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REVIEW AND SYNTHESIS

Oxidative stress in ecology and evolution: lessons from avian studies

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Abstract

Although oxidative stress is a central topic in biochemical and medical research, the number of reports on its relevance in life-history studies of non-human animals is still low. Information about oxidative stress in wild birds may help describe functional interactions among the components of life-history traits. Currently available evidence suggests that oxidative stress may impart an important physiological cost on longevity, reproduction, immune response or intense physical activity. Given the gaps in our present knowledge, it is still premature to attempt to draw definitive conclusions and basic questions (e.g. how is oxidative stress generated and how do organisms cope with it?) have yet to be fully explored under natural conditions. Therefore, caution is needed in developing hypotheses or drawing general conclusions until additional data become available to perform more rigorous comparative analyses.

Keywords

Ageing, antioxidants, exercise, free radicals, life history, membrane unsaturation, metabolism, oxidative damage, reproduction, senescence.

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LIFE HISTORY, STRESS RESPONSE AND OXIDATIVE STRESS

Life histories of animal species may differ notably in fecundity (low vs. high), maturation rate (slow vs. fast), or extrinsic mortality (low vs. high) and how these traits are associated with longevity (e.g. live fast, die young vs. live slow, die old). Identification of the proximate causes underlying such variations and how these traits are associated with stress response is a pivotal topic in current ecology and evolutionary biology.

Stressful conditions are an important ecological and evolutionary force modulating adaptive responses of natural populations (Romero 2004; Korte *et al.* 2005). Indeed, individuals in a population are continually challenged with stressors of differing nature. On one hand, stress response is, *per se*, of adaptive nature as it allows the organism to cope with stressful challenges, no matter of what nature, and to maintain or restore body homeostasis. On the other hand, stress response may be inadequate, resulting in deleterious consequences for the health and for the biological fitness of the individual.

So far, there has been considerable research on the stress response mediated by glucocorticoid hormones release (Romero 2004). Alongside this work on stress hormones, notably fewer ecological studies have analysed another unequivocal component of stress and its links with life-history variation: oxidative stress.

The discovery that reactive oxygen species (ROS) can damage biological tissues (Gershman *et al.* 1954) and modulate ageing (Harman 1956) aroused great interest in the physiological bases of redox homeostasis, i.e. how organisms regulate the balance between pro-oxidants and antioxidants (e.g. Beckman & Ames 1998; Finkel & Holbrook 2000; Dröge 2002; Halliwell & Gutteridge 2007).

A disturbance in the balance between pro-oxidants and antioxidants in favour of the former, leading to damage, gives rise to oxidative stress (Sies 1991). Such damage is referred to as oxidative damage, i.e. damage to biomolecules caused by pro-oxidants (Halliwell & Gutteridge 2007). However, not all damage caused by oxidative stress is directly oxidative in nature. Secondary damage to biomolecules can also result from oxidative stress-related changes, such as in ion levels. In general, oxidative stress may result from (1) increased production of pro-oxidants; (2) diminished antioxidant levels; (3) depletion of essential dietary metal cofactors, such as selenium, magnesium or zinc, which

potentiate the activity of antioxidant enzymes; and (4) failure of repair or replacement systems.

In 1999, von Schantz *et al.* (1999) emphasized the importance of oxidative stress in sexual selection and, more generally, in animal ecology and evolution. Since then, few studies have addressed this issue in natural populations.

My intention in this review is to outline the link between oxidative stress and life-history traits. Examples from avian research are especially emphasized throughout the article, notably because birds have evolved unique molecular adaptations to protect themselves against oxidative stress (Fig. 1).

While previous reviews on oxidative stress have focussed mainly on cellular and molecular aspects (e.g. Beckman & Ames 1998; Finkel & Holbrook 2000; Dröge 2002), in this article, I have summarized studies on adaptations evolved by animal species (birds, in particular) to cope with oxidative stress and studies on the nexus between oxidative stress and life history. In addition, some basic concepts of oxidative stress physiology are presented in Boxes 1 and 2.

ANTIOXIDANTS AND OXIDATIVE STRESS IN A LIFE-HISTORY PERSPECTIVE

Quantification of free radical production, oxidative damage and antioxidant capacity can provide new insights into understanding interspecific variation in styles of life history. However, we need a better basic understanding before any comparative questions can be adequately answered. Oxidative stress differences between sexes or populations of the same species, and changes in the oxidative status of an individual across the phases of its life cycle are just some points needing more intense investigation.

It is still not clear how and to what extent life history and oxidative stress are associated. However, life-history variation seems to be associated with antioxidant parameters, some of which are highly conserved across animal groups.

In birds, the serum or plasma antioxidant capacity shows large differences between species (Corsolini *et al.* 2001; Cohen *et al.* 2007). Two studies showed that variation in the

The "bird" paradox

	Mammals	Birds
• Ageing rate	Faster	Slower
• Rate metabolic rate	Lower	Higher
• Lifetime energy expenditure	Lower	Higher
• Body temperature	Lower	Higher
• Blood glucose level	Lower	Higher
• Ratio between oxygen radical production and oxygen consumption	Higher	Lower
• Oxidative damage	Higher	Lower
• Cell membrane unsaturation	Higher	Lower
• Life history for a given body size	Faster	Slower

Figure 1 Birds are strikingly long-lived compared to their mammalian counterparts, living up to three times longer than mammals of equivalent body mass. The slow ageing rates typical of birds are paradoxical, given their high metabolic rate (double that of mammals, with lifetime energy expenditures up to 15 times higher than mammals), body temperature (*c.* 3 °C higher than mammals) and blood glucose levels (two- to fourfold higher than mammals), which should accelerate tissue damage and physiological senescence. Possible explanations for the higher longevity of birds compared to their mammalian counterparts are that birds have (1) a lower ratio of oxygen radical production and oxygen consumption, (2) an exceptional cellular resistance to oxidative stress, (3) a lower mitochondrial and nuclear DNA oxidative damage, (4) a lower cell membrane unsaturation and (5) a slower life history for a given body size (Ku & Sohal 1993; Barja *et al.* 1994; Pamplona *et al.* 1996, 2005; Herrero & Barja 1997b, 1998, 1999; Pérez-Campo *et al.* 1998; Ogburn *et al.* 2001; Samuels 2005; Hulbert *et al.* 2007; Lambert *et al.* 2007; Pamplona & Barja 2007; Vleck *et al.* 2007; Jones *et al.* 2008).

BOX 1

A free radical is any biochemical species capable of independent existence that contains one or more unpaired electrons, which make free radicals very unstable (half-life at 37 °C: superoxide $O_2^{\bullet -}$, 1×10^{-6} s; hydroxyl $^{\bullet}OH$, 1×10^{-9} s). As electrons have a very strong tendency to exist in a paired rather than an unpaired state, free radicals can accept electrons from other atoms, converting those atoms into secondary free radicals, thus setting up a chain reaction that may cause substantial biological damage through an oxidative cascade process.

Oxygen-containing free radicals are basically by-products of adaptation to aerobic life. Molecular oxygen O_2 is essential for the survival of aerobic organisms, as energy production depends on oxygen as the final acceptor of electrons in mitochondrial electron transport. The respiratory chain, however, generates free radicals that leak from mitochondria, causing oxidative damage to macromolecules.

For example, superoxide anion ($O_2^{\bullet -}$) is the first intermediate of the reduction of O_2. To cope with it, animals have evolved several types of superoxide dismutase (SOD), an antioxidant enzyme that eliminates superoxide anion by catalysing a dismutation reaction

$$2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (1)$$

Superoxide dismutase works synergistically with H_2O_2 removing enzymes, such as catalase and glutathione peroxidase.

Hydroxyl radicals ($^{\bullet}OH$) are important pro-oxidants, mainly derived from the breakdown of H_2O_2 via the Fenton reaction

$$M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + {}^{\bullet}OH + {}^{-}OH \quad (2)$$

(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+}) or by the interaction of superoxide with hydrogen peroxide via the Haber–Weiss reaction

$$O_2^{\bullet -} + H_2O_2 \rightarrow O_2 + {}^{-}OH + {}^{\bullet}OH \quad (3)$$

Peroxidation products can also have pro-oxidant properties. For example, the toxicity of hydroperoxides (ROOH) is promoted by the presence of metals, such as iron (Fe^{2+} and Fe^{3+}) and copper (Cu^+ and Cu^{2+}). These metals catalyse the cleavage of ROOH, leading to the generation of two highly reactive and histolesive pro-oxidants, the alkoxyl ($R–O^{\bullet}$) and alkylperoxyl ($R–OO^{\bullet}$) radicals, which favour the oxidative cascade process.

BOX 2

To maintain redox homeostasis, organisms have evolved a detoxification system that uses several endogenous (e.g. enzymes) or exogenous (e.g. vitamin E) antioxidant compounds, i.e. any substance that delays, prevents or removes oxidative damage to a target molecule. Enzymatic and non-enzymatic antioxidants have different functions, but differences can also occur within the same category. These two forms of antioxidants can detoxify the biological system in very different ways, acting at different stages in the oxidative sequence. Moreover, the levels and composition of antioxidants differ from tissue to tissue and cell type to cell type. Another important point that should not be ignored is that antioxidants are connected to each other, synergistically coping with pro-oxidants. For example, carotenoids repair vitamin E radicals and, in turn, the resulting carotenoid radicals are repaired by vitamin C. Such synergistic protection provided by carotenoids and vitamins C and E strongly depends on a balance among these compounds (Palozza 1998). In fact, an increase in the concentration of one of those at a level beyond which the antioxidant pool cannot effectively recycle radicals might disturb this balance, reducing antioxidant effectiveness (Krinsky & Yeum 2003; Costantini *et al.* 2007c).

The following is a brief summary of the functions of antioxidants.

(1) Quenching $^1O_2$, singlet oxygen, which can react with lipids (e.g. LDL) to produce peroxides.

(2) Binding metal ions to avoid generation of reactive species and decomposition of peroxides to radicals.

(3) Scavenging (reducing the concentration of) reactive species capable of abstracting hydrogen atoms (e.g. $^{\bullet}OH$) prevents the initiation of the oxidative cascade;

(4) Chain breaking, i.e. reacting with chain-propagating radicals, such as alkoxyl and alkylperoxyl radicals, prevents continued hydrogen abstraction from biomolecules.

(5) Compared to low-molecular weight antioxidants, the activity of enzymes is mainly intracellular. In the enzymatic network, SOD catalyses the dismutation of $O_2^{\bullet -}$ to O_2 and H_2O_2, catalase CAT catalyses the direct decomposition of H_2O_2 to H_2O and ground-state O_2 when the levels of H_2O_2 are high, and glutathione peroxidase GPx catalyses the reduction of H_2O_2 and hydroperoxides when the levels of H_2O_2 are low. Antioxidant enzymes constitute a first line of defence against pro-oxidants. Expression of genes regulating antioxidant enzymes may increase in response to stress. However, increased synthesis of enzymes requires

Box 2 (Continued)

additional supply of some dietary metal cofactors, such as selenium, magnesium or zinc, which enhance the activity of antioxidant enzymes. Therefore, food quality or availability could affect adaptive response to oxidative stress.

As a final protective mechanism against DNA damage and mutations caused by reactive oxygen species, animals have evolved apoptosis, a form of programmed cell death in multicellular organisms. Apoptosis may occur when a cell is damaged beyond repair, preventing it from threatening the integrity of the body and from sapping further nutrients from the organism. Failure of oxidatively damaged cells to undergo apoptosis may actually contribute to development of pathologies.

antioxidant capacity of wild nestling Eurasian kestrels *Falco tinnunculus* (Costantini & Dell'Omo 2006b) and nestling yellow-legged gulls *Larus michahellis* (Rubolini *et al.* 2006) may be explained by environmental components (e.g. food quality) rather than genetic ones. A more recent comparative study on wild birds showed that higher plasma micromolecular antioxidant levels were generally characteristic of more rapid development, lower survival rate, smaller body size, larger clutch size and higher mass-adjusted metabolic rate (Cohen *et al.* 2008). The results were partly consistent with the hypothesis that antioxidant levels evolve to mirror free radical production.

Previous comparative studies on vertebrates have shown that maximum lifespan negatively correlates with several enzymatic and non-enzymatic antioxidants (not corrected for basal metabolic rate) measured in different types of cells or tissues (Pérez-Campo *et al.* 1998). The suggested lack of positive association between antioxidants and maximum lifespan is further supported by the failure of antioxidant supplementation to extend lifespan (Herbert 1994; Hulbert *et al.* 2007). These results suggest that levels of antioxidant defences do not explain interspecies differences in maximum longevity, but rather reflect the degree of oxidative stress experienced by the organism. However, antioxidant defences are still important in increasing the mean lifespan (see Table 3 in Hulbert *et al.* 2007). In most of the reports summarized in Hulbert *et al.* (2007), the increase of antioxidants by either dietary or pharmacological manipulation decreased early death, thanks to a reduced incidence of diseases. Therefore, it appears that antioxidants are important in protecting against oxidative damage from pathological conditions or exogenous damage but seem to be unable to change ageing rate.

ADAPTATIONS TO OXIDATIVE STRESS

Avian studies on antioxidants

In the last 20 years, ecophysiological studies on birds have focussed on the antioxidant pigments underlying sexually selected body colourations (McGraw 2005). Following the proposal of Hamilton & Zuk (1982), Lozano (1994) proposed that carotenoid-dependent ornaments reflect the health and immune condition of males, and for that reason they are used by females to select mates. This hypothesis has been rather influential in carotenoid research, and the number of studies on birds, as well as on other animal groups displaying these colourations, has rapidly increased (Tella *et al.* 2004; Olson & Owens 2005).

Several studies sought also to evaluate whether carotenoids can behave as important antioxidants in birds (Costantini 2006). A recent meta-analysis showed that carotenoids explain $< 0.002\%$ of the antioxidant capacity of birds (Costantini & Møller 2008). This finding supports the suggestion that carotenoid-based colourations might not indicate the body's antioxidant capacity but rather reflect (1) the concentrations of other colourless antioxidants (e.g. vitamins A, C and E), which reduce the oxidative decolouration of carotenoids, making them available for sexual signalling; and/or of (2) other functions of carotenoids, e.g. in the immune system or in embryonic development (Hartley & Kennedy 2004). Regardless of what carotenoid-based colourations actually imply, the relative contribution of carotenoids to antioxidant capacity seems to be low. However, given the lipophilic nature of carotenoids, these molecules could still be important as antioxidants for protecting phospholipids in cell membranes, or for their possible participation in vitamin E recycling, which is important for antioxidant defences. These points warrant future experimental studies.

In the light of these recent findings, the importance of other molecules as antioxidants in birds should be addressed. For example, studies of ascorbate and its transporters or of tocopherols and their metabolism might be informative.

Unlike carotenoids, ascorbic acid can be synthesized or obtained in the diet in a species-specific manner (e.g. Del Rio 1997). Because a biochemical trade-off may emerge when a limited supply of a specific antioxidant is being shunted among different functions or tissues, the ability to synthesize a specific antioxidant *de novo* may be important because it could impose different costs among species, leading to the evolution of different trade-off strategies.

Vitamin E is a generic descriptor used for all tocol and tocotrienol derivatives and includes α-, β-, γ- and δ-tocopherol and α-, β-, γ- and δ-tocotrienol (Surai 2002). Studies on captive animals show that vitamin E is an important antioxidant and that it may improve protection mediated by other antioxidants against lipid peroxidation (Surai 2002). However, we do not know to what extent vitamin E availability limits antioxidant defences and physiological functions of free-living animals. So far, only one study addressed some of these points in a wild bird species (de Ayala *et al.* 2006). The authors found that supplementation of vitamin E at intermediate physiological doses enhanced feather growth and condition of nestling barn swallows (*Hirundo rustica*), whereas higher physiological doses did not enhance offspring quality compared to a control treatment.

Glutathione is another relevant antioxidant warranting attention. One study showed that plasma glutathione (total glutathione and oxidized/reduced glutathione ratio) may be a reliable marker of environmental stress in wild great tits, *Parus major* (Isaksson *et al.* 2005).

Another molecule with antioxidant properties is uric acid. In birds, uric acid is the main nitrogen waste product, whereas in mammals it is the major end product of purine metabolism.

The antioxidant role of uric acid has been suggested for both captive birds (Iqbal *et al.* 1999; Klandorf *et al.* 1999; Hõrak *et al.* 2007) and wild ones (Cohen *et al.* 2007). Cohen *et al.* (2007) found that circulating uric acid was highly correlated with levels of circulating antioxidants in 526 individuals from 92 bird species, suggesting that some of the circulating antioxidant capacity can be accounted for by uric acid.

In view of the antioxidant properties of uric acid and its presence in several animal taxa, this compound may be of special interest for biologists in years to come. It would be interesting, for example, to evaluate the causes underlying the high variation in circulating levels of uric acid and its relationships with antioxidant defences and longevity. Human beings, one of the longest living mammalian species, have high levels of uric acid compared with other mammalian species because they lack urate oxidase (uricase), the enzyme involved in the conversion of hypoxanthine to xanthine and xanthine to allantoin. Birds have uric acid concentrations three times higher than humans, sometimes well above the limit of its theoretical solubility. Like humans, birds lack urate oxidase. Therefore, the presence of allantoin in their plasma has been suggested to indicate a direct oxidation of uric acid by free radicals (Grootveld & Halliwell 1987; Tsahar *et al.* 2006).

As uric acid is affected by protein metabolism, could the food quality and quantity affect the body's oxidative status and explain between-species differences in uric acid levels? Protein restriction may extend maximum longevity in rodents, although to a lower extent than caloric restriction, and excessive dietary protein intake gives rise to pathologies (Benevenga & Steele 1984). However, protein malnutrition during growth and development increases oxidative damage to brain lipids and proteins and worsens brain antioxidant activity in humans and rodents (Benevenga & Steele 1984). It is difficult to say to what extent effects of protein intake on the oxidative status are caused by changing levels of uric acid or, for example, by the methionine content of proteins. In fact, the effects of protein restriction on oxidative stress and longevity seem to be specifically due to the lowered methionine intake (Pamplona & Barja 2007). Studies are, therefore, needed to disentangle the importance of these components.

One more unclear point is whether the high levels of uric acid observed in individuals affected by pathologies associated with high oxidative stress are a compensatory response or a signal of the pathologies themselves. Very high levels of plasma uric acid cause gout, i.e. deposition of urates within the body, in both birds and mammals. Renal disorders and protein over supplementation are two of a plethora of potential causes for the increase of uric acid.

To answer all these questions using wild birds as a model, at least three methods could be successfully employed: intramuscularly or intravenously administration of urate oxidase, the enzyme responsible for the degradation of uric acid; supplementation of allopurinol (4-hydroxypurinol), a promising drug similar to urate oxidase used for the treatment of gout; change of dietary protein intake, e.g. by food supplementation or brood size manipulation. As far as the first two methods are concerned, two recent studies on captive bird species showed that urate oxidase decreases the uric acid concentration more effectively than allopurinol (Poffers *et al.* 2002a,b). Although no side effects associated with the administration of these drugs were observed, pilot studies on captive animal models are necessary.

Another important component of antioxidant defences are enzymes. So far, our knowledge of antioxidant enzymes in natural populations is quite poor (e.g. Berglund *et al.* 2007; Rey *et al.* 2008).

A general limitation for analyses of antioxidant enzymes in wild vertebrates is that their activity in the blood (the tissue most usually available for ecological studies) is limited or absent for most of them. The use of other fresh tissues, such as liver or brain, raises ethical objections, especially for studies dealing with threatened species. One possible solution to the problem of tissue sampling could be to sample tissues by biopsy.

A recent study showed that ultrasound-guided liver biopsy in birds is generally possible and with careful application can be considered a relatively risk free and diagnostically sensible addition to liver diagnosis in birds (Zebisch *et al.* 2004). However, validation of this method for oxidative stress analysis is lacking.

As both serum and plasma adequately reflect stressful situations, biochemical assays that measure the blood oxidative profile could be appropriate for field studies on oxidative stress in vertebrate species. However, several markers are needed to adequately assess oxidative stress in biological systems. Moreover, further studies are essential to better determine to what extent markers of serum or plasma oxidative stress reflect the body's oxidative profile in wild animals. This point is important because tissues may show different responses to factors that regulate antioxidants and differences in antioxidant distribution or susceptibility to oxidative stress (Surai 2002).

Level of membrane unsaturation

Membrane fatty acid composition is an important factor that influences tissue susceptibility to ROS and may explain lifespan differences between species (Pamplona *et al.* 1996, 2002; Hulbert 2005; Hulbert *et al.* 2006a,b; Hulbert *et al.* 2007).

Lipids are important targets of oxidative damage, but not all lipids have the same susceptibility to ROS. Indeed, saturated fatty acids are much less susceptible to peroxidation than unsaturated ones.

Hulbert *et al.* (2002a,b) showed that bird phospholipids have fewer *n*-3 polyunsaturates and more *n*-6 polyunsaturates than mammalian ones. This point is relevant because, in general, polyunsaturated *n*-3 fatty acids are more susceptible to oxidative damage than *n*-6 ones. Therefore, these differences between birds and mammals have been proposed to explain the longer lifespan of birds (Pamplona *et al.* 1996, 2002).

Differences in the membrane peroxidation index may help explain lifespan differences between the two rodent species, the naked-mole rat (*Heterocephalus glaber*) and the mouse (*Mus musculus*) (Hulbert *et al.* 2006a). The naked-mole rate is a very important exception to the paradigm 'fewer free radicals, longer lifespan'. In fact, the naked mole rate is the longest-living rodent with a maximum lifespan in captivity of *c.* 30 years, which is much higher than expected for its body size (Buffenstein 2005). Despite its extraordinary longevity, naked mole-rat mitochondria produce more free radicals than mouse (maximum lifespan = 4 years) 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). How can we reconcile the high free radical production with the long lifespan of naked-mole rats? A recent study from Hulbert *et al.* (2006a) suggested that differences in membrane phospholipid composition may explain why naked-mole rats live so long. The authors showed that the peroxidation indices of the naked-mole rat, calculated from muscle and liver mitochondrial membranes, are lower than in mice. Moreover, membrane composition did not appear to change significantly with age, conferring to the naked-mole rat a high tolerance to oxidative stress throughout its entire life.

Similar conclusions were drawn from a more recent comparison between two bird orders, Procellariformes (seabirds) and Galliformes (fowl). The 3.8-fold greater predicted longevity of seabirds was associated with elevated contents of monounsaturates and reduced contents of polyunsaturates and, consequently, a reduced peroxidation index in heart membrane lipids, compared with fowl (Buttemer *et al.* 2008).

These findings support the 'membrane pacemaker' theory of ageing (Hulbert 2005), a recent extension of the 'oxidative stress' theory of ageing, and they suggest that membrane fatty acid composition could be another important mechanism that evolved to cope with the detrimental effects of pro-oxidants. However, these results do not allow us to conclude whether such a relationship proves causality. Therefore, experimental determinations of alterations in membrane fatty acid composition as a function of age or oxidative stress are needed to clear this picture. For example, the question of whether dietary changes in polyunsaturated fatty acid content alter membrane composition and its susceptibility to oxidative damage could be ecologically relevant. In fact, individuals and populations of the same species may show significant dietary differences caused by oscillations in food availability, habitat quality or individual feeding habits, which could modulate their stress resistance.

OXIDATIVE STRESS AS A PHYSIOLOGICAL COST

Several factors may affect the balance between pro-oxidants and antioxidants. Below, I have reviewed three ecologically relevant inducers of oxidative stress (reproduction, immune response, physical activity).

Oxidative stress as a cost of reproduction

The cost of reproduction is a central paradigm of life-history theory (Bell 1980; Stearns 1992; Roff 2002). Resources, such as antioxidants and nutrients, allocated to reproduction are no longer available for self-maintenance, so a high investment in breeding performance is

expected to lower future fecundity or survival perspectives (Rose & Bradley 1998; Zera & Harshman 2001). A recent study of a long-lived seabird (common guillemot, *Uria aalge*) showed that the costs of early reproduction may actually lower later reproductive performance, determining reproductive senescence (Reed *et al.* 2008). More specifically, the more chicks guillemots 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.

Brood size manipulations, such as enlargement or reduction of the brood size, are a classic way of modifying the breeding effort of parent birds under both captive and natural conditions. Such brood manipulation studies have shown that parents must cope with increased energetic demands when rearing enlarged broods, resulting in a trade-off between investment in current and future reproduction (e.g. Williams 1966; Drent & Daan 1980; Gustafsson & Sutherland 1988; Nilsson 2002).

Investing much energy in reproduction can be very demanding in terms of reduced longevity. For example, parent kestrels (*F. tinnunculus*) changed their work rate during the nestling phase in response to brood size manipulation, 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).

The idea that investing most of the total energy available to an organism is necessarily very demanding, such as in terms of reduced longevity, cannot be generalized to all animal species. One exception is human beings. Females invest higher amounts of energy in reproduction, such as during pregnancy or lactation, than males, but live longer on average. Females generally have a greater antioxidant capacity than males (Halliwell & Gutteridge 2007, p. 159–160; Viña *et al.* 2006). However, the mortality rates of mothers of twins are higher than those of mothers of singletons (Haukioja *et al.* 1989). Another potential exception to the 'cost of reproduction' paradigm comes from rodents. Mitochondria from female rats generate half the amount of hydrogen peroxide than those of males and have higher levels of mitochondrial reduced glutathione (Borras *et al.* 2007).

Recent research has suggested that oxidative stress, rather than energy demand, may underlie the cost of reproduction. Studies on invertebrates (Salmon *et al.* 2001; Wang *et al.* 2001) and captive birds (Alonso-Alvarez *et al.* 2004a, 2006; Wiersma *et al.* 2004; Bertrand *et al.* 2005; Table 1) found a link between reproduction and free radical resistance/antioxidant protection. Studies on birds also found that the breeding effort had affected circulating antioxidant defences in a sex-related manner, suggesting that males and females could have different strategies to cope with an increased workload. For example, male zebra finches (*Taeniopygia guttata*) showed higher resistance

Table 1 Summary of studies examining the effects of breeding effort on redox state

Study	Effect on pro-oxidants/antioxidants	Predicted effect on the oxidative state	Reference
Increase of breeding effort by enlarging the brood size	Red blood cells were more susceptible to haemolyse when exposed to an *in vitro* free radical attack (KRL test)	+	Alonso-Alvarez *et al.* (2004a)
Increase of breeding effort by enlarging the brood size	Decreased activity of superoxide dismutases and glutathione peroxidases	+	Wiersma *et al.* (2004)
The reproductive effort was manipulated by either allowing or preventing pairs to breed	Pairs laying a large number of eggs had red blood cells more susceptible to haemolyse when exposed to an *in vitro* free radical attack (KRL test) than pairs laying few eggs	+	Bertrand *et al.* (2005)
Birds were engaged in several breeding events	Birds that engaged in a higher number of breeding events had a weaker red blood cell resistance to oxidative stress (KRL test), and red blood cell resistance to oxidative stress predicted short-term mortality (but not longevity) and was related with a parabolic function to age	+	Alonso-Alvarez *et al.* (2006)

All these studies were carried out on captive zebra finches. 2,2′-azobis-(aminodinopropane) hydrochloride is used as the free radical source. +, imbalance towards pro-oxidants.

Table 2 Summary of studies examining the effects of the immune response on redox state. 2,2′-azobis-(aminodinopropane) hydrochloride was used as the free radical source

Species	Study	Effect on pro-oxidants/antioxidants	Predicted effect on the oxidative status	Context	Reference
Sula nebouxii	Males injected with LPS	Increase of the oxidative damage (TBARS)	+	Wild	Torres & Velando (2007)
Falco tinnunculus	24-26-day-old nestlings injected with PHA	Increase of the oxidative damage (d-ROMs test) and decrease of the serum antioxidant capacity (OXY-adsorbent test)	+	Wild	Costantini & Dell'Omo (2006a)
Alectoris rufa	Males injected with PHA	No any effects on lipid peroxidation (TBARS) in red blood cells and plasma antioxidant capacity (Randox TAS test)	0	Captivity	Perez-Rodriguez *et al.* (2008)
Taeniopygia guttata	Adults injected with LPS	No any effects on the susceptibility of red blood cells to haemolyse when exposed to an *in vitro* free radical attack (KRL test)	0	Captivity	Alonso-Alvarez *et al.* (2004b)
T. guttata	Adults injected with LPS	Red blood cells were more susceptible to haemolyse when exposed to an *in vitro* free radical attack (KRL test)	+	Captivity	Bertrand *et al.* (2006)
T. guttata	Adults injected with PHA	No any effects on the susceptibility of red blood cells to haemolyse when exposed to an *in vitro* free radical attack (KRL test)	0	Captivity	Alonso-Alvarez *et al.* (2007)
Carduelis chloris	Males injected with SRBC and PHA	No any effects on the plasma antioxidant capacity (TEAC test and BIOXYTECH AOP-490 test)	0	Captivity	Hõrak *et al.* (2006)
C. chloris	Males injected with PHA	Increase of the oxidative damage and of the plasma antioxidant capacity (BI-OXYTECH AOP-490 test)	−/0/+	Captivity	Hõrak *et al.* (2007)
Parus major	Count of leucocytes in females incubating their second clutches	Nonlinear relationship between two markers of plasma antioxidant capacity (TEAC test and BIOXYTECH AOP-490 test) and respectively the heterophile number and the heterophile/lymphocyte ratio (index of avian stress); specifically, the antioxidant capacity was higher in individuals showing lower and higher values of both the leucocytic markers of inflammation	non-linear	Wild	Tummeleht *et al.* (2006)

LPS, lipopolysaccharide; PHA, phytohaemagglutinin assay; SRBC, sheep red blood cells; TAS, total antioxidant status; +, imbalance toward pro-oxidants; −, imbalance towards antioxidants; 0, no imbalance.

of red blood cells to an *in vitro* free radical attack than females (Alonso-Alvarez *et al.* 2006). However, as all these studies were carried out on zebra finches, it is difficult to generalize. In addition, the avian studies only evaluated the effects on antioxidants, regardless of whether rearing offspring causes oxidative damage. Therefore, it is premature to conclude that antioxidant defences were overwhelmed by increased free radical production caused by the high workload. Consequently, measuring only the antioxidant capacity may not be sufficient to infer anything about free radical production and oxidative stress.

Oxidative stress as a cost of immune response

The relationship between life-history decisions and parasitism may be modulated by a trade-off between investment in life-history traits and immune response (Sheldon & Verhulst

1996; Norris & Evans 2000). Activation of an immune response may require the organism to cope with increased free radical production due to increased oxygen O_2 uptake and consumption (respiratory burst).

Heterophils (neutrophils in mammals), macrophages, B and T lymphocytes activated during the inflammatory response release pro-oxidant chemicals, which kill pathogens. Overproduction of pro-oxidants, however, may damage host tissues (immunopathology) if not effectively counteracted by the organism's antioxidant defences.

Recent studies on birds (Table 2) have investigated the effects of the immune response on the redox balance, but they have yielded contrasting results. Some of them showed significant changes in the levels of oxidative damage and antioxidants following an immune challenge, while others did not. There are at least three different explanations for these seemingly contradictory results: (1) different methods and experimental schedules were used, making comparison of the studies difficult; (2) there are differences in how species cope with the immune response and (3) they may reflect different capabilities of the protective machinery, perhaps depending on the phase of the life cycle. In the following, each of these explanations is addressed.

First, some studies measured antioxidant capacity only, which is not sufficient to infer anything about immune response-related oxidative damage and oxidative stress. In addition, these studies used different markers to quantify the oxidative status and different methods to elicit the immune response. Several biochemical markers have been developed to measure oxidative damage and the antioxidant capacity of a tissue. While an array of different markers is useful to assess oxidative stress in a biological system, some problems arise because the specificity of the method to be used or its underlying biochemical rationale may make comparison of results difficult. Moreover, differences may emerge between hydrophilic and lipophilic assays of antioxidant capacity. However, a discussion of the problems arising from using different markers of oxidative stress is beyond the scope of the present review and many comprehensive reviews of this topic are already available (e.g. Prior & Cao 1999; Dotan *et al.* 2004; Yeum *et al.* 2004). A second explanation implies that species with different life histories, intensities of sexual selection, histories of parasite-mediated selection or feeding habits have evolved different mechanisms to cope with immune response-related oxidative stress. A third explanation implies that immunopathological effects (an infection-induced immune response that damages host tissues) could arise only when compensatory mechanisms are unable to cope with the disease, such as during chronic inflammation. For example, the capability of the protective machinery may vary from one species to another depending on the phase of the life cycle (e.g. nestlings have immature buffer systems, Costantini *et al.* 2006, 2007b) or according to the age of the individual, which is known to affect the immunocompetence of birds (see review in Lavoie 2006).

Oxidative stress and physical activity

Although direct evidence for ROS production during physical activity is scarce, there is a large body of literature providing indirect support that exercise may increase oxidative stress. For example, isolation of mitochondrial fractions showed that long-lasting exercise strongly increases the amount of damaged mitochondria (Di Meo & Venditti 2001).

Early studies suggested that the transition of mitochondria from state 4 (resting) to state 3 (respiratory active, producing ATP) is not accompanied by a proportionate increase of free radical production (Loschen *et al.* 1971). This would mean that tissue damage caused by mitochondrial ROS during exercise is of minor importance. However, studies using electron spin resonance techniques found increases in free radical production in rat and human muscles during exercise (Davies *et al.* 1982; Barja 1992). This led to the 'exercise' paradox. Subsequent studies addressing this paradox clarified the picture. An *in vitro* study (Herrero & Barja 1997a) on rat and pigeon heart and non-synaptic brain mitochondrial free radical production showed that (1) exercise may cause some oxidative damage and tissue activation because state 3 mitochondria produce free radicals with complex I-linked substrates (peptides representative of mitochondrial respiratory complex I: pyruvate + malate, glutamate + malate) and (2) oxidative damage is not massive during exercise because the mitochondrial free radical leak strongly decreases over the states 4 to 3 transition. This pattern is also explained by the decrease in local partial pressure of oxygen (p O_2) near the mitochondria (due to the large rate of O_2 consumption) and the lowering of the degree of electronic reduction of the respiratory chain during the states 4 to 3 transition. Therefore, even if oxygen consumption increases in skeletal muscles by as much as 20 times or more than during the resting phase, mitochondrial ROS production does not increase in proportion to O_2 consumption, avoiding massive oxidative damage.

The decreased free radical leak in state 3 may have evolved as a protective mechanism against severe oxidative stress that aerobic organisms experience during exhausting activities (Herrero & Barja 1997a; Barja 2007). One recent study suggested that moderate exercise might itself be considered as an antioxidant (Gomez-Cabrera *et al.* 2008). However, an *in vivo* study showed that, while mitochondrial free radical production did not increase, flies (*Drosophila melanogaster*) allowed to fly

throughout life had altered membrane fatty acid composition, higher lipid peroxidation and mortality rates, and decreased median and maximum lifespans compared to controls (Magwere *et al.* 2006).

The study of migratory animals can provide insights into mechanisms that evolved to cope with exhausting exercise. Migration is a very demanding activity involving several physiological challenges, such as increased energy expenditure, in animals ranging from invertebrates (Rankin & Burchsted 1992; Magwere *et al.* 2006) to vertebrates (Jonsson *et al.* 1997; McWilliams *et al.* 2004; Costantini *et al.* 2007a).

Flying birds may undergo oxidative stress during long flights (Costantini *et al.* 2007a, 2008). For example, although pigeons flying a short distance (*c.* 60 km) showed no significant change in oxidative damage or serum antioxidant capacity compared to control birds, after flights of *c.* 200 km they had increased levels of oxidative damage (+54%) and decreased levels of serum antioxidant capacity (−19%) (Costantini *et al.* 2008).

Given these points, two hypotheses can be advanced: (1) individuals experiencing their first migratory flight (even within the same migratory journey) might suffer higher oxidative stress than trained ones, such as adults; and (2) migratory species should be able to cope with flight-induced oxidative stress better than non-migratory ones as they have evolved more effective defence systems. As regards the second point, arctic terns are a relevant example. In fact, terns migrate annually between Polar Regions and live more than 30 years. How can we reconcile these very long and physiologically demanding flights with the long lifespan of terns? One reason may be that membrane fatty acid composition and uncoupling proteins play a relevant role in affecting susceptibility to flight-related oxidative damage.

RESEARCH PERSPECTIVES AND CLOSING REMARKS ON OXIDATIVE STRESS

The physiological and biochemical machinery underlying oxidative stress is a very complex system involving many different compounds and mechanisms. Given this, using a marker of total tissue antioxidant capacity, rather than markers that measure the activity of individual antioxidants, may be a good way to measure the overall biological effect of an oxidative challenge on antioxidants. However, some of these assays are poorly defined and not specific, while reliable methods are available to measure well-defined individual antioxidants, such as vitamin E and glutathione. Thus, how many markers of oxidative stress, and which ones, should be used? Studies on oxidative stress suggest that using more than a single oxidative biochemical marker would be the best way to obtain insight into such systems. The use of a marker of antioxidant capacity should always be associated with a marker of oxidative damage. This approach may also be important to evaluate functional relationships between the different components that determine the redox state. In fact, such relationships could determine biochemical integration, i.e. evolution could be seen not only as a change of the overall oxidative system of an organism but also as a change of patterns of covariation of its multiple components. Many recent findings on the link between oxidative stress and life history were based on a single biochemical marker or measurement of single antioxidants. Hence, caution is called for before making hypotheses and drawing conclusions.

One way to strengthen ecological studies of oxidative stress would be closer collaboration with biochemical and medical disciplines. Ecological studies need to be complemented by *in vitro* and *in vivo* laboratory models, taking into account that results obtained from *in vitro* models may not necessarily translate completely into *in vivo* ones. On the other hand, biochemical studies can benefit from those on animal models under natural (but also captive) conditions, which would provide them with opportunities to test hypotheses on organisms living under natural conditions and to place the evolutionary meaning of mechanisms underlying oxidative stress in a more functional context. Ecotoxicological studies represent a good example of integration between ecological and biochemical fields. However, they often ignore the life-history traits of the species under investigation and the problem of statistical dependence in comparative analyses due to phylogenetic relatedness between species.

Finally, central questions that should be addressed in future research under natural conditions are: (1) How is oxidative stress generated? (2) How do organisms reduce the damaging effect of oxidative stress? (3) Which antioxidants do organisms really need to help quench free radicals? (4) What are the fecundity and survival perspectives of individuals that differ in the level of oxidative stress? Given the paucity of studies addressing these questions under natural conditions, studies of natural vertebrate populations may ultimately provide an important link between laboratory research and our understanding of the natural history and evolution of basic mechanisms of oxidative stress. Natural populations are, in fact, continually exposed to changing environments and genetic backgrounds that may jeopardize body homeostasis. Therefore, understanding how organisms regulate the levels of pro-oxidants and antioxidants may be of evolutionary and ecological importance. It will be necessary to answer all these basic questions before we can draw valid conclusions.

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