Review

Oxidative damage, ageing, and life-history evolution: where now?

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The idea that resources are limited and animals can maximise fitness by trading costly activities off against one another forms the basis of life-history theory. Although investment in reproduction or growth negatively affects survival, the mechanisms underlying such trade-offs remain obscure. One plausible mechanism is oxidative damage to proteins, lipids, and nucleic acids caused by reactive oxygen species (ROS). Here, we critically evaluate the premise that ROS-induced oxidative damage shapes life history, focussing on birds and mammals, and highlight the importance of ecological studies examining free-living animals within this experimental framework. We conclude by emphasising the value of using multiple assays to determine oxidative protection and damage. We also highlight the importance of using standardised and appropriate protocols, and discuss future research directions.

Life-history theory, physiology, and ageing

Although a Darwinian demon is hypothesised to maximise all aspects of fitness simultaneously [1], in reality it is evident that life-history traits, such as fecundity and lifespan, show an inverse relation. To explain these patterns, life-history theory assumes that resources are limited and animals maximise their fitness by trading costly activities off against one another [2,3]. Given that perpetuating the germ line is a key aim, reproductive activity may be given priority in this allocation of resources, but this can only be achieved by reducing investment in resources allocated towards somatic protection and maintenance. Within this theoretical framework, the disposable soma theory (DST; see Glossary) was proposed to help explain the evolution of ageing [4]. Ageing is most normally thought of as an accumulation of molecular and cellular damage, leading to functional decline and, ultimately, the increased risk of disease and death with advancing age [5]. Conceptually, the DST assumes that investment in reproduction or growth diverts key resources, such as energy, away from somatic maintenance. Natural selection should shape life histories to maximise reproductive fitness for the environment of an organism, given this fundamental physiological constraint [4]. The risk of environmentally caused mortality (e.g., predation or infection) is classically considered as being central to understanding the degree to which natural selection favours early reproduction and fast growth over maintenance [4]. For example, under conditions of high age-independent predation risk, investing in somatic maintenance to the detriment of early reproduction or rapid growth is likely to be a less successful strategy, because few individuals investing in maintenance will

Glossary

Catalase: antioxidant enzymes that catalyse the dismutation of hydrogen peroxide into oxygen and water.
Comet assay: an assay that uses single-cell gel electrophoresis to measure DNA damage in individual cells.
Dismutation: a redox reaction whereby a chemical species is simultaneously reduced and oxidised to form two distinct products.
Disposable soma theory (DST): first postulated by Thomas Kirkwood in 1977, it suggests that longevity is determined through the regulation of longevity assurance mechanisms, which provide an optimal compromise between reproduction and growth on the one hand and somatic maintenance on the other.
Free radical: an atom or group of atoms that contains an unpaired electron and, therefore, are unstable and highly reactive. Free radicals are natural byproducts of aerobic metabolism.
Glutathione (γ-glutamyl-cysteinyl-glycine): the most abundant low-molecular-weight thiol in animal cells. It participates in a range of cellular reactions and is a potent antioxidant that can scavenge free radicals and other reactive oxygen species (ROS).
Glutathione peroxidise: antioxidant enzymes that converts hydrogen peroxide to water and reduces lipid hydroperoxides to their resultant alcohols.
Hydroxynonenal: a reactive and toxic aldehyde produced following lipid peroxidation of cellular components, such as membranes.
Malondialdehyde: a marker of lipid peroxidation formed primarily following the decomposition of various polyunsaturated lipids by ROS. It is highly reactive and can act as a ROS.
Metabolomics: the systematic measurement and analysis of metabolites.
Mitochondrial respiratory steady-state: the prevailing oxygen consumption by mitochondria when maintained on a particular metabolic substrate.
Mitochondrial uncoupling: the loss of coupling between the rate of electron transport within the respiratory chain (respiration) and ATP production (oxidative phosphorylation).
Protein carbonyls: markers of protein oxidation that are formed when carbonyl groups (aldehydes and ketones) are produced on protein side chains following oxidation.
Proteomics: the large-scale analysis of proteins, particularly their structure and function.
Proton leak: the movement of protons (H+ ions) across the inner mitochondrial membrane. This process does not contribute to ATP synthesis.
Reactive oxygen species (ROS): molecules or ions formed by the incomplete one-electron reduction of oxygen; not necessarily free radicals.
Redox reaction: oxidation–reduction reactions that primarily involve the transfer of electrons between two chemical species.
Superoxide dismutase: antioxidant enzymes that catalyse the dismutation of superoxide into oxygen and hydrogen peroxide.
Thiobarbituric acid: a reagent used to determine lipid peroxidation. It reacts with compounds such as malondialdehyde to form a fluorescent red adduct.
Transcriptomics: the examination of mRNAs within a genome.

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survive to sexual maturity [4]. Consequently, the DST predicts that, under such conditions, individuals should grow fast, reproduce early and at a high rate, but with the cost of a relatively unprotected soma, leading to rapid ageing and a comparatively short lifespan.

This, in turn, leads to the idea that life-history variation in ageing rate across species and/or populations lies on a fast–slow continuum [6] from 'live fast, die young' to 'live slow, die old', with natural selection operating to position a species or a population on this continuum depending upon its ecological context. Age-related declines in demographic rates (survival probability and reproductive performance traits) have been widely observed in free-living vertebrates [7,8], and are increasingly well documented in free-living invertebrates (e.g., [9]). Evidence of physiological deterioration with age in natural populations is also steadily increasing, including body mass loss [10], altered muscle structure [11,12], osteoarthritis [13], and changes in immune phenotype [14]. Evolutionary theory predicts that variation in life-history decisions during development and in early adulthood should underpin variation in ageing rates among individuals within a population [8,15]. Long-term, longitudinal studies of wild birds and mammals suggest that environmental conditions in early life are associated with ageing patterns later on in adulthood [16–18]. As predicted, more rapid ageing and shorter lifespan are associated with previous investment in energetically expensive activities associated with successful reproduction [17,19–21]. However, the exact nature of the constraints acting on different life-history strategies is currently unclear.

Recently, there has been growing recognition of the importance of physiological rather than ecological costs as the key causal mechanisms [22–24], and a growing awareness of the need to include studies of behaviour and physiology to understand ecology and life histories [6]. Ecological costs of increased parental effort, for example, include elevated exposure to predators or infective agents in the environment. Physiological costs would include, for instance, reduced immune function and withdrawal of calcium from bones, leading to elevated risk of fracture [3]. Over the past few years, there has been a surge of interest in the potential role of oxidative damage as a mediator of life-history trade-offs. In this review, we examine this idea and discuss the potential pitfalls in measuring markers associated with oxidative damage and suggest how best these might be avoided.

Oxidative damage as a life-history cost

One plausible physiological cost incurred during metabolically expensive activities is elevated damage to cellular components by highly reactive chemical species derived directly as byproducts of metabolism. The free radical theory [25] or oxidative stress theory of ageing (OSTA

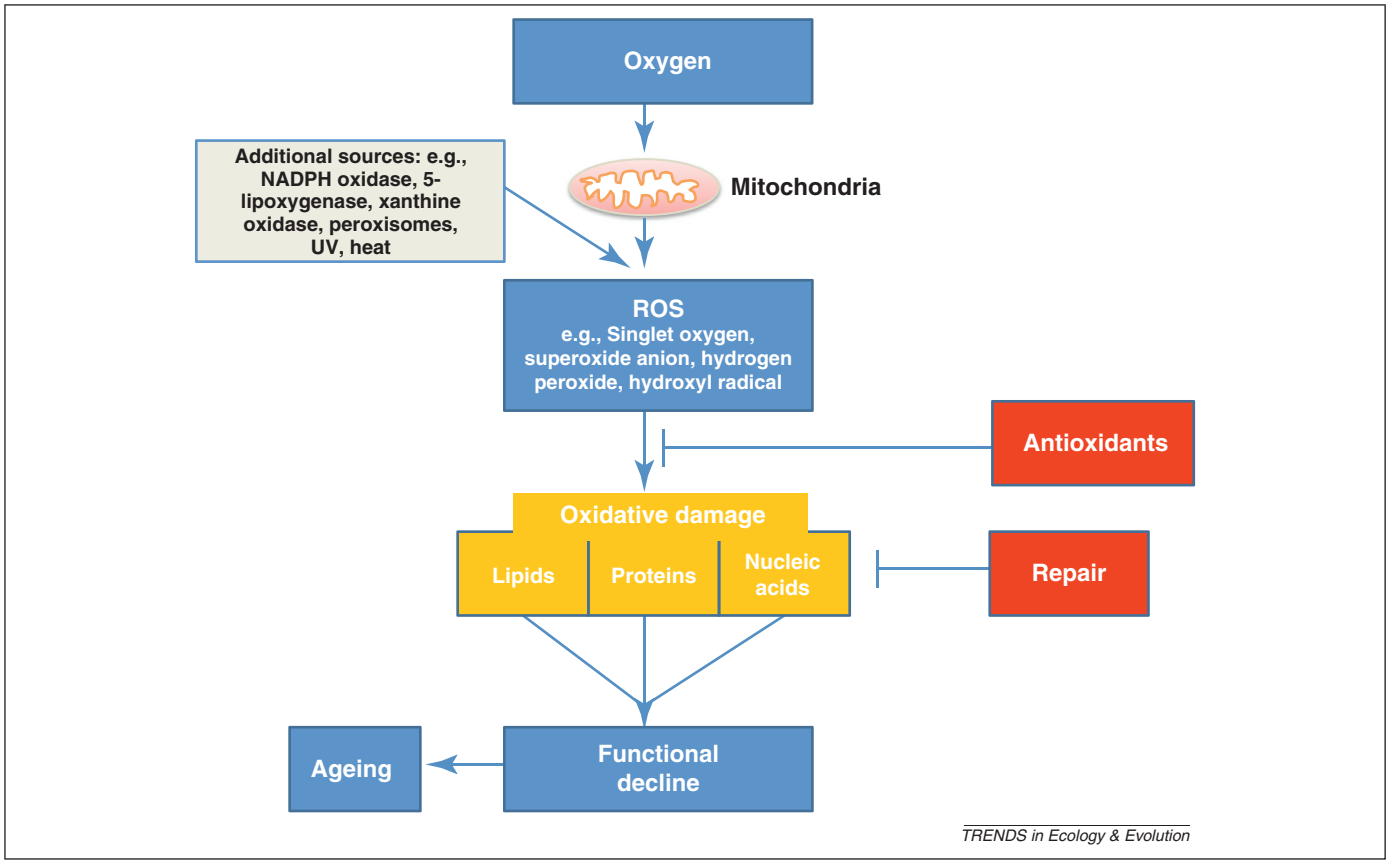

Figure 1. Schematic showing premise behind the oxidative stress theory of ageing (OSTA). Reactive oxygen species (ROS) are produced primarily within mitochondria during oxidative phosphorylation, although they are also produced by additional endogenous and exogenous factors. ROS are subsequently neutralised by a network of endogenous and exogenous antioxidants, although some ROS always evade these protective systems. It is these ROS that induce oxidative damage to cellular components, such as lipids, proteins, and nucleic acids. Although mechanisms exist to repair oxidatively-damaged biomolecules, some damage remains. The OSTA hypothesises that it is this oxidative damage that causes cellular attrition, physiological decline, ageing, and ultimately death.

[26]) hypothesise that free radicals damage various cellular components (e.g., lipids, proteins, and DNA), that the rate and/or amount of damage increases with advancing age, and that this damage causes ageing. Free radicals, such as superoxide (O_2^-), are generated primarily as byproducts of mitochondrial oxidative phosphorylation. Such radicals can be involved in subsequent chemical reactions producing additional molecules [i.e. ROS and reactive nitrogen species (RNS)] that can also elicit damage, but are not necessarily free radicals themselves [26,27]. Therefore, ROS is a collective term for radical and nonradical agents. Biological systems have well-described protection and repair systems (Figure 1) to help mitigate oxidative damage [26–28], although these are not 100% effective. Therefore, it is feasible that ROS-induced oxidative damage is a key metabolic mechanism that acts to mediate life-history trade-offs in wild animals [22,24,29,30]. The idea that a higher metabolic rate produces more ROS, leading to faster ageing, has been used by advocates of the rate-of-living (ROL) theory of ageing [31] to help explain the inverse relation between metabolic rate and longevity. However, empirical support for the ROL theory has recently waned [32–34], although some support still exists interspecifically for birds [35], but not mammals [36], following appropriate accounting for phylogeny and body mass.

Critically, ROS are not simply generated in direct proportion to oxygen (O_2) consumption; that is, a twofold increase in O_2 consumption does not necessarily double ROS production. Mitochondrial ROS production depends on many factors, including proton leak, mitochondrial uncoupling, and the prevailing mitochondrial respiratory steady-state [37], with different tissues producing ROS at different rates [38]. Indeed, under many circumstances, mitochondrial ROS production is highest when animals have their lowest energy expenditure, which might explain the reported positive associations between high metabolic rate and longevity [33,39]. Moreover, 'outlier' species, which live exceptionally long lives, have evolved particular mechanisms to cope with the effects of ROS-induced oxidative damage (Box 1).

What can free-living animals reveal about ageing?

Although the findings in model organisms are impressive [5,40,41], several lines of research suggest that there is no cause–effect relation between oxidative damage and ageing (reviewed in [30,42]). It is also unclear whether findings from the laboratory generalise to less protected and less benign environments, and to longer-lived organisms with slower life histories [29]. Laboratory 'model' organisms, such as mice, have been selectively bred for rapid growth, short generation times, early reproduction, and high fecundity. Their genetics and life histories have been altered by artificial selection and so it might be difficult to generalise as to whether life-history trade-offs or genetic variants observed in these organisms have wider ecological relevance. Also, physiological and fitness costs of particular life-history choices are profoundly dependent on environmental conditions. Costs might not appear, or might be different, in laboratory animals. Evolutionarily relevant costs might only manifest under natural conditions, when resources are finite and ecological factors, such as extrinsic mortality risk, are at play [22,24,29,30,43]. Model organisms are by default small and thereby likely evolutionarily to have faced high mortality rates. As a consequence, they might not have needed to invest heavily in somatic protection against oxidative damage [44]. Recently, there has been significant focus on using 'nontraditional' animal models, both within the laboratory and under free-living conditions, to identify the mechanistic basis of the physiological costs of different life-history strategies [29].

Does oxidative damage shape life-histories? Evidence from nontraditional models and free-living animals

A significant amount of recent research effort has aimed to integrate physiology and ecology to understand whether oxidative damage is a plausible life-history cost [22,24,45]. However, findings from both laboratory and field studies are somewhat ambiguous with regard to whether ROS-induced oxidative damage mediates life-history trade-offs. Captive reproducing female house mice (*Mus musculus domesticus*) had less hepatic oxidative damage (malondialdehyde and protein thiols) compared with non-breeding

Box 1. Radical species: exceptions to the rule?

Several animal species appear to refute the OSTA, although perhaps for these same reasons they might help identify the mechanisms underlying ageing. Undoubtedly, the best described 'outlier' is the naked mole rat (NMR; *Heterocephalus glaber*), a eusocial subterranean rodent with a maximum lifespan approaching 30 years. NMRs maintain reproductive and physiological function as they age, show no demonstrable age-related increase in morbidity or mortality and do not suffer from cancer [85]. However, they produce comparable amounts of ROS to similarly sized mice (approximate lifespan 3 years), have relatively short telomeres and unremarkable antioxidant levels, and experience relatively high levels of oxidative damage [85]. The key to their exceptional longevity might be how they mitigate and cope with oxidative damage; NMRs maintain protein quality and stability throughout their life, their cells resist experimental tumorigenesis and are also resistant to some, but not all, types of chemical challenge [85]. Excitingly, the NMR genome has been sequenced [86], and so this information should help understand why they are just so 'peculiar'.

The ability of animals and their cells to resist ROS is not only observed in NMRs, but is also correlated with longevity in birds [87], wild-derived [88] and genetically mutant captive mice [89], bats [90], and in the ocean quahog (*Arctica islandica*) [91]. In addition, the olm (*Proteus anguinus*), a cave-dwelling salamander, combines a long life [92] with unremarkable antioxidant protection [93]. Of course, ROS-induced oxidative damage might be a private (specific to particular evolutionary lineages) rather than a public (shared across evolutionary lineages) determinant of ageing [94], and this might explain these outliers. Those species that have undergone selection for exceptionally long lifespan, driven by ecological factors, might have evolved in tandem specific mechanisms that mitigate oxidative damage. The absence of such mechanisms in most animal species, which have relatively short lifespan, perhaps suggests that in general the fitness costs of oxidative damage are of relatively minor importance in determining the evolution of life histories.

We suggest that studying members of 'Methuselah's Zoo' [29] and examining precisely how they mitigate the impact of ROS-induced oxidative damage on ageing rate and pathology is critical to understanding the mechanisms underlying ageing. In addition, it would also be useful to identify commonalities (and differences) in life histories among these outlier species compared to close relatives with shorter lifespan.

females [46]. Similarly, lipid oxidative damage was lower (kidney and muscle) and protein oxidative damage unaltered (kidney, muscle, and heart) in breeding female bank voles (*Myodes glareolus*) relative to non-breeding females [47]. These data suggest that reproductively active females invoke protective mechanisms to limit oxidative damage, and indeed the antioxidant glutathione was increased in livers of reproducing house mice [46]. Life-long cold exposure in captive short-tailed field voles (*Microtus agrestis*) significantly increased metabolic rate relative to warm-exposed animals, but had negligible impact on tissue oxidative damage or antioxidant levels [32]. This increase in metabolic rate also had no impact on longevity, implying that physiologically mediated costs associated with increased energy expenditure do not affect oxidative damage or survival. In captive zebra finches (*Taeniopygia guttata*), increased reproductive effort was associated with reduced antioxidant protection (e.g., [45,48]), although neither study assayed oxidative damage or survival. Captive zebra finches from experimentally reduced broods also had greater cellular (erythrocyte) resistance to oxidative stress than did birds from large broods [48]. This enhanced cellular resistance was correlated with higher lifetime reproductive output, but reduced lifespan [49]. Overall then, these studies paint a rather confused picture of the manner in which oxidative damage is related to reproductive effort.

One exciting approach to examine how differences in ageing rates and levels of ROS and oxidative damage have evolved in the wild has involved common garden experiments, which take different ecotypes from the wild and compare them under controlled laboratory conditions. For example, western terrestrial garter snakes (*Thamnophis elegans*) have evolved long or short lifespans in response to low or high extrinsic mortality, respectively [50]. Neonates of the long-lived ecotype had more efficient mitochondria and antioxidant defences than did those of short-lived snakes, although their DNA was more damaged by UV exposure [50]. However, DNA damage was repaired more efficiently in the long-lived ecotypes. It was also shown that captive-bred long-lived colubrid snakes generated less ROS (H_2O_2) within liver mitochondria compared with shorter lived species, despite whole-animal metabolic rates and mitochondrial efficiency being unaffected [51].

Ageing and ROS-induced oxidative damage in free-living animals

Surprisingly few studies have examined whether oxidative damage is altered with age or reproductive effort in free-living animals [29]. Most research examining parameters relating to ROS-induced oxidative damage and ageing in free-living animals has been undertaken in birds and mammals. Oxidative status [the ratio between reactive oxygen metabolites (ROMs; hydroperoxides generated primarily by lipid peroxidation) and total antioxidant capacity (TAC)] was elevated in young female collared flycatchers (*Ficedula albicollis*) relative to older birds, although no age effect was observed in males [52]. Oxidative status was also elevated in younger relative to older Eurasian kestrel chicks (*Falco tinnunculus*) between 9 and 31 days of age, with the levels of ROMs also lower in older nestlings [53]. The resistance of erythrocytes to ROS-induced damage in greater flamingos (*Phoenicopterus ruber roseus*) was higher in young adults compared with both immature and old individuals [54]. Similarly, erythrocyte resistance to ROS increased in early life but declined in later life in female Alpine swifts (*Apus melba*), although no age-related decline was seen in male birds [55]; erythrocyte resistance was also associated with a greater survival to the next breeding season in males, but not females [55]. In addition, a relatively high plasma TAC was predictive of long-term survival in barn swallows (*Hirundo rustica*), with antioxidant protection having high repeatability within individuals both within and between years [56]. Therefore, although resistance to oxidative stress and increased antioxidant levels may be predictors of survival in some bird species, less evidence supports the premise that oxidative damage increases with age.

Fewer studies have examined the impact of age on oxidative damage and antioxidant protection under free-living conditions in non-avian species. In water shrews (*Sorex palustris*) and short-tailed shrews (*Blarina brevicauda*), an age-related increase in antioxidant enzymes (catalase, glutathione peroxidise, and superoxide dismutase) was observed in skeletal muscle [12]. Although lipid peroxidation levels increased with age, other oxidative damage markers were either unaltered or decreased by age [12]. By contrast, plasma lipid oxidative damage levels were highest in lambs relative to older age classes in Soay sheep (*Ovis aries*) [57]. Thus, these data are again unsupportive of a key tenet of the OSTA; that oxidative damage increases with age.

Reproduction and ROS-induced oxidative damage in free-living animals

Reproduction in many animals is associated with a significant increase in energy requirements [58], and investment in reproduction can negatively impact on subsequent survival [17,23,59,60]. However, reproduction is not generally associated with increased oxidative damage [24]. In adult free-living Soay sheep, no relation between lipid oxidative damage and reproductive effort was reported [57]. Similarly, no effect of reproduction on lipid peroxidation was observed in female eastern chipmunks [61]. Average annual reproductive rates in Leach's storm petrels (*Oceanodroma leucorhoa*) were negatively correlated with TAC [62]. This suggests that high reproductive rates impact on antioxidant protection, as reported in captive zebra finches [45,48]. However, this relation did not hold for savannah sparrows (*Passerculus sandwichensis*) [62]. Female alpine swifts with greater erythrocyte resistance to ROS laid larger clutches that were more likely to hatch successfully [55]. Egg size was also positively correlated with plasma TAC in female collared flycatchers [52]. Similarly, great tits (*Parus major*) given experimentally enlarged broods showed lower erythrocyte resistance to ROS compared with individuals with experimentally reduced or unmanipulated broods [63], again in agreement with captive studies [48]. However, by contrast, an experimental increase in foraging rate and chick provisioning in Adélie penguins (*Pygoscelis adeliae*) increased plasma TAC [64] and had no effect on plasma hydroperoxides. Offspring

Box 2. Challenges for field studies

Significant difficulties exist in examining oxidative damage in wild animals, notably sampling individual animals and offspring, repeatedly identifying and sampling the same individuals and capturing sufficient individuals of a known age and sex. Most studies undertaken in the laboratory and field are cross-sectional in design, which can confound age effects due to the selective disappearance of specific phenotypes within a population [95], thus making it difficult to separate intraindividual ageing rates from interindividual heterogeneity within free-living populations [8]. The collection of sufficient and relevant biological material is also complicated when studying endangered populations or individuals from long-term studies, where experimental manipulations might be considered unethical, too invasive, or might conflict with other objectives. Consequently, most studies examining oxidative damage in wild animals collect blood. Although this is important, it might reflect immediate measures of the state of an individual, as opposed to damage within tissue, which perhaps might give better insights into long-term processes [24]. A single study using a multi-assay approach (Box 3) has reported that oxidative damage and antioxidant levels in plasma might provide reliable information on these same parameters in tissues (e.g., heart, skeletal muscle, and liver [75]). However, whether this is generally the case is still open to question.

Sample collection in the field can also be challenging due to the remoteness of study sites and the absence of appropriate laboratory facilities. Oxidative damage markers, by their very nature, are prone to oxidation and so there is a requirement to have equipment close at hand to preserve sample integrity. Blood samples should be cooled quickly following collection and centrifuged rapidly to prevent lysis of red blood cells, because lysis can affect spectrophotometric absorbencies and haem can, under certain conditions, exert pro-oxidant effects [96]. Where possible, samples should be aliquoted to minimise the number of subsequent freeze and thaw cycles. For specific assays, samples should also be frozen in liquid nitrogen immediately after collection and stored at −80°C. Researchers should always test that their sampling and storage methods are appropriate for each particular assay before starting any experiment. The 'shelf life' of biological material can also vary, for example, being reduced in samples with high levels of polyunsaturated fatty acids and/or low concentrations of antioxidants. Excitingly, the requirement to freeze samples might soon be a thing of the past, with companies (e.g., Biomatrica®) now offering technology derived from extremophiles whereby biological samples can be stored at room temperature for several months without degradation.

from extra-pair matings enjoy various phenotypic advantages over those from within-pair matings [65], and great tit chicks from extra-pair matings had lower plasma lipid peroxidation relative to within-pair mating offspring [66].

Social status and life-history decisions might also impact on oxidative damage. In cooperatively breeding Seychelles warblers (*Acrocephalus sechellensis*), subordinate non-helping females had poorer body condition and elevated plasma ROMs relative to subordinate female helpers and dominant females [67]. Low-quality territories are also associated with elevated plasma ROMs in this species [68], perhaps due to increased foraging effort within poor-quality habitats. Accelerated early growth can have negative consequences later in life [69–71], and so compensatory growth, in the context of life-history theory, might also induce oxidative damage. Few studies have examined this in free-living organisms and the findings are contrasting. Whereas faster growth rate in Soay sheep was correlated with elevated plasma lipid oxidative damage [57], no effect of growth rates on damage was reported in red-winged blackbirds (*Agelaius phoeniceus*) [72].

Whereas some studies support the idea that ROS-induced oxidative damage mediates life-history trade-offs, others fail to demonstrate such effects. We suggest that the lack of agreement can, at least in part, be traced to methodological differences between studies, that is, the use of heterogeneous sampling protocols (Box 2) and assorted assays (Box 3). Caution must be taken when discussing the role of oxidative damage in a life-history context, if oxidative damage *per se* has not been determined [24,29]. ROS-induced oxidative damage occurs when ROS production exceeds the ability of the protection and repair systems that mitigate against ROS. Therefore, measures of TAC in isolation cannot inform about ROS-induced

Box 3. ROS-induced oxidative damage: does a gold standard exist?

A long-standing question in oxidative biology is what assay is most appropriate. Currently, no gold standard exists [28], although what is unequivocal is that the route from ROS production to oxidative damage is complex. At the very minimum, measures of oxidative damage should be performed (e.g., protein carbonyls and lipid peroxidation), because, unlike antioxidants, these measure the outcome of oxidative stress. Many studies have determined TAC in blood. Although this approach has some advantages (e.g., requiring small sample volumes, ease of use, and providing an index of the capacity to withstand ROS attack), several issues exist. Not least, it is nonspecific and the 'antioxidant' response is restricted to one particular ROS generator, which might not biologically be the most relevant [24]. In addition, although several commercial kits are available to measure TAC, they do not appear to measure exactly the same antioxidants [97]. Therefore, TAC assays should ideally be run alongside antioxidant enzyme activity assays (e.g., superoxide dismutase, glutathione peroxidase, and catalase).

Lipid peroxidation, determined by malondialdehyde levels via derivatisation of thiobarbituric acid reactive substances, is also used extensively. However, commercial spectrophotometric kits should be avoided because thiobarbituric acid is not 100% specific for malondialdehyde, and can be produced during sample preparation and affected by diet [24,28]. Consequently, this nonspecificity can overestimate the amount of oxidative damage present. However, HPLC-based techniques are less prone to experimental artefacts and have greater specificity [28,98].

We suggest that researchers should aim to collect multiple measures wherever possible. Oxidative damage to proteins (carbonyls and thiols), lipids (e.g., F2 isoprostanes, 4-hydroxynonenal, malondialdehyde by HPLC, and hydroperoxides) and/or DNA (e.g., 8-hydroxy-2′-deoxyguanosine, 8-hydroxyguanosine, and the Comet assay), in conjunction with antioxidant protection and/or measures of redox status (e.g., superoxide dismutase, glutathione peroxidase, catalase, glutathione, reduced: oxidised glutathione, TAC, and vitamin E), and/or cellular stress resistance. This multi-assay approach also appears to give better insights into what is happening at the tissue level, even when only plasma is measured [75].

Assays to determine repair mechanisms (e.g., proteasome activity and DNA base-excision repair) are run routinely in many laboratories and, therefore, are also accessible to ecologists. Interpreting data from multiple measures of protection, damage, and repair and relating these to life-history traits will benefit from multivariate statistical techniques, such as factor analysis [99]. We suggest that using such a combinatory approach will provide the best hope for understanding the role of ROS-induced oxidative damage in determining life histories.

oxidative damage [24,73]. Different ROS, antioxidants, and repair mechanisms exist that do not act in isolation and that react and compensate for one another in a bewildering fashion [24,27,29,74]. Care must also be taken when reaching conclusions relating to oxidative damage when a single tissue or damage to a single macromolecule is determined. Tissues generate specific types and levels of ROS, and have different types (and relative concentrations) of antioxidants and repair mechanisms [24]. Consequently, they are all likely to experience different rates of oxidative damage, which may also be specific to a particular life-history trait. Moreover, the probable consequences of lipid damage might be different to the probable consequences of DNA damage. We do not imply any criticism of previous work, given that, in many cases, our own studies to date have included such types of isolated measurement (e.g., [57,61]). On the bright side, using a multi-assay approach (Box 3) in laboratory rats demonstrated that markers of oxidative damage and antioxidant protection were correlated between plasma and several tissues (skeletal muscle, liver, and heart), although some tissue- and marker-specific differences were observed [75]. In addition, plasma ROM levels correlated with 4-hydroxynonenal (a marker of lipid peroxidation) in wild alpine marmots (*Marmota marmot*) [76].

Where now?

Although significant logistical and technological challenges exist for researchers examining ROS-induced oxidative damage (Box 3), widespread interest exists in whether ROS-induced oxidative damage has a role in shaping the life histories of free-living animals. To date, much research has concerned small animals living in protected pathogen-free environments with abundant resources and few environmental pressures. The clear benefits of such studies include an ability to perform experimental manipulations and make multiple measurements of the traits of interest. Yet, the shortcomings of such models are that trade-offs might be less obvious compared with animals dealing with limited resources combined with other pressures in the wild. The scepticism resulting from laboratory studies regarding the role of oxidative damage in ageing does not necessarily reflect a lack of importance in mediating life-history phenomena in natural settings. Long-term studies using natural populations with extensive databases of life-history traits and repeated measures are likely to provide unprecedented opportunities to test these predictions [15]. It is clear that meaningfully testing hypotheses of ageing and life history in natural populations demands longitudinal data and analyses at the within-individual level [40]. Such studies in the context of oxidative damage are lacking. Costs of early life-history decisions and the ageing process occur, of course, across the lifetimes of individuals. Individual phenotypic heterogeneity due to variation in resource availability, early life conditions, or genetic background is considerable in wild animals. This among-individual variation will bias estimates and lead to erroneous conclusions about the life-history costs and ageing, unless within and between individual processes can be dissected and explored separately with longitudinal data and appropriate statistical tools [40].

What is evident from this review, and highlighted elsewhere (e.g., [29]), is that despite the widespread interest in the potential role that ROS might have in life-history, few studies have determined ROS-induced oxidative damage *per se* in free-living animals (e.g., [12,57,76]). We suggest that significant progress will only be made if ecological researchers use assays to determine simultaneously antioxidant protection, oxidative damage, and, ideally, also repair. Although single assays, in single tissues (or blood) might be easier to perform, we suggest that these approaches have led to the current state of confusion (see also [8]). Studies using a range of assays reflecting damage, protection, and repair across multiple tissues and multiple macromolecules, will provide the most significant insights. We suggest that only by following relevant experimental protocols (Box 2) and through adopting a multi-assay approach of appropriate assays (Box 3) will one get close to understanding the role of ROS-induced oxidative damage in shaping life history. Of course, many long-term field studies necessitate nonterminal procedures (Box 2), but using biopsies to collect tissue might help circumvent this. There is also a requirement for studies that not only quantify oxidative damage, but also simultaneously measure additional parameters that are likely to be of importance, such as metabolic rate [50,51,61], mitochondrial function [51], telomere dynamics [44,77], and/or physiological condition [11,50,51,78]. Such studies are clearly starting to be made and we encourage ecologists to embrace these approaches, and collaborate more expansively with molecular biologists and biogerontologists where possible. Field experiments manipulating brood size, litter size, and/or diet will continue to be informative. Studies using non-model organisms or ecotypes within laboratory settings (e.g., [32,46,50,51]) are also likely to continue being important, because they will test the generality of laboratory findings in semi-natural or natural conditions, and test evolutionary and ecological ideas in the laboratory. Field ecology in isolation, although producing important correlative data, might not move the field forward sufficiently in terms of cause and effect with regard to the key questions highlighted in this review. However, it is clear that such approaches are starting to percolate across disciplines (e.g., [12,32,50,51,78,79]). We suggest that only multidisciplinary research can elucidate the cause and effect relations between oxidative damage, ageing, and life-history evolution, if they do in fact exist.

Comparative approaches [80], particularly those using methodologies and analytical tools (e.g., [81]) rarely used in ecology, should help uncover whether ROS-induced oxidative damage is an important cost to free-living animals. In particular, due to its declining expense, next-generation sequencing and network analysis [81] should unearth genetic differences between and within species [81], and identify pathways linked to somatic protection. In theory, researchers should be able to identify a signature of somatic protection, that is, a 'protectome', shaped by natural selection. Metabolomic and proteomic approaches will also help identify metabolites and enzymes associated with oxidative damage, both of which can be used non-invasively on blood and urine [81]. Such approaches will complement more traditional assays (Box 3) to obtain

unparalleled insights into the role of ROS and somatic protection in the lives of free-living animals. Many of the assays highlighted in Box 3 can be undertaken easily on blood samples, which many researchers find themselves limited to (e.g., protein carbonyls, 8-hydroxy-2′-deoxyguanosine, and 4-hydroxynonenal). It is also conceivable that ROS-induced oxidative damage is not a key mediator of life-history trade-offs across diverse taxa, hence the equivocal picture painted by our review of laboratory and field studies. Excluding oxidative damage, other molecular mechanisms exist that may drive functional decline and ageing (e.g., [5,14,23,40,41,82–84] and that may mediate life-history trade-offs. Field researchers need to also start incorporating these ideas into their studies.

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