

THE ECOLOGY OF ANTIOXIDANTS & OXIDATIVE STRESS IN ANIMALS

Ecological, biomedical and epidemiological approaches to understanding oxidative balance and ageing: what they can teach each other

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Summary

1. Oxidative stress and antioxidants have been studied in a number of disciplines, but these disciplines have not always been informed by each other's work.
2. Here, we discuss the strengths and weaknesses of oxidative stress and antioxidant research in the areas of (i) ecology, (ii) ageing research, (iii) epidemiology, and (iv) physiology of model organisms, with an emphasis on what ecologists can learn from and bring to other fields.
3. We find that physiologists provide an essential role in clarifying basic mechanisms, but that many of their findings are context-dependent. Ecologists and epidemiologists bring strengths in understanding the relevance of context, whether it is across species, environments, or diets. Ageing research has helped to provide a clear theoretical framework for all fields and has thus spurred much of the research to date.
4. Comprehensive understanding of the complexity of oxidative balance systems will rely on integration of knowledge of physiological pathways from studies of model organisms, knowledge of long-term interactions of many parameters from epidemiological studies, and knowledge of specificity and generality of results across species and conditions as gleaned from ecological studies.
5. Studies of ageing have helped to show that all fields of antioxidant/oxidative stress research should focus not on individual markers of oxidative damage or antioxidant status, but on how they integrate into oxidative balance systems. Free radicals can have beneficial roles in signalling as well as causing damage and should not be interpreted out of context.

Key-words: ageing, antioxidant, ecology, epidemiology, interdisciplinary, oxidative stress/balance/damage, physiology

Introduction

Physiological ecologists have become interested in oxidative balance – i.e. homeostasis with respect to oxidative stress and antioxidant levels – as a way to understand variation in individual fitness (within species) and variation in life history traits (across species) (Monaghan, Metcalfe & Torres 2009). If proper maintenance of oxidative balance is important for health, and if there is variation across individuals, then individuals that fail to maintain this balance may be less healthy and thus less fit; alternatively, individuals may trade-off maintenance of oxidative balance (and thus long-

term health) with current reproduction. At an interspecific level, traits such as longevity and pace of life may be associated with physiological strategies along this trade-off continuum. Indeed, many studies have shown relationships between markers of oxidative balance (including free radical levels, antioxidant levels, oxidative damage measures, etc.) and suspected fitness or life-history components such as immune function, development rate, reproduction, and annual survival (Alonso-Alvarez *et al.* 2007; Cohen *et al.* 2008b; Hōrak *et al.* 2007; Wiersma *et al.* 2004).

In spite of a sound theoretical framework, results to date have been less straightforward than many had anticipated. Many studies have reported patterns of interest, but effects and patterns appear to be species-specific (e.g. Cohen, Hau &

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Wikelski 2008a), and it is becoming clear that the interpretation of oxidative balance markers and their relevance for fitness and life histories depends on many factors that are difficult to account for simultaneously, including hormone levels, immune function, stress, species, diet, season, habitat, and so forth (Monaghan, Metcalfe & Torres 2009; Costantini & Verhulst 2009). Accordingly, further progress may depend on understanding the complex dynamics of multiple factors simultaneously, a great methodological challenge.

Ecological studies of oxidative balance have been discussed in detail in the previous articles of this issue, but many other fields have also taken an interest in understanding both the mechanisms and implications of free radical damage. Biochemists have helped characterize the key molecules involved and their properties, as well as devise ways to measure them (e.g. Re *et al.* 1999). Laboratory physiologists and geneticists have used model organisms to show roles and pathways involved (e.g. Gohil *et al.* 2004). Exercise physiologists have studied how oxidative status affects physical performance and vice versa (Ji, Stratman & Lardy 1988). Ageing scientists have looked into oxidative damage as one of the key physiological mechanisms of the ageing process (Barja 2000). And epidemiologists have examined large-scale consequences for human health of dietary antioxidants and exposure to sources of damage (Miller *et al.* 2005).

Each of these fields began to study oxidative balance for reasons often unrelated to the other fields – for example, studies of carotenoid pigmentation as a sexually selected signal in birds are probably of limited interest to non-ecologists, and studies of the effects of vitamin supplementation on long-term human health are only of indirect interest to non-epidemiologists. However, progress in each field has resulted in greater awareness of the gaps in our current knowledge, gaps that are often common across fields and unanswerable by each field independently of the others. To properly understand the consequences of carotenoid pigmentation for fitness components, we need to know not only how carotenoids are used, but what the physiological consequences of carotenoid levels are for other aspects of physiology and health, under a variety of conditions. To make sense out of conflicting results from epidemiological studies of supplements, we need to understand what factors are likely to mediate the physiological effects of ingested micronutrients.

In particular, a key shared finding across disciplines has been an inability to generalize results broadly. Vitamin deficiency in rats sometimes causes decreased endurance and sometimes does not (Gohil *et al.* 1986; Packer *et al.* 1989). Dietary antioxidants are good for human health in some studies, bad in others (Gey *et al.* 1991; Miller *et al.* 2005). Oxidative damage levels inversely correlate with lifespan in some studies but not in others (Morley & Trainor 2001; Navarro *et al.* 2005). The relationship between oxidative status and immune function differs across bird species (Costantini & Møller 2009). Free radicals may be harmful in some situations, but not in others (de Magalhães & Church 2006). And the shared interpretation has been that there are complex physiological networks of free radicals, antioxidants and

repair mechanisms, all regulated or affected by many other factors both internal and external to the organism.

A key goal of all fields has become to understand how these systems work, and how they are affected by factors such as diet, stress, genetics, and species. For this reason, we refer throughout this article to oxidative balance systems rather than to free radicals, oxidative damage, or antioxidants, except when the more specific concepts are appropriate. We use the term oxidative stress to indicate levels of free radical production or damage beyond normal control mechanisms and with potential fitness consequences, i.e. a loss of balance in the direction of too many free radicals. A biochemically more precise definition [such as damage rather than reactive oxygen species (ROS) levels] is difficult without better knowledge of what each measure means in a given context for a given species (see Hōrak & Cohen 2010). We note that fitness strategies may vary, and in some cases sustaining more damage may be a better strategy, but for ease of reference we will discuss fitness consequences of loss of balance, with the implicit assumption that the current versus future reproduction trade-off is being held constant.

The goals of this article are to (i) summarize the general knowledge of oxidative balance generated by several different fields and how it relates to ecology and evolution; and (ii) examine the shared questions and how each field can contribute to a greater comprehensive understanding. In particular, we focus on ecology, epidemiology, ageing studies, and laboratory physiology with model organisms.

Ecological and evolutionary approaches

Ecological interest in oxidative balance is largely focused on understanding the links between oxidative status and fitness, on the assumption that poor control of oxidative stress leads to poor health and could lower fitness (Monaghan, Metcalfe & Torres 2009). Elucidating this relationship should thus help us understand how physiology evolves in relation to fitness components and how competing selective pressures may play out over evolutionary time, which in turn can shed light on the ageing process (Holmes & Martin 2009). Because fitness itself is hard to measure, most studies have examined more specific fitness components, particularly sexually selected traits and various measures of immune function, as well as life-history traits. A consistent challenge that has arisen is the difficulty of measuring and characterizing complex physiological systems (Hōrak & Cohen 2010), including both oxidative balance and immune systems.

Some ecological questions can be difficult to test with controlled experiments. Manipulations are often unfeasible (e.g. of an ecosystem), and the systems studied are diverse enough that results may not be generalizable. As a result, ecologists and evolutionary biologists – while continuing to use experiments when possible – have developed and used many tools for understanding complex variation, including the comparative method for phylogenetic analyses (Felsenstein 1985; Harvey & Pagel 1991) and various analytical methods for observational studies. Controlled experiments have remained

as an essential tool, but are often not viewed as sufficient for many questions. Because of the difficulty in characterizing complex oxidative balance systems, such comparative approaches are often necessary, and can be applied well beyond ecological studies. For example, results of epidemiological studies on oxidative balance may well vary across cultures due to factors such as diet and lifestyle (Gey *et al.* 1991). For many purposes, cultures could be viewed as analogous to species and analysed in similar ways. The ability to analyse complex variation across systems is one of the great strengths of ecological approaches.

Another advantage of ecological studies is the range of study systems available. For example, species with very specialized and unique diets may have evolved novel physiological ways of dealing with very low or high intake of certain nutrients, an under-explored avenue of inquiry. Results from ecological studies should also prove useful to researchers on humans and model organisms. To start with, many studies from the ecological literature would likely be of equal interest to other researchers, but are not likely to be found. For example, Alonso-Alvarez *et al.* (2007) showed that increasing the growth rate of nestling zebra finches by altering parental workload resulted in decreased resistance of red blood cells to an oxidative challenge, a result that might be of interest to obstetricians and neonatologists. Beyond such direct implications, understanding how oxidative systems vary across species and ecological conditions can help shape informative questions. As shown below, substantial effort has been invested in understanding how dietary micronutrients affect health in humans, with often ambiguous results. Ecological studies have shown that intake of certain antioxidants often has somewhat unpredictable effects on the levels of other antioxidants and on immune parameters, depending on which antioxidants and which species are being studied (e.g. Costantini & Dell'omo 2006; Hōrak *et al.* 2007; McGraw & Ardia 2003). An understanding of these results might have changed the questions posed about micronutrients and human health, leading to less simplistic hypotheses. Lastly, studies on model organisms are generally designed to either give us information about humans or about general processes; such inferences are problematic when there is substantial variation across species. The ecological literature can provide guidance as to which sorts of results from model organisms are likely to be generalizable and to aid in making useful inferences even when generalization is not possible.

However, ecological approaches alone also have substantial weaknesses. Novel measurement techniques may not be quickly transmitted to fields like ecology, where adoption can sometimes be slow. For example, lipid peroxidation can be measured much better by new isoprostane assays than by the traditional Thiobarbituric acid reactive substances (TBARS) assay (Liu, Stern & Morrow 1998); nonetheless, many ecological studies continue to use TBARS (Sodergren 2000). Ecologists often work on poorly characterized systems and thus face substantial challenges doing detailed biochemical and genetic studies. It is also difficult to gather the sample sizes available to epidemiologists. Study organisms are usually not

nearly as well understood as humans or model organisms. Many ecological studies are not able to include terminal sampling, limiting the sorts of questions that can be addressed. For these reasons, ecological studies are often better suited to examining variation across species and conditions but less suited to detailed examination of any specific system. For example, we might want to use a model organism such as mice to study how a series of subtle changes in diet and conditions affects levels of various oxidative balance biomarkers across many tissues. This could be done using a careful series of controlled experiments. However, in order to interpret the results, we would want to understand the extent to which the results were likely to be particular to mice or to the laboratory environment; this might be achieved by performing a small subset of the experiments on a variety of other species, including some in the wild.

It is also likely that the physiological roles of markers of oxidative balance evolve based on the needs of species, their diet, and reliance on alternative physiological mechanisms. A summary of the ecological roles of vitamin E in this context is provided in Appendix S1 as an example. This principle of evolving biochemistry and physiology creates an important bridge between ecology and other fields: other fields must consider physiology in the light of evolution and how it has affected the study of species (including humans), and ecologists must consider details of the biochemistry to understand how to interpret oxidative balance measures in a given study species. Likewise, biogerontologists seeking to understand ageing would do well to view it in light of how physiology evolves (Holmes & Martin 2009).

Epidemiological approaches to micronutrients and oxidative balance

Epidemiology grew out of the medical profession as doctors strove to understand population-level patterns that affected their patients. Accordingly, epidemiological studies of oxidative balance have primarily been conducted with discrete patient outcomes in mind. Relevant epidemiological approaches include long-term cohort studies where individual patients are followed over time; cross-sectional studies on the covariation of parameters of interest; and clinical trials (including everything from qualitative research to randomized controlled double-blind trials), which measure the effects of interventions (Rothman, Greenland & Lash 2008). Two somewhat separate branches of epidemiology have contributed to oxidative balance studies: nutritional studies of micronutrient effects on health (e.g. Miller *et al.* 2005) and biomarker studies of how oxidative stress fits into the pathology of ageing-related diseases such as metabolic syndrome, cardiovascular disease, Alzheimer's disease, and cancer (e.g. Butterfield & Lauderback 2002).

Relevant studies from nutritional epidemiology have focused on how micronutrients – as measured by dietary intake, serum levels, or supplements – have affected disease and mortality outcomes. However, results have often been inconsistent and puzzling. For example, many early

observational studies showed apparent health benefits of high dietary or serum vitamin E or supplements (Gey *et al.* 1991; Knekt *et al.* 1994; Stampfer & Rimm 1995; Wright *et al.* 2006). However, more recent meta-analyses of randomized controlled trials have shown potentially harmful effects (Bjelakovic *et al.* 2007; Miller *et al.* 2005). These discrepancies could be due to many factors, including failure of observational studies to properly control socio-economic and health consciousness differences in who takes supplements or biases in who gets recruited into different study types. What is clear, however, is that results of such studies have been much less straightforward than originally hoped: there appear to be either differences in effect that are quite sensitive to the population studied, or persistent measurement or confounding issues.

Epidemiological studies of oxidative stress in disease have usually focused on identifying associations between biomarkers of oxidative damage such as 8-hydroxy-2'-deoxyguanosine and disease outcomes such as cardiovascular disease or cancer (e.g. Vulimiri *et al.* 2000; Collins *et al.* 1998). Often, strong associations are found, but the interpretation is less clear: is oxidative stress causing disease, or is some other factor causing both? Most likely, oxidative stress can be harmful, but can also be exacerbated by other harmful processes, making precise quantification of its role problematic. Nonetheless, extensive long-term data bases of biomarkers and health outcomes have now been collected in many studies – including the Framingham Heart Study, the Women's Health and Ageing Study, and the Baltimore Longitudinal Study of Ageing, to name a few (Guralnik *et al.* 1995; Kannel & Mcgee 1979; Shock *et al.* 1984) – raising the potential for sophisticated statistical analyses of the interplay of many physiological factors in the ageing process over time, at least for those with access to the data. This is one of the more promising avenues for understanding the role of oxidative balance in ageing, at least in humans. Humans are an excellent study species because of the possibility of detailed environmental and social information, the availability of large sample sizes, large blood samples not feasible for small animals, and the ease of repeated sampling of individuals; this is of course balanced by a need to avoid harmful interventions, the length of time needed to follow a generation, and the logistical burdens associated with following ethical guidelines.

There are also a number of challenges to epidemiological studies of oxidative balance. First, it is nearly impossible to fully control diet in human studies, but there is great potential for interactions among diet components. Second, diet depends on many amorphous factors, including social class and culture, which are likely to affect health outcomes on their own; these factors are notoriously difficult to control for statistically. Randomized trials can control for these two problems, but also make it impossible to study the effects of such factors, and there are likely important interactions being missed. Third, effects are not likely linearly dose-dependent, and may depend on intake mode, so that results from studies of supplements cannot be compared directly with results based on surveys of diet or serum measurement. Fourth,

because most studies are conducted on otherwise healthy people, and because health-threatening interventions are not possible, most effect sizes are fairly small, necessitating a very large sample size to detect.

From an epidemiological perspective, the main goals of most studies have been practical: to provide recommendations on dietary intake or the advisability of supplements, or to identify clinical markers for use by physicians to predict disease. These are certainly laudable goals, but in our view, an opportunity has often been missed to use the same data sets to inform basic science. The same data that show whether to recommend a vitamin supplement (and the complexity of when this might be advisable) can provide potential insight into the factors that mediate disease and ageing processes. For example, longitudinal data sets on vitamin E intake and disease outcomes will often include other measures such as circulating vitamin E levels, oxidative stress markers, and inflammatory markers. These additional markers can be used to assess not just if vitamin E intake affects disease outcomes, but whether such effects are mediated by oxidative stress and inflammation: in other words, to look at pathways. Such implications are not completely ignored by the epidemiological literature, but we believe that there has been a tendency for the medical community to view basic science as the province of laboratory researchers and controlled experimental approaches, and thus to disregard useful information arising from population-level observational studies. In particular, the nature of oxidative balance systems is such that neither experimental nor observational approaches alone are sufficient to disentangle the convoluted relationships and modifying factors, and that the rich data sets collected in epidemiological cohort studies are precisely what are needed to help inform future research directions.

Ageing and oxidative balance

APPROACHES TO OXIDATIVE BALANCE IN THE AGEING LITERATURE

The ability to genetically manipulate individual components of antioxidant mechanisms has given researchers a powerful tool to further study the roles of ROS in ageing and disease (de Magalhães 2005). Multiple experiments have been carried out in laboratory model organisms – typically in mice – in which antioxidant genes were deleted or their levels manipulated. In some cases, such manipulations alter levels of oxidative stress and even result in pathology, but no genetic manipulation of antioxidants has been shown to alter the process of ageing (de Magalhães & Church 2006; Perez *et al.* 2009a). Of course, these experiments only focus on individual pathways for the most part, but it is still significant to observe that by and large manipulations of ROS and even of oxidative stress in model organisms (and mice in particular) fails to support the free radical theory of ageing. One recent work focused on overexpressing two major antioxidant enzymes in mice and found no effects on lifespan (Perez *et al.* 2009c). Another recent work showed that overexpression of an

antioxidant (superoxide dismutase) in mice, while resulting in a decreased lipid peroxidation and increased paraquat resistance, does not alter lifespan or age-related pathology (Jang *et al.* 2009). Similarly, mice deficient in two antioxidant enzymes exhibited increased levels of oxidative damage at the level of DNA and protein oxidation as well as increased incidence of neoplasms but not a shorter lifespan (Zhang *et al.* 2009). Taken together, the data from laboratory model organisms have led a number of researchers to suggest that the free radical theory of ageing – at least as originally suggested by Harman – is incorrect or at least insufficient (de Magalhães & Church 2006; Gems & Doonan 2009; Lapointe & Hekimi 2009; Perez *et al.* 2009a).

Reactive oxygen species were initially seen as toxic by-products of metabolism, but it is now clear that ROS are employed in multiple cellular functions, including defence against pathogens and as signalling molecules in multiple processes such as apoptosis, cellular growth, and proliferation (de Magalhães & Church 2006; Forman, Maiorino & Ursini 2010). One comparative study recently found a negative correlation between ROS production in heart mitochondria and species longevity in mammals and birds (Lambert *et al.* 2007). This is an interesting finding suggesting that long-lived species may produce lower levels of ROS, though in light of the multiple roles of ROS it may be related to differences in other life history traits – since long-lived species also tend to have a lower reproductive output and a slower growth – or in signalling pathways rather than lifespan (de Magalhães & Church 2006).

It should also be pointed out that studies of correlations between antioxidant defences or ROS and animal lifespan have by and large produced contradictory findings (de Magalhães & Church 2006; Finch 1990; Page *et al.* 2010). For example, the long-lived naked mole-rat (*Heterocephalus glaber*) has recently emerged as a model of successful ageing. Surprisingly, naked mole-rats have lower antioxidant activities and higher levels of lipid, DNA and protein oxidation levels than age-equivalent mice (Andziak, O'Connor & Buffenstein 2005; Andziak *et al.* 2006). One recent study reported that mice show an increase in cysteine oxidation with age whereas naked mole-rats do not (Perez *et al.* 2009b). The same work showed that naked mole-rats exhibit less protein ubiquitination and higher proteasome activity. Taken together, the emerging picture is that naked mole-rats have a more pro-oxidative cellular environment than mice (Andziak *et al.* 2006), which contradicts the idea that oxidation has negative physiological consequences, even though it is possible that naked mole-rats evolved mechanisms to counteract the higher levels of oxidation. More generally, these findings suggest that biogerontologists need to start considering how selection and constraints shape physiological systems over evolutionary time, and how this will affect the patterns observed within and across species.

Because of their proposed role in ageing, ROS has also been the subject of studies with human subjects. One recent study highlighting the complexity of ROS mechanisms showed increased levels of oxidative stress (increased

malondialdehyde and decreased glutathione peroxidase levels) in older adults that exercise (Mergener *et al.* 2009). It is well-established that during exercise ROS production increases, as do antioxidant levels, and a number of pathways that maintain intracellular oxidant–antioxidant homeostasis are activated during exercise (Ji 2008). This showcases how increased levels of oxidative damage are not necessarily related to reduce fitness since humans (and animals) have a great ability to adapt to them.

Despite many approaches for manipulating oxidative stress, it seems to have little effect on ageing. One hypothesis is that antioxidant defences are already optimized, and thus ROS are not a causal mechanism of ageing, even if they can be disrupted during ageing (as a secondary effect) and in particular diseases (as the drivers of disease). Activities that increase free radical production are counteracted by antioxidant defences and other mechanisms. In certain diseases, however, these mechanisms may be dysfunctional or insufficient and ROS likely play important roles (de Magalhães & Church 2006). For example, oxidative damage may contribute to the progression of neurodegenerative diseases (Butterfield & Lauderback 2002; Halliwell 2001), as well as other diseases (Valko *et al.* 2007). Therefore, it is possible that some manipulations of oxidative pathways impact on specific diseases and the slight increase in lifespan observed by feeding antioxidants to rodents supports this view. It is also possible that the forgiving laboratory conditions mask physiological weaknesses or susceptibilities in genetically modified strains that might be revealed under more challenging conditions.

A number of studies in ecology have focused on the simplistic view that ROS mediate life-history trade-offs, such as faster growth leading to a higher level of energy expenditure, a higher ROS generation and a shorter lifespan. For example, one study showed that reproduction in birds decreases antioxidant defences, and this has been interpreted as evidence that oxidative stress represents a cost of reproduction and may mediate trade-offs between reproduction and survival (Alonso-Alvarez *et al.* 2004). This is akin to the hypothesis by Harman that ROS mediate species differences in ageing as mediators of effects caused by variation in metabolic rates. The situation appears to be far more complex, however. When the effects of body size and phylogeny are corrected for, metabolic rates have been shown not to correlate with mammalian longevity, and the rate of living theory has been mostly discarded in biomedical research (de Magalhães, Costa & Church 2007; Speakman 2005a,b). While there are circumstances in which higher energy expenditure can be correlated to higher levels of free radical production or oxidative damage, assuming this will lead to a decrease in fitness seems too simplistic, given that organisms possess a great capacity to adapt to higher levels of ROS and damage.

POTENTIAL CONTRIBUTIONS OF ECOLOGY TO BIOMEDICAL STUDIES OF ROS

Even if ROS are not a causal mechanism of ageing, their deregulation appears to play a role in the pathophysiology of

certain diseases and thus understanding oxidative balance could have important biomedical applications. In this context, ecological studies have a great potential to contribute to biomedical knowledge. Specifically, understanding how organisms adapt to oxidative stress can be relevant and ecologists can focus on a much wider variety of environments than what can be achieved in the lab. Understanding how oxidative balance is achieved in a variety of extreme environments and conditions could have biomedical applications. Moreover, ecological studies could help to understand the phenotypic plasticity of organisms, its limits and how health could be impacted by exceeding these limits. Overnutrition, which has an enormous relevance to biomedical research in our modern societies, could be seen as such a case of exceeding the limits of phenotypic adaptation to diet set by a species' evolutionary history. Ecological studies can provide clues about the evolutionarily-determined limits for phenotypic plasticity that affect health in relation to environment (Bateson 2007).

Species that evolved unique mechanisms to cope with oxidative stress in different environments may provide relevant clues about mechanisms relevant to specific diseases, including age-related diseases. In other words, comparative methods could elucidate how organisms evolve in response to different kinds of selection for oxidative homeostasis. The AnAge database of species longevity may be a valuable resource to identify species of interest (<http://genomics.senescence.info/species/>). AnAge also provides over 3000 longevity records and data on life-history traits to serve as a basis for comparative studies of ageing (de Magalhães & Costa 2009). Numerous studies, including studies comparing antioxidants levels and ROS generation across species (Lambert *et al.* 2007; Page *et al.* 2010), have employed this benchmark data set as source of longevity data. Moreover, we would expect the physiological roles of ROS to evolve and thus different ROS and antioxidants could have different physiological roles in different species. Understanding this diversity may have implications in our understanding of disease and ecologists are well-positioned to study how different conditions lead to different roles for ROS.

Ecological studies of oxidative balance in birds have already shown a number of results that should begin to affect the questions biomedical scientists pose. Demonstration of a regulated change in antioxidant levels in response to stress contributes to a picture of homeostatic oxidative balance that may be most harmful when there is dysregulation (Wingfield *et al.* 1998). Complex patterns of correlations among different measures of oxidative balance within and across species are probably an important cautionary parallel for how to measure and interpret biomarkers in human studies across populations (Cohen & McGraw 2009). Carotenoids (including β -carotene) are antioxidants that are used by many bird species as pigments in sexual displays, presumably as an indicator of health status (Hill, Inouye & Montgomerie 2002). In several species, birds with high carotenoid levels appear to be healthier (McGraw & Ardia 2003; McGraw & Gregory 2004). However, many bird species also have very low carot-

enoid levels, and consensus is now that carotenoids contribute only a miniscule amount to the overall molar antioxidant capacity of most birds (Cohen & McGraw 2009; Costantini & Møller 2008). Taken together, these results suggest that the physiological roles of different micronutrients are flexible over evolutionary time, and that overall levels of protection may be much less important than localized protection of key tissues or lipid types.

Although the role of ROS in immune responses is well established, how these differ between species and environments is not yet understood. Therefore, we propose that ecologists also focus on studying inflammation in the context of oxidative balance and life history. There have already been a number of studies looking at oxidative balance in relation to immune function in wild birds (Costantini & Møller 2009), and the results have been mixed and somewhat confusing, perhaps because measuring immune function in the wild is as difficult as measuring oxidative balance. However, inflammation may well have a different role in the ageing process in mammals, and we specifically feel that an effort to understand the relationship between oxidative balance, inflammation, and ageing in the wild would be a productive route for mammalian physiological ecologists.

Laboratory physiology of model organisms

The advantages of laboratory studies on model organisms are perhaps the most generally appreciated. Controlled experiments can be used to precisely pinpoint cause-effect relationships and to disentangle mechanistic pathways, as long as the questions are clear and can be framed in terms of distinct alternatives. Study organisms are well characterized, and can be manipulated in sophisticated ways that take advantage of known genetics and thoroughly validated methodological techniques. Results are straightforward to replicate under similar conditions. The potential drawbacks mostly relate to external validity. Most laboratory species are inbred and short-lived – excellent characteristics in some ways, but hardly representative of other species or wild conditions, and it is hard to know which results might be generalized to other conditions, and which are particular to the laboratory environment (Holmes & Martin 2009). Controlled experiments by definition identify the effects of one factor that differs across groups; when those effects depend on many other factors simultaneously – as is often the case – controlled experiments can give a false sense of certainty when results may not be generalizable.

In terms of oxidative balance systems, studies of model organisms and biochemical pathways have helped to shape our understanding of the shared machinery of free radical production and antioxidants common to most organisms. For example, studies on mice (*Mus musculus*) and fruit flies (*Drosophila melanogaster*) have helped us understand the role of uncoupling proteins in regulating free radical production in mitochondria (Echtay *et al.* 2002; Miwa & Brand 2003). Most of the ageing studies cited above were conducted on model organisms. Lastly, studies of the pathology of oxida-

tive stress and of exercise physiology have relied heavily on rodent models. However, in all these cases, puzzling and contradictory findings have meant that a comprehensive understanding of oxidative physiology has remained elusive. Here, we look at how this has played out in studies of vitamin E in rodent physiology in brief, with a more detailed analysis included in Appendix S1.

Although the antioxidant actions of vitamin E *ex vivo* are unequivocal, a similar role *in vivo* is still intensely debated (Azzi 2007; Brigelius-Flohé & Davies 2007; Traber & Atkinson 2007). The role of vitamin E is most critical in neuromuscular function, and deficiency can result in loss of endurance capacity and eventually ataxia. However, very low levels (in the nanomolar range) are often sufficient, and it can take months of deprivation for adverse effects to become apparent in rats (Gohil *et al.* 1986). Nonetheless, a number of experiments have also failed to demonstrate adverse effects of deprivation in rats (Packer *et al.* 1989; Tiidus & Houston 1993).

From the perspective of oxidative balance, the question is whether any eventual pathology can be attributed to oxidative stress that occurs under conditions of vitamin E deficiency, and this is still not clear. Increased free radical activity has been detected in muscle and liver homogenates both from rats run to exhaustion and from vitamin E-deficient rats with or without endurance stress (Davies *et al.* 1982), but this has not been considered definitive evidence. The evidence for humans is also mixed, with several studies failing to show an effect of vitamin E on human physical performance (Gerster 1991; Sharman, Down & Sen 1971; Takamami *et al.* 2000).

Thus, what appears to be a straightforward question – the physiological role of an essential vitamin, particularly in preventing oxidative stress – has not been easily tractable through experimental approaches. This appears to be because of (i) a wide tolerance for varying levels of the vitamin; (ii) dependence of the adverse effects on particular conditions, including biochemical environment and amount of exercise stress; (iii) different effects of severe depletion and supplementation; (iv) multiple physiological roles of vitamin E; and (v) species-specific utilization patterns. Development of an α -tocopherol transfer protein knock-out mouse has been hailed as a major advance in our ability to answer some of these questions (Jishage *et al.* 2000), but we believe further insight will also come from combining the results of these laboratory studies with the observational approaches described earlier. For example, what is the distribution of vitamin E across tissues of birds during migration, as compared with at other times? How does dietary vitamin E intake associate with tissue levels and health status across species, and how does this depend on the particular metabolic needs of each species? Answering these questions alone will not provide definitive answers, but will increase the ability of laboratory studies to construct the best experiments to answer the most important questions.

Conclusions

A number of fields doing research on oxidative balance have simultaneously been finding that the systems involved are highly complex and difficult to characterize. Relatively simi-

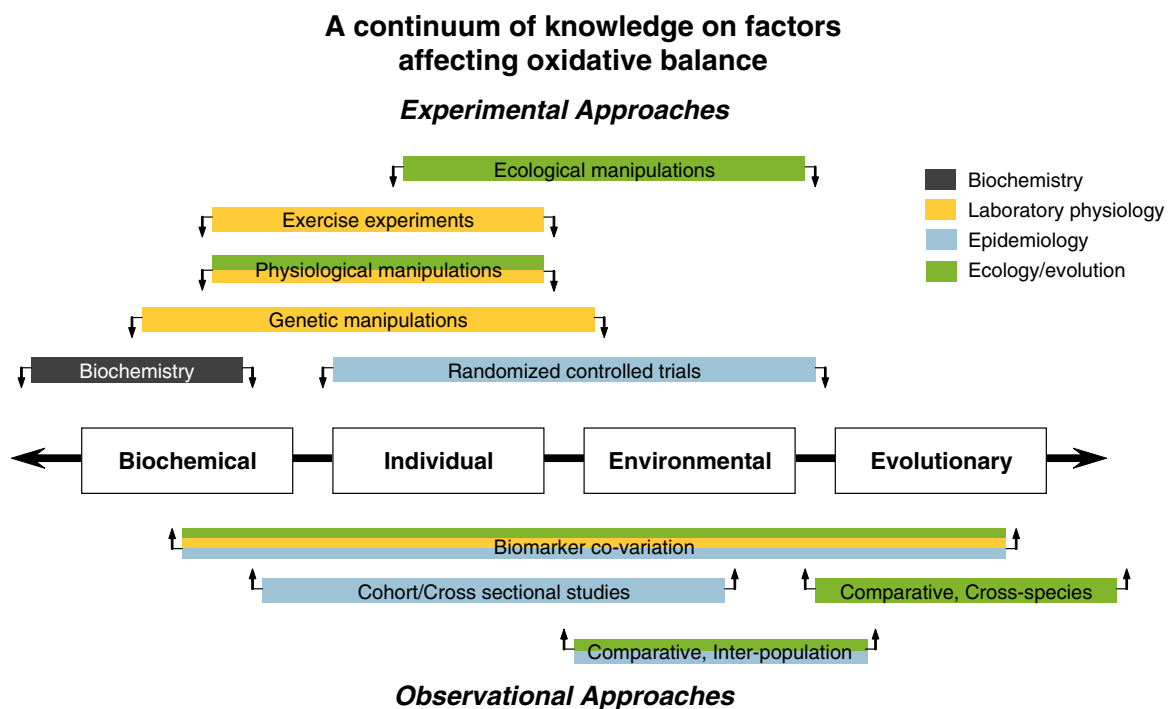


Fig. 1. Conceptual model for how different types of research integrate into a coherent understanding of oxidative balance and the factors that affect it. Factors throughout the continuum can be influenced by other factors from different parts – for example, there is variation in biochemistry across species.

lar studies often produce contradictory results, suggesting that many patterns are context-dependent. Given these difficulties, neither reductionist nor holistic approaches are able to provide a comprehensive understanding of oxidative physiology and its role in ageing, health, and fitness. The lack of knowledge regarding external validity makes experimental results hard to interpret, whereas observational studies suffer from the difficulty in interpreting biomarkers whose function and importance shifts with context. We have outlined here the strengths and weaknesses of several fields involved in the study of oxidative balance, and feel that a comprehensive understanding will require extensive collaboration across disciplines (Fig. 1). This is not a question of interdisciplinary studies being trendy, but rather a question of how to integrate knowledge from many levels of analysis to generate good research questions and approaches. An ideal scenario would be the development of a single large database of hundreds or thousands of physiological markers over time, in many individuals of many species, combined with a pre-specified set of controlled experiments designed to identify the precise interpretation of the markers and their relationships in each species.

The current structure of scientific research, where single researchers or small groups of collaborators pursue discrete grants for relatively limited projects, is not well suited to this type of research. The need for many experiments and many species outstrips the capacity of individual labs or small collaborations to accomplish, but the level of standardization required is probably not feasible without coordination from a central authority. In some ways, this is analogous to the standardization-versus-innovation trade-off across companies in industry; this is a circumstance when some of the innovation of individual labs might be worth sacrificing to achieve a standardized set of measures.

One potential partial solution may be for funding organizations to offer grants for narrowly specified projects – for example, to replicate one successful experiment as closely as possible in other species. Nonetheless, we feel it is unlikely that sufficient coordination will emerge from a top-down structure, and that much of the burden must be on individual researchers to not only become informed across fields, but to establish collaborations and projects that integrate well into the larger goals of disentangling a set of complex processes and how they vary across species, environments, and time scales.

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Supporting Information

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Appendix S1. Vitamin E as an example of progress and difficulties across disciplines.

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