 30 % lower than expected (O'Connor et al. 2002), this does not account for its high longevity. Despite its extraordinary longevity, naked mole rat mitochondria produce more free radicals than mouse mitochondria. Moreover, the activity of glutathione peroxidase is almost undetectable in naked mole rat tissues (Andziak et al. 2005) and, at an early age (7 % of maximum lifespan), naked mole rat tissues have higher levels of oxidative damage to lipids, proteins and DNA than mice (Andziak et al. 2006). Its high longevity might be because of its low peroxidation index, which is a result of it having proportionately large amounts of peroxidation-resistant monounsaturated fatty acids, but low amounts of peroxidation-sensitive polyunsaturated fatty acids, particularly docosahexaenoic acid (Hulbert et al. 2006, 2007). It has also been proposed that naked mole rats have proteins that are particularly resistant to oxidative damage (Pérez et al. 2009). However, both explanations are undermined from the fact that naked mole rats have high levels of lipid peroxidation and protein damage.

In other taxa, the contribution of peroxidation index to longevity appears stronger. For example, humans and echidnas live about 5 times longer and have membrane peroxidation indices much lower than expected for their body size (Pamplona et al. 1996; Hulbert et al. 2008). Among birds, Procellariiformes (petrels and albatrosses) are exceptionally long lived, whereas Galliformes (fowl) have short lifespans. A comparison of myocardial phospholipids from birds of each group revealed significantly lower peroxidation index values in the Procellariiformes than in the Galliformes, due mainly to the proportionately higher monounsaturated fatty acids and reduced polyunsaturated (particularly $n - 6$) in the seabirds compared to the fowl (Buttemer et al. 2008). Interestingly, the 36 % lower average peroxidation index in Procellariiformes was exactly the magnitude expected for the 4.5-fold greater average longevity in the petrels compared to the fowl (Buttemer et al. 2008).

The correspondence between peroxidation index and maximum lifespan might also be affected by any mass-related influences on longevity because the peroxidation index declines with body mass in birds and mammals. Valencak and Ruf (2007) addressed this issue in their analysis of skeletal muscle phospholipids composition of 42 mammalian species. They statistically removed the effect of body mass on maximum lifespan and various lipid measures, and did not find

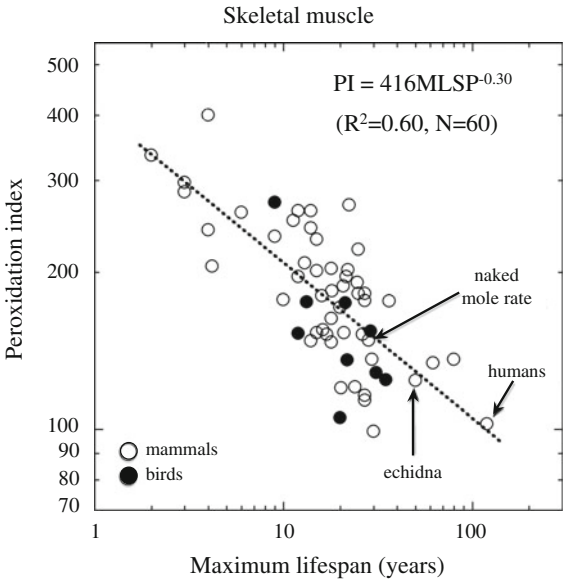


Fig. 10.1 Relationship between skeletal muscle membrane fatty acid peroxidation index (*PI*) and maximum lifespan potential in selected birds and mammals. Reprinted with minimal alterations from Hulbert (2008) with permission from Elsevier

variation in peroxidation index to account for residual differences in maximum lifespan.

The fatty acid desaturation pathway (placement of double bonds in a fatty acid molecule) and the deacylation–reacylation cycle (introduction of polyunsaturated fatty acids into cell membranes) are two important mechanisms that contribute to generate among-species variation in fatty acid profile (Pamplona et al. 2002). Estimates of delta-5 and delta-6 desaturase activities indicated that they are lower in long-lived than in short-lived species (Pamplona and Barja 2003; Pamplona 2008). Accordingly, 22:6$n-3$ and 20:4$n-6$ fatty acids decrease and 18:2$n-6$ and 18:3$n-3$ fatty acids increase, respectively, from short- to long-lived species, since desaturases are the rate-limiting enzymes of the $n-3$ and $n-6$ pathways that synthesise the polyunsaturated fatty acids 20:4$n-6$ and 22:6$n-3$ from their dietary precursors, 18:2$n-6$ and 18:3$n-3$, respectively (Pamplona et al. 2002; Pamplona and Costantini 2011). Importantly, long-lived species have a low degree of membrane fatty acid desaturation based on the redistribution among types of polyunsaturated fatty acids without any alteration in the total (%) polyunsaturated fatty acids content, average chain length, and phospholipid distribution. Hence, desaturation of fatty acids may be used to decrease the sensitivity to lipid peroxidation and lipoxidation-derived damage to cellular macromolecules without strongly altering fluidity/microviscosity, a fundamental property of cellular membranes for the proper function of receptors, ion pumps and transport of metabolites (Pamplona et al. 2002). This would occur because membrane fluidity increases acutely with the introduction of the first and less with the second double bond, whereas additional double bonds have a weak effect on membrane fluidity (Brenner 1984). The susceptibility of fatty acids to oxidative damage increases

with the increase in double bonds. Hence, substitution of fatty acids with four or six double bonds with those having only two or three double bonds allows animals to increase resistance to oxidative damage, while maintaining the membrane fluidity unaltered.

10.2.3 The Uncoupling to Survive Hypothesis of Ageing

One reason for lack of consistency of effects of metabolic rate on lifespan appears to be consequence of an uncoupling between metabolism and generation of free radicals. Specifically, the transition of mitochondria from state 4 (resting) to state 3 (respiratory active, producing ATP) is accompanied by a decrease in free radical production (Loschen et al. 1971). Consequently, oxidative damage cannot be massive during phases of increased metabolic rate because the mitochondrial free radical leak strongly decreases over the states 4 to 3 transition (Herrero and Barja 1997a). Uncoupling proteins are responsible for the decreased leak of free radicals; hence, they potentially represent a biochemical adaptation to mitigate oxidative stress generation (Chap. 1). Importantly, the activity of uncoupling proteins can result in a positive relationship between metabolic rate and lifespan (*uncoupling to survive hypothesis of ageing*; Brand 2000; Speakman et al. 2004), which is completely the opposite of the rate of living theory.

Mice lacking superoxide dismutase 2 (SOD2) usually do not survive more than a few weeks of age due to high levels of mitochondrial oxidative damage in metabolically actives tissues (Lebovitz et al. 1996). Andrews and Horvath (2009) confirmed the lethal consequence of SOD2 deficiency, but noted that the time of death depended on the expression of the uncoupling protein 2 (UCP2), with SOD2-deficient mice survived only 7 days if lacking UCP2 expression ($UCP2^{-/-}$), 14 days if heterozygous for UCP2 and 21 days if homozygous ($UCP2^{+/+}$). This confirms both the capacity for mitochondrial oxidative damage to affect longevity and the ability of uncoupling proteins to mitigate its deleterious potential.

The possibility that increases in uncoupling proteins might explain among-species differences in maximum lifespan has been inferred from a study examining the mitochondrial characteristics of two metabolically distinct cohorts from a population of outbred mice that differed in lifespan (Speakman et al. 2004). Because the longer living mice had a higher UCP3 content than the other cohort, an active debate has followed regarding the involvement of uncoupling proteins in increasing uncoupling of longer-lived species, with the assumption that an increase in uncoupling necessarily leads to longer lifespans. Studies that transgenically overexpressed UCP1 in murine skeletal muscle showed that lifespan was increased considerably in high-fat diet-fed UCP1 transgenic mice compared with wild types (Keipert et al. 2011), although overexpression of UCP1 did not reduce oxidative stress (Keipert et al. 2013).

It is generally forgotten that measurements of basal proton flux rates in mitochondrial membranes in mammals (Porter and Brand 1993) and birds (Brand et al.

2003) showed a significant increase in respiratory control ratio (RCR) as body mass increases. The RCR is the ratio of the oxygen consumption rate at maximum ATP synthesis (state 3 respiration with non-limiting ADP) to the non-phosphorylating respiration rate (state 4 in the presence of ATPase inhibition; therefore, proton leak). Thus, the mitochondrial membranes of smaller species are more uncoupled than those of larger species since the non-phosphorylating respiration rates decrease more strongly than the phosphorylating respiration rates as body mass increases, yet larger species usually live longer (Buttemer et al. 2010).

Critical assessment of the extent to which among-species differences in maximum lifespan is accounted for by UCP variation will require quantification of mitochondrial UCP content and how this affects formation of reactive species under physiological conditions known to provoke high rates of superoxide production. In this regard, it is important to note that Lambert et al. (2007) examined mitochondrial formation of reactive species under the same conditions as those in experiment from Talbot et al. (2004), which activated UCP3 expression in rats. If mitochondrial UCP activation did occur in the species examined by Lambert et al. (2007), then the lack of statistically significant differences in formation of reactive species among some species with very different maximum lifespans may raise doubt about the contribution of uncoupling proteins in affecting among-species differences in longevity (Buttemer et al. 2010). This conclusion may also apply to taxa beyond vertebrates. For example, the induction of oxidative stress by cadmium exposure and hypoxia-reoxygenation failed to activate the uncoupling protein 5 in eastern oysters (*Crassostrea virginica*), suggesting that this protein does not have any important antioxidant role in this bivalve species (Kern et al. 2009).

10.2.4 The Cell Senescence-Telomere Hypothesis of Ageing

The *cell senescence-telomere hypothesis of ageing*, as coined by Weinert and Timiras (2003), likely finds its roots in 1965, when Hayflick (1965) proposed that the replicative capacity of cells is limited and this would be the reason for cell senescence. In the early 1990s, Harley et al. (1990) showed that telomeres shorten during ageing of human fibroblasts. This finding suggested that the shrinking of telomeres might be an important mechanism responsible for replicative senescence. Telomeres are structures composed of repetitive nucleotide sequences located at the ends of chromosomes. Telomeres protect the ends of the chromosome from deterioration or from fusion with neighbouring chromosomes. Telomeres shorten with each replication event, and when telomeres shorten to a critical length, they induce a permanent arrest in the cell cycle through a process called cellular senescence (Hornsby 2003; Haussmann and Marchetto 2010). The rate of telomere loss is, however, not constant, rather it varies during the lifetime (Heidinger et al. 2012) and accelerates when the organism is exposed to stressful events (Epel et al. 2004). The enzyme telomerase can limit the reduction in telomere length, so slowing down the process of cell senescence. While its activity

is suppressed in most adult human tissues (Wright and Shay 2001), telomerase activity is maintained throughout the lifespan in other animal taxa (sea urchins, Francis et al. 2006; fish, Hatakeyama et al. 2008; birds, Haussmann et al. 2007; small mammals, Gorbunova and Seluanov 2009). For example, Lund et al. (2009) measured telomere length and telomerase activity in heart, gills, kidney, spleen, liver and intestine in zebrafish (*Danio rerio*) at 3, 6, 9, and 24 months of age. Telomeres did not appreciably shorten throughout the lifespan of the zebrafish in any organ, even when cells were under the highest pressure to divide after fin-clipping experiments. All aged (2 years old) tissues examined also expressed active amounts of telomerase activity, which might have contributed to buffering telomere shortening (Lund et al. 2009).

In the last years, there has been a growing interest of ecologists in telomere dynamics (Nakagawa et al. 2004; Monaghan and Haussmann 2006; Haussmann and Marchetto 2010; Monaghan 2010), although most of the work was focussed on birds. Telomere dynamics has been found to be related to survival and lifespan in tree swallows *Tachycineta bicolor* (Haussmann et al. 2005), Alpine swifts *Apus melba* (Bize et al. 2009), jackdaws *Corvus monedula* (Salomons et al. 2009), zebra finches *T. guttata* (Heidinger et al. 2012) and American redstarts *Setophaga ruticilla* (Angelier et al. 2013); lifetime reproductive success in dunlins *Calidris alpina* (Pauliny et al. 2006); physiological stress in humans (Epel et al. 2004) or house mice *Mus musculus* (Kotrschal et al. 2007); growth in rats (Jennings et al. 1999), European shags *Phalacrocorax aristotelis* and wandering albatrosses *Diomedea exulans* (Hall et al. 2004) and zebra finches (Heidinger et al. 2012); and infection in house mice (Ilmonen et al. 2008). There is therefore good support for a link between telomere dynamics and lifestyle, and for the role of telomeres in shaping life history variation. Such evidence also supports indirectly the importance of oxidative stress as a constraint to life history evolution. This is because oxidative stress accelerates telomere loss because oxidative damage to telomeric DNA is repaired less well than elsewhere in the chromosome (von Zglinicki 2002). For this reason, it was suggested that telomere-shortening measurements may also serve as a parameter of chronic oxidative stress (Houben et al. 2008).

10.2.5 The Redox Stress Hypothesis of Ageing

The *redox stress hypothesis of ageing* states that ageing-associated functional losses are primarily caused by a progressive shift in the redox state of the cells, which leads to overoxidation of redox-sensitive protein thiols and the consequent disruption of the redox-regulated signalling mechanisms (Sohal and Orr 2012). The available evidence raises some considerable doubts about the role of oxidative damage generation as the sole mechanism driving the ageing process; in recent years, various authors proposed that oxidative stress should be defined as a pro-oxidant shift in the thiol redox state and the resulting dysfunction of redox-sensitive proteins (Jones 2006). Sohal and Orr (2012) raised further points

against the simple link between oxidative damage and ageing. For example, the steady-state levels of modified macromolecules are a snap-shot of the dynamic balance between the processes of degradation, repair, and biosynthesis rather than a measure of the cumulative damage (Sohal and Orr 2012). Also, it is unknown whether the age-related increase in the steady-state amounts of oxidised macromolecules necessarily entails significant decreases in the net amount/abundance of the unmodified molecules (Sohal and Orr 2012). Studies on oxidative protein damage also showed that not all proteins lost functionality after being carbonylated (Yarian et al. 2005). It is therefore unclear to what degree parameters of oxidative damage reflect functional impairment.

On the other hand, Sohal and Orr (2012) highlighted how the oxidation of protein thiols causes dysfunction for various basic cellular activities and that the cellular redox state becomes increasingly more pro-oxidising in the latter part of life (e.g. increases in oxidised glutathione or protein mixed disulphides). Hence, the loss in reducing power (thiol groups) would be the main mechanism underlying senescence. This implies that oxidative damage specific to thiols rather than generic oxidative damage would drive cell senescence.

This apparent ambiguity in the relationship between oxidative damage and lifespan is also likely the result of dissociation between ageing and healthy ageing (Salmon et al. 2010). Oxidative damage appeared to promote the emergence of diseases and so to reduce the health span (period of life free of diseases) and to have little effect on lifespan (Salmon et al. 2010).

10.3 Evolutionary Hypotheses of Ageing: Antagonistic Pleiotropy and Disposable Soma

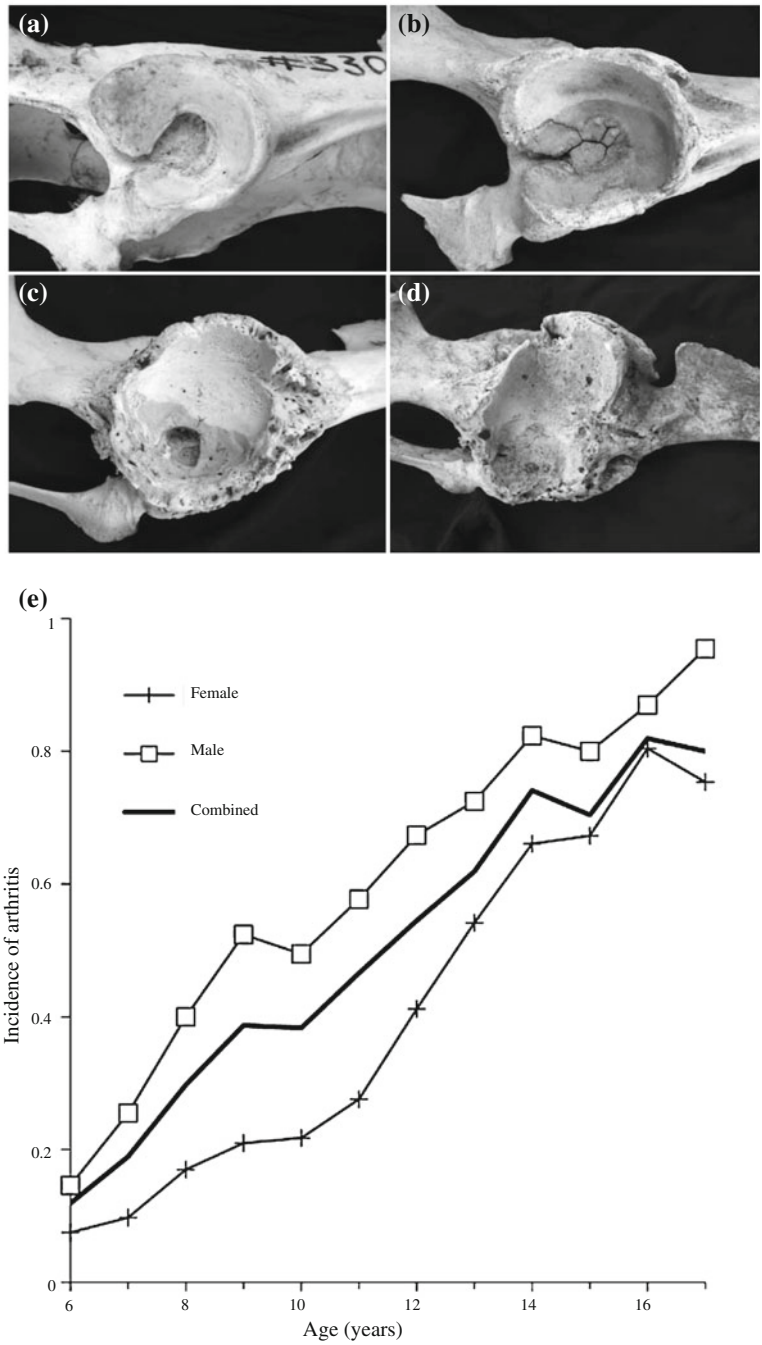
It was likely the German biologist August Weissman who was the first to examine why senescence evolved. His idea was that evolving senescence is beneficial for populations because it removes old and unproductive individuals (Wiessman 1889), but this did not provide any mechanism by which senescence should achieve such goal. Today, we have two main, albeit connected, hypotheses for the evolution of senescence that provide a theoretical framework under which their biological relevance can be tested.

The *antagonistic pleiotropy hypothesis of ageing* posits that alleles with beneficial effects on survival or reproduction early in life can be favoured by natural selection even when they are detrimental in old individuals (Williams 1957). Hence, natural selection would favour the evolution of life histories in which senescence has a detectable fitness cost within the natural life expectancies of organisms, as long as the genes responsible for such a cost confer fitness benefits in early life (Nussey et al. 2013). The cost of senescence would be low because it would emerge at a phase of lifetime (old age) when the force of natural selection is expected to be low. This hypothesis was derived from the Medawar's *hypothesis of mutation accumulation* (1952).

Predictions of the *disposable some hypothesis of ageing* (Kirkwood 1977; Kirkwood and Rose 1991) are quite similar to the antagonistic pleiotropy hypothesis, but rely on the phenotype rather than on the genotype. It predicts that, while natural selection optimises trade-offs in the allocation of limited resources among self-maintenance and other activities, they inevitably come at a cost for the soma. Given that the force of selection decreases with age, natural selection would favour higher investment of resources in early reproduction and costs for the soma would emerge in the long-term. Accumulation of damage (cost) would then cause senescence.

The environmental conditions are very important for the disposable soma hypothesis (but also for the antagonistic pleiotropy) because they may make the demands for resources of different body functions more or less conflicting. The risk of predation is, for example, very important. Although protection against oxidative damage is needed as exemplified by experiments in transgenic mice, natural selection would not favour high investment in antioxidant protection in an environment where the rate of extrinsic mortality was high. There is in fact no reason to invest resources in a long-lasting soma if chances of being predated are very high. In a seminal article, Austad (1993) showed that female Virginia opossums (*Didelphis virginiana*) from a population having four to five thousand years of history of reduced exposure to predators had retarded senescence compared to opossums from a population exposed to predators. Senescence might also be more rapid for one of the two sexes if it were more exposed to extrinsic mortality risks (Williams 1957). Among-species variation in lifespan may also be dependent on the evolution of protective mechanisms against predators. In a comparative study of 1,193 species of chemically protected and non-chemically protected fish, snakes and amphibians, Blanco and Sherman (2005) found that, independent of body size, species with longer maximum lifespans were usually those equipped with anti-predator chemical defences.

Implicit in both the antagonistic pleiotropy and the disposable soma hypotheses of ageing is a connection between the rate of senescence and life history traits. Various comparative analyses of senescence have been carried out so far, and although different estimates of senescence were used, they provided support for life history connections to senescence (e.g. Møller 2006; Munshi-South and Wilkinson 2006; de Magalhães et al. 2007; Jones et al. 2008; Ricklefs 2010; Wasser and Sherman 2010). For example, Jones et al. (2008) examined senescence rates in annual individual fitness using a dataset of 19 terrestrial vertebrate species. They found that the onset and rate of senescence in reproduction and survival are tightly associated with generation time, so that senescence is embodied in the life history strategy of a given species. Overall, data showed that mammals senesce faster than birds because they have a faster life history for a given body size; however, for a given pace of life, birds and mammals senescence at similar rates (Jones et al. 2008). Using a database of 426 terrestrial vertebrate species, Ricklefs (2010) showed that the actuarial senescence (i.e. the rate of increase in adult mortality) is higher in those species that become sexually mature earlier. Ricklefs (2010) argued that age at maturity might reflect the extrinsic mortality, which, in


(a)
#330
(b)
(c)
(d)
(e)
Female
Male
Combined
Incidence of arthritis
0
0.2
0.4
0.6
0.8
1
6
8
10
12
14
16
Age (years)

◀ **Fig. 10.2** Right pelvic (coxofemoral) joints of moose (*A. alces*), illustrating the progressive bony deterioration associated with osteoarthritis. **a** Normal joint capsule with open acetabular fossa, which contains the ligament of the head of the femur **b** yields to closure of the acetabular fossa, cartilage deterioration and dorsal eburnation (bone-on-bone articulation) **c** then dorsal displacement of joint articulation from biomechanical weight-bearing forces and finally **d** continued displacement as a prelude to dislocation. **e** Incidence of osteoarthritis in relation to sex and age of moose. Data were collected from 1959 to 2008. Reproduced with minimal alternations from Peterson et al. (2010) with permission of Wiley

turn, influences selection on mechanisms that postpone physiological and actuarial senescence. Møller (2006) and de Magalhães et al. (2007) obtained similar results to those of Ricklefs (2010) using the maximum reported lifespans, respectively, adjusted or not for sampling effort rather than the actuarial rate of ageing.

Longitudinal field studies are now also providing robust support for the expectations of both hypotheses, i.e. that senescence does occur in natural animal populations (Monaghan et al. 2008; Nussey et al. 2008, 2013). For example, studies of female red deer *Cervus elaphus* (Nussey et al. 2006), common guillemot *Uria aalge* (Reed et al. 2008) and great tits *Parus major* (Bouwhuis et al. 2010) have found that high fecundity or reproductive performance in early adulthood was associated with a more rapid decline in reproductive performance later in life. Also, American red squirrels *Tamiasciurus hudsonicus* that started reproducing earlier in life had shorter life expectancies (Descamps et al. 2006). Various authors have also found evidence showing that poor conditions in early life may exacerbate the rate and onset of decline in survival and reproductive performance (Nussey et al. 2007; Reed et al. 2008). Importantly, Peterson et al. (2010) provided evidence for a link between early-life experiences and the emergence of a degenerative disease during senescence in a wild mammalian species. Moose (*Alces alces*) that experienced poorer nutritional conditions in early life were more likely to develop osteoarthritis and also had reduced life expectancies (Fig. 10.2). Moreover, moose with osteoarthritis were more likely preyed on by wolves (*Canis lupus*), suggesting a potential connection between senescence of prey and the population ecology of predator–prey systems (Peterson et al. 2010).

10.4 Antioxidant Mechanisms and Longevity in a Comparative Framework

10.4.1 Correlative Evidence

Much research has examined the relationship between some aspects of antioxidant protection and among-species variation in maximum lifespan (Table 10.1 in Pamplona and Costantini 2011). Many studies tend to converge to the conclusion that long-lived species are those that have lower reactive species production (Fig. 10.3) (Herrero and Barja 1997b, 1998; Brunet-Rossinni 2004; Lambert et al.

Table 10.1 Summary of studies examining the relationships between parameters of oxidative damage or antioxidant protection and survival in free-living birds

Species	Age	Tissue	Biomarker	Effect	Study
Pygoscelis adeliae	Adult	Plasma	OD—hydroperoxides	No, there was no difference between birds that returned to the colony and those that did not	Beaulieu et al. (2011)
Pygoscelis adeliae	Adult	Plasma	ANT—non-enzymatic antioxidant capacity	No, there was no difference between birds that returned to the colony and those that did not	Beaulieu et al. (2011)
Phalacrocorax aristotelis	Nestling/ Adult	Plasma	OD—Malondialdehyde	Yes, lower in nestlings that had higher recruitment probability as adults	Noguera et al. (2011)
Acrocephalus sechellensis	Adult	Plasma	OD—hydroperoxides	No, it did not predict the probability of an individual being alive in the next year	van de Crommenacker (2011)
Acrocephalus sechellensis	Adult	Plasma	ANT—non-enzymatic antioxidant capacity	No, it did not predict the probability of an individual being alive in the next year	van de Crommenacker (2011)
Geothlypis trichas	Adult	Red blood cells	OD—DNA damage	Yes, lower in males that returned the next year	Freeman-Gallant et al. (2011)
Hirundo rustica	Adult	Plasma	ANT—non-enzymatic antioxidant capacity	Yes, high levels positively predicted long-term survival	Saino et al. (2011)
Apus melba	Adult	Whole blood	ANT—resistance of red blood cells to haemolysis induced by an in vitro exposure to a pro-oxidant molecule	Yes, higher in males, but not in females, that returned the next year	Bize et al. (2008)
Parus major	Nestling/ Adult	Red blood cells	ANT—glutathione peroxidase	Yes, higher survival to first winter and recruitment of nestlings with intermediate levels	Norte et al. (2008)
Parus major	Nestling	Red blood cells	ANT—glutathione peroxidase	Yes, lower in nestlings that successfully fledged	Koivula et al. (2011)

(continued)

Table 10.1 (continued)

Species	Age	Tissue	Biomarker	Effect	Study
Parus major	Nestling	Red blood cells	ANT—glutathione reductase	No	Koivula et al. (2011)
Parus major	Nestling	Red blood cells	ANT—glutathione-S-transferase	No	Koivula et al. (2011)
Parus major	Nestling	Red blood cells	ANT—catalase	No	Koivula et al. (2011)
Parus major	Nestling	Red blood cells	ANT—superoxide dismutase	No	Koivula et al. (2011)
Parus major	Nestling/ Adult	Plasma	OD—hydroperoxides	No, it did not predict post-fledging survival	De Coster et al. (2012)
Parus major	Nestling/ Adult	Plasma	ANT—non-enzymatic antioxidant capacity	No, it did not predict post-fledging survival	De Coster et al. (2012)
Parus major	Nestling	Whole blood	ANT—resistance of red blood cells to haemolysis induced by an in vitro exposure to a pro-oxidant molecule	Yes, higher in nestlings that successfully fledged	Losdat et al. (2013)
Parus major	Nestling	Whole blood	ANT—resistance of red blood cells to haemolysis induced by an in vitro exposure to a pro-oxidant molecule	No, it did not predict local recruitment	Losdat et al. (2013)
Sturnus vulgaris	Nestling	Plasma	OD—hydroperoxides	Yes, lower in birds that successfully fledged	Costantini (unpublished data)
Sturnus vulgaris	Nestling	Plasma	ANT—non-enzymatic antioxidant capacity	No	Costantini (unpublished data)
Sturnus vulgaris	Nestling	Plasma	ANT—thiols	No	Costantini (unpublished data)

All biochemical analyses were carried out on whole blood or its components plasma and red blood cells, separately. *OD* oxidative damage; *ANT* antioxidant

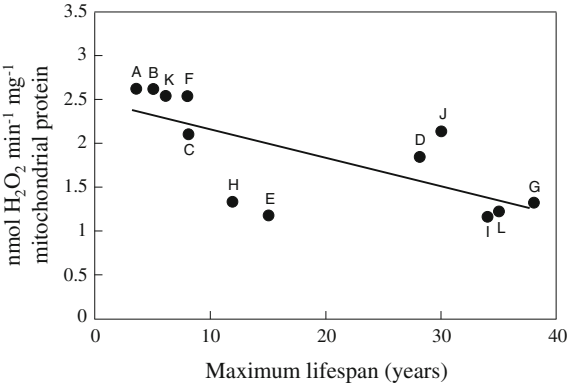


Fig. 10.3 Negative relationship between hydrogen peroxide production by heart mitochondria and maximum lifespan in various species of birds and mammals. $R^2 = 0.54$, $P < 0.007$; A. mice (*Mus musculus*), B. rat (*Rattus norvegicus*), C. white-footed mouse (*Peromyscus leucopus*), D. naked mole rat (*Heterocephalus glaber*), E. Damara mole-rat (*Crypomis damarensis*), F. guinea pig (*Cavia porcellus*), G. baboon (*Papio cynocephalus*), H. Brazilian free-tailed bat (*Tadarida brasiliensis*), I. little brown bat (*Myotis lucifugus*), J. ox (*Bos taurus*), K. Japanese quail (*Coturnix japonica*), and L. domestic pigeon (*Columba livia*). Reproduced with minimal alternations from Buttemer et al. (2010), redrawn from Lambert et al. (2007), with permission of Wiley

2007; Sasaki et al. 2008), have lower concentrations of endogenous antioxidants (e.g. Lopez-Torres et al. 1993; Perez-Campo et al. 1994) or have molecules in cell structures that are more resistant to damage than those of short-lived species (Hulbert et al. 2007; Pamplona and Costantini 2011). However, some of these studies suffer from the fact that are based on a limited number of species, are of correlative nature and did not control for body mass or phylogenetic relatedness among species. Related species share an evolutionary history; hence, they are not independent from each other. This lack of independence generates pseudoreplication and inflation of degrees of freedom. These issues might explain why there are apparently important exceptions. For example, the olm (*Proteus anguinus*), a small cave salamander, has evolved an extreme life history strategy with a predicted maximum lifespan of over 100 years, an adult average lifespan of 68.5 years and an age at sexual maturity of 15.6 years (Voituron et al. 2011). Neither its basal metabolism nor oxidative damage level and activities of antioxidant enzymes explain why this species sits as an outlier in the relationship between amphibian size and longevity (Issartel et al. 2009; Voituron et al. 2011).

Resistance of mitochondrial DNA to oxidative damage has been suggested to be a critical determinant of lifespan. Nucleotide sequence may therefore be expected to be a target of selection because of the differing sensitivities of nucleotides to oxidation (e.g. guanine is most easily oxidised, Bjelland and Seeberg 2003). Support for this scenario came from a comparative study (76 mammalian species, 14 bird species and 4 invertebrate species) analysing the relationships among lifespan, proportion of the differing nucleotides in one strand

of the mitochondrial DNA molecule, and the free energy (Samuels 2005). The free energy is a measure of the binding energy between the two DNA strands, which is affected by the nucleotide sequence; the more negative the free energy is, the less likely is the spontaneous separation of the two strands through thermal fluctuations (Samuels 2005). Short-lived species had higher abundances of adenine and thymine and lower abundance of cytosine than long-lived species, while the abundance of guanine tended to be low across all species. The free energy was lower in long- than in short-lived species, and this likely conferred to long-lived species a DNA less susceptible to mutations because a low free energy would limit the opening of the double strands of mitochondrial DNA and so their exposure to reactive species (Samuels 2005). This study, however, did not control for phylogeny.

Nabholz et al. (2008) provided some evidence for a relationship between mutation rate of mitochondrial DNA and maximum lifespan. They examined the variation in lineage-specific mitochondrial mutation rate (cytochrome *b* sequence) across 1,696 mammalian species and they sought to uncover the reasons for the among-species variation in mitochondrial DNA substitution rate. They found that rates of mutation of mitochondrial DNA were lower in long-lived species and this association held significant with or without control for phylogeny (Nabholz et al. 2008). Rodentia was the fastest evolving order (0.176 substitution/site/million years) and its rate of mutation was 20 times faster than Cetacea, which was the slowest evolving order. Interestingly, there was also high within-order variation, especially in orders with short-lived species. For example, Rodentia included very fast (e.g. Muridae) and very slow evolving (e.g. Bathyergidae) families. Results of this study suggested that long-lived species tend to have mitochondrial DNA more resistant to damage (hence less susceptible to mutation) although this does not prove a cause–effect relationship.

Mechanisms that prevent mutation may be many (e.g. antioxidant enzymes, repair systems, lipid composition) and the most important can be expected to be under stronger selective pressure in long- than in short-lived species (Jobson et al. 2010). In order to identify genes that can slow down ageing, Jobson et al. (2010) compared the evolution of non-synonymous (alter the amino acid sequence of a protein) and synonymous (do not alter the amino acid sequence of a protein) substitutions of around 5.7 million codon sites across 25 mammalian species. Genes involved in regulation of lipid composition and vitamin C binding have undergone increased selective pressure in long-lived species, while genes involved in DNA replication/repair or antioxidant activity (e.g. antioxidant enzymes, uncoupling proteins) have not (Jobson et al. 2010). Of the vitamin C-binding genes examined, most were hydroxylation enzymes that selectively utilise vitamin C for biosynthesis and stabilisation of collagen's triple helix structure (Jobson et al. 2010). Given the importance of collagen for the soma, low selective pressure on vitamin C-binding genes in short-lived species is in agreement with the expectation that these species have been selected to prioritise investment in reproduction at a cost for self-maintenance.

Oxidative stress also appears to be an important selective agent in bacteria. Torres-Barceló et al. (2013) found that resistance to oxidative stress correlated positively with the mutation rate in the absence of stress, suggesting the existence of direct benefit of mutator alleles in the pathogenic bacterium *Pseudomonas aeruginosa*. The increase in resistance was attributable to elevated levels of catalase secretion, at least by the *mutS* mutator strain. Exposure to hydrogen peroxide caused an increase in mutation rate in the wild-type strain, but mutators did not display stress-induced increases in mutation rate as a result of their intrinsic resistance to hydrogen peroxide (Torres-Barceló et al. 2013). Direct competition experiments between mutators and wild-type confirmed the nature of "public good" of the protective enzymes, as both strains shared the benefits of having high catalase levels when exposed to the stress. Hence, exposure to a pro-oxidant molecule has the potential to generate direct selection for an elevated mutation rate in *P. aeruginosa* (Torres-Barceló et al. 2013).

Expression of heat shock proteins also appears to be associated with lifespan. Measures of constitutive levels of these proteins in brain, heart and liver of 12 mammalian and one avian species ranging in maximum lifespan from 3 to 30 years showed that their expression was highly correlated with maximum lifespan (while controlling for body mass and phylogeny), indicating that higher basal levels of heat shock protein expression are characteristic of longer-lived species (Salway et al. 2011).

Protein composition is also very important because amino acids differ in their propensity to oxidation, with cysteine and methionine being the most sensitive to oxidative damage (Chap. 1). Resistance of proteins to oxidative damage has been proposed to be an important mechanism explaining the surprising long lifespan of naked mole rats (Pérez et al. 2009) and bats (Salmon et al. 2009). Moosmann and Behl (2008) compared the genome sequences across 218 animal species, including 95 mammals, 14 birds, 10 reptiles, 12 amphibians, 39 fish and 48 invertebrates. The analysis revealed that long-lived species synthesise respiratory chain complexes that are depleted of mitochondrial cysteine. The relationship between maximum lifespan and mitochondrial cysteine depletion was also significant after correction for body mass and phylogeny. In an additional analysis, Moosmann and Behl (2008) found that aerobic species had a decreased content in genomic cysteine as compared to anaerobic species and the significant relationship between maximum lifespan and cysteine in anaerobic species (e.g. some nematodes, parasitic platyhelminthes) did not emerge. Interestingly, in nematodes with a biphasic aerobic–anaerobic lifestyle, cysteine appeared to predict the duration of the aerobic phase of life. Overall, these results suggested that there might have been a selection against cysteine presence in mitochondrial DNA-encoded proteins of long-lived species because cysteine thiyl radicals would promote irreversible protein cross-linking. To do so natural selection might have increased the amino acid substitution rates in mitochondrially encoded proteins (e.g. cytochrome b, which influences the rate of reactive species generation), which were found to be higher in long-lived mammalian species than in short-lived ones (Rottenberg 2006, 2007a) or in long-lived songbirds than in short-lived ones (but not in other avian

taxa; Rottenberg 2007b). On the other hand, selection switched to an anaerobic metabolism those longed-lived species that maintained high cysteine levels. In contrast to Moosmann and Behl (2008), a later analysis of mitochondrial genomes across 168 mammalian species also found a negative correlation between longevity and cysteine usage, but, while it was significant without control for phylogeny, it was not when phylogeny was taken into account (Aledo et al. 2011).

As regards with methionine, Aledo et al. (2011) tested the hypothesis that the usage of methionine could also influence lifespan. They examined the mitochondrial genomes of 168 mammalian species, representing 24 different taxonomical orders. Mitochondrial-encoded proteins from short-lived species resulted to contain higher abundances of methionine than long-lived species, and this result held after control for body mass variation and phylogeny. However, the relationship between longevity and methionine was not significant across all orders. Results also suggested that it was the addition of methionine to proteins in short-lived species rather than the loss of methionine from proteins in long-lived species to explain the higher abundance of methionine in species that liver shorter (Aledo et al. 2011). Importantly, while nuclear-encoded mitochondrial proteins are richer in methionine, a correlation between longevity and usage of methionine occurred only for those proteins located in the inner mitochondrial membrane, that is where most reactive species are generated. These results were interpreted as supportive for an antioxidant role of methionine in short-lived mammals: species that live shorter suffer more oxidative stress; hence, they selectively accumulate methionine to protect mitochondria. This hypothesis is based on the evidence showing that oxidised methionine can be reduced back by methionine sulfoxide reductases (Stadtman et al. 2002; Bender et al. 2008). However, an opposite interpretation may also be reasonable: mammals that live shorter do so because the high accumulation of methionine makes them to suffer more oxidative stress. If this were the case, the next question would be what adaptive advantages short-lived species may have gained from using more methionine than long-lived species. One reason might be that methionine helped to sustain the high reproductive activity typical of short-lived species with a high pace of life. For example, supplementation of methionine enhanced population density, recruitment and reproductive activity in wild hispid cotton rats *Sigmodon hispidus* (Webb et al. 2005).

Finally, we cannot rule out the hypothesis that repair of oxidised methionine might have energetic costs that are paid to some degree in terms of reduced longevity. Experimental tests of these hypotheses on a large and diverse range of species are certainly needed. This is actually a general need of comparative studies of ageing mechanisms. For example, invertebrates have been subject of a limited number of studies, which is surprising given their taxonomic richness, variation in life histories and biomass. Although a general relationship among body size, metabolism and longevity does not appear to occur in marine invertebrates, they are generally characterised by low rates of metabolism and reactive oxygen species formation associated with lower antioxidant enzyme activities compared with vertebrates (Buttemer et al. 2010; Philipp et al. 2012). Despite the high phenotypic diversity and enormous genetic distance among invertebrate clades, the oxidative

stress theory of ageing is generally applicable to invertebrates (e.g. Philipp et al. 2006; Abele et al. 2009; Begum et al. 2009). For example, the bivalve *Artica islandica* is the longest-living non-colonial organism; it can live over 500 years of age (Butler et al. 2013). Mitochondria of *A. islandica* produced significantly less hydrogen peroxide than those of the two short-lived species *Mya arenaria* (maximum longevity = 28 years) and *Spisula solidissima* (maximum longevity = 37 years) under various conditions of mitochondrial respiration (Munro et al. 2013). Similarly, studies on sea urchins suggested that negligible senescence is accompanied by a lack of accumulation of cellular oxidative damage with age and that maintenance of antioxidant capacity and proteasome enzyme activities may be important mechanisms to mitigate damage (Du et al. 2013).

A major caveat of most comparative studies is that measures of mean or maximum lifespan include both the pre- and post-reproductive phases. Various studies showed that the post-reproductive lifespan may be pronounced (Dagg 2009). For example, guppies (*Poecilia reticulata*) from high-predation environments live longer than guppies from low-predation environments because they have a longer reproductive lifespan, which is the component of the life history that makes a direct contribution to individual fitness (Reznick et al. 2006). There were no differences among populations in post-reproductive lifespan, which is as predicted since there can be no contribution of this segment of the life history to an individual's evolutionary fitness (Reznick et al. 2006). In a study on wild *Drosophila melanogaster*, females had a post-reproductive lifespan that on average made up 40 % of total lifespan (Klepsatel et al. 2013). Importantly, post-reproductive lifespan was not correlated with reproductive fitness components, supporting the hypothesis that this period is a highly variable, random "add-on" at the end of reproductive life rather than a correlate of selection on reproductive fitness (Reznick et al. 2006; Klepsatel et al. 2013). In fact, selection should favour the evolution of an extended post-reproductive lifespan only whether post-reproductive individuals contribute to their inclusive fitness in some way, either by caring for their own young, other kin, or grand-offspring, as for example appears to occur in primates or elephants (Dagg 2009). Findings of these studies also suggest that measures of total lifespan commonly used in comparative studies may be inflated with the addition of a period of life over which individuals (and hence their antioxidant mechanisms) are under a waning force of selection.

10.4.2 In Vitro Evidence

Studies on cell cultures provided contrasting evidence in favour of a role of oxidative stress as promoter of senescence. Harper et al. (2011) tested primary fibroblast cultures from 35 species of free-living birds for their resistance to multiple forms of cellular stress. They found that cell lines from longer-lived species were more resistant to death caused by cadmium, paraquat, hydrogen peroxide, methyl methanesulfonate and metabolic inhibition induced in

low-glucose medium than short-live species, and, overall, all avian fibroblasts were more resistant than rodent fibroblasts (Harper et al. 2011). Cell lines from longer-lived species also proliferated more rapidly than cells from short-lived species, although there was no correlation between proliferation and cell resistance. Importantly, results were largely consistent even after accounting for the influence of body mass and phylogeny (Harper et al. 2011). Taken together, these results supported the idea that resistance to oxidative stress influences longevity. This conclusion was in agreement with previous results obtained from Ogburn et al. (2001). It was also suggested that the extreme oxidative damage resistance of long-lived birds requires active gene transcription and translation (Ogburn et al. 2001).

Another study on avian fibroblasts concluded that maintenance of active quality control mechanisms is more important than production of reactive species in influencing the in vitro ageing process (Strecker et al. 2010). In vitro populations of cells derived from chickens and pigeons did not show any age-related increase in reactive species levels or accumulation of oxidative damage to mtDNA or proteins. Moreover, and in disagreement with other studies (Ogburn et al. 2001; Harper et al. 2011), fibroblasts from the long-lived pigeon were not more resistant to oxidative stress than fibroblasts from the short-lived chicken. These results were also in disagreement with previous studies on mammalian cells, which found a significant accumulation of oxidative damage molecules with age (Chaudhuri et al. 2006; Unterluggauer et al. 2007). Strecker et al. (2010) proposed that cells did not lose resistance to oxidative stress with age because the autophagosomal/lysosomal pathway was not downregulated in old avian cells and overexpression of autophagy proteins improved mitochondrial fitness and enhanced resistance against oxidative stress-induced apoptosis. Hence, autophagy might act as an important pathway regulating ageing in avian cells.

10.5 Does Oxidative Stress Level Predict Survival in Wild Animals?

We know little about whether oxidative stress impacts on survival in natural animal populations. The few studies published so far that looked at the relationship between survival and parameters of oxidative damage or antioxidant status were mostly limited to birds and were of correlative nature, which limit conclusions and generalisations. Studies examined the relationship between survival and oxidative stress in various ways, such as performing a comparison of biochemical measures between adults that returned the next breeding season and those that did not; nestlings that successfully fledge and those that did not; nestlings that after fledging recruited the next breeding season and those that did not recruit. Sample sizes are often low and estimates of survival also have some limits. For example, survival may vary with age and season; birds may disperse in other areas; birds

with high oxidative stress might be more susceptible to being predated, so any effects of oxidative stress on survival would not come through an effect on physiological senescence; adults may not adequately represent the all variation in resistance to oxidative stress because individuals with low resistance might have died early in life (selective disappearance). Moreover, all biochemical measures are limited to blood. Although the oxidative status of blood may reflect that of other tissues depending on the molecule measured (Argüelles et al. 2004; Veskoukis et al. 2009) and is sensitive to oxidative challenges (Isaksson 2010; Costantini et al. 2011), we also know that physiological ageing may vary among cells (Passos et al. 2007) and tissues (Herndon et al. 2002). Results have been therefore quite mixed (Table 10.1). While some studies found that birds with high oxidative damage levels did not survive, others did not find such a relationship. Differences also emerged when looking at the same parameter of oxidative damage. Data on antioxidant parameters are also unclear. For example, while Norte et al. (2008) found that nestling great tits (*P. major*) with low or high glutathione peroxidase activity did not recruit the next season, Koivula et al. (2011) found that nestling great tits with high activity of glutathione peroxidase were less likely to survive until fledging. That high glutathione peroxidase activity is associated with lower chances of survival might indicate that those birds needed to upregulate this enzyme to cope with higher production of early derivatives of oxidative damage (i.e. hydroperoxides). Nestling starlings with higher plasma hydroperoxides actually had lower probability to fledge (Costantini unpublished data; Table 10.1).

Using longitudinal data, Saino et al. (2011) showed that high levels of non-enzymatic antioxidant capacity of plasma positively predicted long-term survival in wild barn swallows (*Hirundo rustica*) while controlling for any confounding effects of individual age. The effect of antioxidants on viability was relatively strong, as an increase in one standard deviation in antioxidant capacity was positively associated to a ca. 20 % change in annual survival (Saino et al. 2011). Using the same antioxidant assay as that used for barn swallows, Beaulieu et al. (2013) found that Gentoo (*Pygoscelis papua*) and Adélie penguins (*Pygoscelis adeliae*) from increasing populations had higher plasma non-enzymatic antioxidant capacity than penguins from decreasing populations and this was true when considering each penguin colony independently or irrespective of species, location and levels of plasma oxidative damage. Other studies that used the same antioxidant assay as that used in previous studies did not, however, find any associations between antioxidant status and survival (e.g. Beaulieu et al. 2011; van de Crommenacker 2011). In contrast, a study on wild-caught spotted snow skinks (*Niveoscincus ocellatus*) found females with high circulating non-enzymatic antioxidants showed a decreased probability of survival to the following season (Isaksson et al. 2011).

10.6 Hormesis Promotes Longevity

Experiencing short episodes of mild stress (e.g. radiation, caloric restriction, thermal stress, physical effort) at some point in life has been repeatedly found to increase longevity in various species of invertebrates and vertebrates (Rattan 2008; see also Chap. 2). For example, mice (*M. musculus*) exposed to very low doses of radiation in early life lived longer than controls (Caratero et al. 1998). Similarly, exposure of nematodes (*Caenorhabditis elegans*) in early life to heat stress, hyperbaric oxygen or juglone (a chemical that generates reactive species) significantly increased subsequent resistance to the same challenge, resulting in a longer lifespan (Cypser and Johnson 2002). As with radiation exposure, research on various insect species demonstrated that lifespan extension was stimulated following exposure to levels of ionising radiation higher than those usually experienced in the wild (Calabrese 2013).

Of the various stressors that were shown to induce hormetic responses, heat stress has been one of the most used so far. The reason for this focus on temperature is not only with the ease of implementing heat stress protocols, but also because high temperatures activate evolutionarily highly conserved stress response pathways, such as the heat shock protein response (Rattan 2008). From an ecological viewpoint, it is also a naturally occurring cause of stress as many organisms are exposed to episodes of high environmental temperatures from which they cannot escape. A recent meta-analysis reviewed all the literature on the life extension promoted by thermal hormesis (Lagisz et al. 2013). The analysis included data on twelve species from nine genera of invertebrates: *Drosophila*, *Bicyclus*, *Helicoverpa*, *Trichogramma*, *Aphidus*, *Ophraella*, *Panstrongylus*, *Caenorhabditis* and *Saccharomyces*. The main findings of the study were that the 12 invertebrate species responded to heat shock in a similar way; heat shock temperature had the largest influence on experimental outcomes, with higher temperatures decreasing the likelihood of any life extension; longer durations of heat shock decreased the chance of a hormetic response; more heat shock repeats decreased the likelihood of life extension; longer periods of recovery between repeated heat shocks were found to increase the likelihood of life extension; hormetic effects on longevity were stronger in young than adults. Results of the meta-analysis confirmed the importance of individual age at which the mild stress is experienced and of the strength/duration of heat stress as determinants of life extension promotion typical of hormesis.

A recent study suggested that, for early-life hormetic phenotypic adjustments to be beneficial, the early environment needs to be predictive to some extent of the adult environment (Costantini et al. 2014) (Fig. 10.4). Environmentally primed stress resistance in early life may therefore carry survival costs if the subsequent environment does not match that to which the adjustment was made (Costantini et al. 2014). This may explain both why the stress response system is not maintained in an upregulated state and why the hormetic response has evolved.

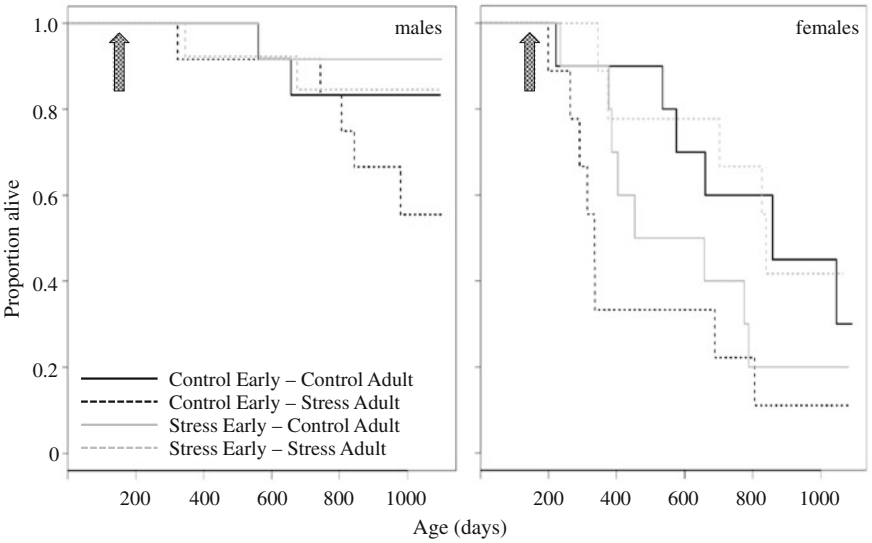


Fig. 10.4 Survival up to three years of age of control zebra finches or those exposed to mild heat stress before sexual maturity in relation to their treatment in adulthood. The effects on survival of hormetic priming to mild heat stress in early life were contingent on whether heat stress was encountered again in adulthood. The timing of the adult temperature challenge is shown as an arrow. Reproduced from Costantini et al. (2014)

10.7 Sex Differences in Lifespan, Ageing and Hormesis

There are various mechanistic explanations of why males and females differ in lifespan. For example, the effects of recessive deleterious mutations occurring on X or Z (depending on the species) chromosome are not counterbalanced by alleles on the second chromosome in the heterogametic sex, resulting in increased mortality (Trivers 1985; Tower 2006). A more evolutionary view of the sexual dimorphism in lifespan and ageing predicts that sexes resolve the trade-off between reproduction and survival differently resulting in sex-specific optima for lifespan (Maklakov and Lummaa 2013). Several empirical studies provided support for sex-specific selection as a force shaping the evolution of lifespan and ageing (Carranza et al. 2004; Clutton-Brock and Isvaran 2007; Maklakov et al. 2007; Nussey et al. 2009). This is not totally surprising because males and females differ in life history strategies. Males and females also differ in many important physiological systems that may speed up or slow down cell senescence. For example, some studies on birds or mammals showed that females tend to have lower levels of oxidative damage and higher levels of antioxidants (Casagrande et al. 2011; Viña et al. 2011). Studies on invertebrates also showed that females may suffer less oxidative stress than males. For example, once the sexual maturity is reached, females of the tarantula *Brachypelma albopilosa* may live until 20 years, while males have a life expectancy of

1–2 years (Criscuolo et al. 2010). Compared to males, females had lower production of reactive species and oxidative damage and higher levels of non-enzymatic antioxidants (Criscuolo et al. 2010). In contrast, studies on alpine swifts (*A. melba*), Galápagos land iguanas (*Conolophus subcristatus*), pigeons (*Columba livia*), barn swallows (*Hirundo rustica*), rats (*Rattus norvegicus*) or decorated crickets (*Gryllodes sigillatus*) showed that females have higher oxidative stress than males (Bize et al. 2008; Costantini et al. 2009; Kamper et al. 2009; Costantini 2010; Rubolini et al. 2012; Archer et al. 2013). These studies also showed that sexual dimorphism in oxidative balance is not consistent throughout time, but it depends on the stage of life cycle or life history of the species.

Males and females also differ in the hormesis of longevity. The way sexes resolve the trade-off between longevity and reproduction might explain why beneficial effects of hormesis on longevity tend to be more common in males than in females (Salmon et al. 2001; Le Bourg 2005; Sørensen et al. 2007).

10.8 Univariate and Multivariate Systems in the Study of Ageing

Research on senescence has provided considerable insight into the mechanisms underlying the decrease in genetic and physiological performance with age. Although some mechanisms may certainly be more important than others in driving the process of ageing, we should recognise that ageing is the result of the interaction of multiple mechanisms. In 1996, Kowald and Kirkwood have called for a synthesis of formerly distinct hypotheses into a *network theory of ageing*. It states that it is the collective set of maintenance processes that together determines the organism's ability to preserve homeostasis by protecting against the many endogeneous and exogeneous agents that can cause damage (Kowald and Kirkwood 1996). It integrates the contribution of, for example, defective mitochondria, aberrant proteins, free radicals and antioxidant enzymes. Importantly, the explanatory and predictive power of this theory is potentially much higher than that of individual hypotheses of ageing as shown by model simulations. Using a multivariate approach, Cohen et al. (2013) showed that simultaneous dysregulation in multiple physiological systems is associated with ageing and that abnormal physiological states are associated with increasing risk of mortality. Predictive power of models also increased as more variables were incorporated into the calculation (Cohen et al. 2013). Although these results did not clarify cause–effect relationships, they highlighted the importance of the predictive power of multivariate systems, which would make them preferable to using univariate approaches.

Multivariate approaches and network analyses are already a reality in gene expression analyses. For example, Brink et al. (2009) used microarrays to investigate the age-dependence of transcriptional changes of genes in the insulin signalling, oxidative phosphorylation and glutathione metabolism pathways in mice. Results showed that age- and tissue-specific patterns of transcriptional

changes are consistent with the metabolic stability–longevity principle, which states that it is the ability of cells to maintain stable concentrations of reactive oxygen species and other critical metabolites to be the prime determinant of lifespan. This study also suggested that it is not the rate of production of reactive species that determines the rate of ageing, but the dynamic stability of concentration of reactive species and of other metabolites that regulate normal cell functions (Brink et al. 2009). Further studies on gene and metabolic networks suggested that these systems can become less integrated as the individual ages, so becoming noisier and less stable because of a decrease in the effectiveness of communication among functional units (Csermely and Soti 2006; Xue et al. 2007; Southworth et al. 2009; Soltow et al. 2010). These results strengthen the importance of moving from a reductionist to a more holistic view of the ageing process.

10.9 Conclusions

Empirical evidence is often supportive of the prediction that long-lived species produce less reactive species, have lower oxidative damage levels and antioxidants, and have molecules more resistant to oxidative damage (Hulbert et al. 2007; Buttemer et al. 2010; Pamplona and Costantini 2011). Much of this evidence is, however, based on comparative studies limited to a few species and that often lack of control of body mass or phylogeny. Moreover, estimates of lifespan used in comparative studies also include the post-reproductive lifespan, a period over which forces of selection are relaxed (Reznick et al. 2006; Klepsatel et al. 2013), and may not adequately reflect the ageing rate, since lifespan depends on both ageing rate and intrinsic vulnerability (Kowald 2002). Maximum lifespan is routinely used as an estimate of longevity because it is supposed to be proportional to genetic limitations on longevity potential and it might reflect the species' rate of physiological ageing (Finch and Pike 1996; de Magalhães et al. 2005). However, it might also be influenced by mortality rates, which are independent from ageing-related mortality rates. It is also influenced by the sample size: the bigger it is, the higher is the chance of having a very old individual. Another relevant limit of comparative studies is that they usually consider baseline values of oxidative status parameters, while studies using stress-induced values or testing resistance to stress are rare (Torres-Barceló et al. 2013).

Studies on transgenic animals have also limitations because they do not provide a realistic approach. Moreover, experimentally increasing tissue antioxidants through dietary supplementation, pharmacological induction or transgenic techniques moderately increased mean life expectancy but did not change the maximum lifespan (Sanz et al. 2006; Hulbert et al. 2007). Effects of antioxidant supplementation on longevity have also been shown to be extremely difficult to predict because they depend on complex interactions among antioxidants. Snell et al. (2012) showed that of 60 two-way combinations of selected antioxidants, only seven (12 %) produced significant life extension in the rotifer *Brachionus*

manjavacas, a species with a lifespan of 10–15 days. Moreover, supplementation of single antioxidants and none of the 20 three- and four-way antioxidant combinations yielded significant effects on lifespan (Snell et al. 2012). However, all of these manipulations of antioxidant status were limited to a few species (humans and mostly laboratory strains). Moreover, some of the species investigated so far are extremely short-lived, which suggests that they were selected for investing more in reproduction rather than in using antioxidants to extend their life. Evidence of increases or decreases in damage or antioxidant protection (e.g. Costantini et al. 2012; Guerra et al. 2012; Sosnowska et al. 2014) with age are also mostly based on cross-sectional studies, which may suffer from interference of phenotypic plasticity or selective disappearance. Therefore, we now need studies that (1) track the oxidative stress and life histories in natural animal populations over the individual lifetime; (2) assess the link among antioxidants, oxidative damage and senescence in wild strains under controlled laboratory conditions using experimental settings (e.g. common garden experiments) that are somehow seminatural (e.g. with food that is not provided ad libitum or is costly to obtain in order to face animals with resource-based trade-offs; various combinations of social competition); (3) apply techniques to control for phylogeny bearing in mind that different methods of phylogenetic reconstruction can yield conflicting phylogenies, some methods are inadequate because are based on a phenetic analysis and not on a phylogenetic one, and some phylogenies are unresolved and unstable (e.g. Cracraft 1987). It is also very important to test hypotheses under different environmental conditions because environmentally dependent effects may have significant ecological implications. For example, there may be high heterogeneity in the quality of the environment in which animals live, and there may also be seasonal and annual variation (see Chap. 9). In particular, the interaction between environmental quality and phase of the life cycle (e.g. reproduction, migration and hibernation) of the species may be relevant in setting up the specific needs for antioxidants and the magnitude of the oxidative challenges the individual is exposed to. For example, water temperature appears to be an important modulator of ageing in marine ectotherms, such as bivalves or molluscs; species that live at low water temperatures tend to live longer than species with similar life histories that live at higher temperatures (Philipp et al. 2012).

We also need studies that incorporate a wider range of taxa than that used so far, with a special attention to those species with indeterminate growth (e.g. fish and reptiles), very short (e.g. adult mayflies live from a few minutes to a few days) or long lives (e.g. Great Basin bristlecone pines, *Pinus longaeva*, live more than 4,500 years, Lanner and Connor 2001) and those that withstand environmental extremes (e.g. those species that live in abyssal, arid or polar areas). Species like the *Hydra vulgaris* might also offer a unique opportunity to study mechanisms (e.g. constant renewal of the tissues of body) that some species have evolved to escape senescence (Martinez 1998). Incorporation of a wider range of taxa than that used so far in comparative studies may for example be relevant for studies examining the relationship between reactive species production and lifespan. But these studies will also need to take into account various others important aspects.

There may be in fact among-species differences in free radical generation during energy production that might explain variation in longevity. However, the generation of reactive species also varies with metabolic substrate, and the among-species differences in reactive species generation may not be so marked when different cell types are used (Montgomery et al. 2011, 2012). Moreover, it may be a specific class of reactive species (e.g. those derived by lipids, Montgomery et al. 2011) rather than the overall production to impact on senescence. And this is not all. Mechanistic hypotheses of ageing are still proliferating (e.g. fading electricity in De Loof et al. 2013; autophagy in Lionaki et al. 2013) and the number of mechanisms advocated to be important is going to become out of control. There is clear need to develop new lines of research aimed to clarify the importance of each single mechanism and to merge them in multivariate and network analyses in order to have a more holistic approach to the study of ageing (e.g. Brink et al. 2009; Southworth et al. 2009; Cohen et al. 2013). Identification of bystander mechanisms that probably do not contribute much to loss of function is clearly a major challenge scientists need to face in the years to come. But this is really needed to put some order in the continuous paradigm shifts and controversies in the ageing research (Sanz et al. 2006; Salmon et al. 2010; Sohal and Orr 2012; Barja 2013; Gems and de la Guardia 2013; Gems and Partridge 2013).

Quantitative genetic research has revealed that there is significant and heritable variation in lifespan and hormesis of longevity (Rose and Charlesworth 1981; Tanaka 1993; Wang et al. 2006; Rodriguez et al. 2012; Reynolds and Phillips 2013). The number of studies is, however, very small. We are therefore in need of studies that quantify the natural genetic variation and heritability of longevity in order to assess whether longevity and hormesis are a target of natural selection.

Until we address these issues, caution has to be taken in making inferences on the senescence mechanisms and long-term effects of oxidative stress on survival in wild animals.

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