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COMMENTARY

Understanding diversity in oxidative status and oxidative stress: the opportunities and challenges ahead

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ABSTRACT

Oxidative stress may be of profound biological relevance. In this Commentary, I discuss some key issues faced by the emerging field of oxidative stress ecology, and seek to provide interpretations and solutions. First, I show that the way in which we define oxidative stress has far-reaching implications for the interpretation of results, and that we need to distinguish between (1) a biochemical definition in terms of the molecular outcomes of oxidative stress (e.g. generation of oxidative damage) and (2) a biological definition in terms of the fitness consequences for the organism (e.g. effects on fertility). Second, I discuss the dangers of comparing different tissues and markers. Third, I highlight the need to pay more attention to the cross-talk between oxidative stress and other important physiological costs and functions; this will allow us to better understand the mechanistic basis of fitness costs. Fourth, I propose the 'redox signalling hypothesis' of life history to complement the current 'oxidative stress hypothesis' of life history. The latter states that oxidative damage underlies trade-offs because it affects traits like growth, reproduction or cell senescence. By contrast, the redox signalling hypothesis states that a trade-off between signalling and biochemical oxidative stress underlies the regulation of reactive oxygen species production and their subsequent control. Finally, I critically appraise our current knowledge of oxidative stress ecology, highlighting key research themes and providing an optimistic overview of future opportunities for the discipline to yield considerable insight into the ecological and evolutionary meaning of oxidative stress.

KEY WORDS: Antioxidants, Life history, Redox, Reproduction, Senescence, Signalling

Introduction

There is considerable evidence across a wide range of taxa that oxidative (or redox) status (see Glossary) plays a central role in biological processes. The redox system (see Glossary) is highly conserved across animals, but there are also significant differences among taxa; for example, in the amount of pro-oxidants generated or the types of antioxidants (see Glossary; Costantini, 2014; Halliwell and Gutteridge, 2015).

Over the last two decades, there has been much interest in understanding the diversity in pro-oxidant generation, antioxidant responses and molecular oxidative damage (see Glossary), as well as the fitness consequences and over what time scales such effects on fitness occur (e.g. Costantini, 2008, 2014; Isaksson et al., 2011a; Speakman et al., 2015; Vágási et al., 2019). For example, how does oxidative status vary as a function of species, population, sex, age, developmental stage, genotype, life history, season, habitat or abiotic factors? Does oxidative status affect the adaptiveness of species to environmental changes? Is oxidative stress significantly linked to other physiological costs or to life-history variation?

That reactive oxygen species (ROS; see Glossary) may be of profound biological relevance is exemplified by the fact that animals have evolved a number of antioxidant, detoxification and repair mechanisms to control ROS-mediated oxidation of biomolecules and avoid accumulation of oxidative damage or disruption of redox signalling (see Glossary; Costantini, 2014; Halliwell and Gutteridge, 2015). In bacteria and invertebrates, there is strong support for the idea that oxidative stress plays a significant role in the physiological adaptive response to abiotic stressors or as a mediator of life-history trade-offs (e.g. Costantini, 2014; Beaulieu et al., 2015; Janssens and Stoks, 2018). In vertebrates, although significant heterogeneity among species exists, there is good evidence that a higher growth rate results in greater oxidative damage and that oxidative damage itself may constrain growth strategy (Smith et al., 2016). However, results appear more conflicting when we come to the role of oxidative stress in determining lifespan or reproductive outcomes (e.g. Alonso-Alvarez et al., 2004, 2017; Isaksson et al., 2011b; Noguera et al., 2012; Stier et al., 2012; Costantini, 2014; Beaulieu et al., 2015; Costantini and Dell'Omo, 2015; Costantini et al., 2015; Blount et al., 2016; Costantini et al., 2016, 2017; Herborn et al., 2016; Colominas-Ciuró et al., 2017; Marasco et al., 2017; Merkling et al., 2017; Boonekamp et al., 2018; Casagrande and Hau, 2018; Losdat et al., 2018; Viblanc et al., 2018; Vágási et al., 2019). It is not trivial to assess the ecological and evolutionary relevance of oxidative stress, particularly in free-ranging animals. In the wild, it is difficult (or even impossible) to control variation in abiotic factors or exposure to contaminants, which have a significant effect on oxidative status. Also, field studies often use blood samples to measure oxidative damage or antioxidant levels. This raises issues about the generality of the results, because both the generators of ROS and the antioxidant systems are highly dynamic and compartmentalised; in addition, oxidative damage to different tissues potentially results in different fitness outcomes.

It is reasonable to expect that oxidative stress might significantly determine life-history variation, at least under certain environmental conditions or in particular species. Prior work has highlighted some unresolved issues about the links between oxidative stress and life histories, e.g. what is the functional significance of oxidative damage and why do different tissues respond differently to particular experimental manipulations (Costantini, 2014; Speakman et al., 2015)? However, this work has overlooked factors that might explain some of the apparent disagreement among the experimental results (e.g. how we define oxidative stress, when we measure oxidative damage, comparability of tissues).

In this Commentary, I discuss key theoretical and mechanistic aspects that we need to consider when defining oxidative stress and that are important for the interpretation of experimental results. I highlight some often-neglected features of the molecular response

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Glossary

Antioxidant
Any mechanism, structure and/or substance that prevents, delays, removes or protects against oxidative non-enzymatic chemical modification (damage) to a target molecule (Pamplona and Costantini, 2011). Molecular antioxidants may be enzymatic (e.g. glutathione peroxidase, superoxide dismutase, catalase) or non-enzymatic (e.g. glutathione, vitamin C, tocopherols, protein thiols).

Marker of oxidative status
Any biomolecule that (1) is part of the redox system, (2) provides information about individual oxidative status and (3) is measurable with dedicated laboratory methods (e.g. chromatography, ELISA, kinetic assay).

Oxidative damage
Non-enzymatic chemical modification of a given biomolecule (e.g. lipid, protein, DNA) caused by ROS.

Oxidative (redox) status
The amount of pro-oxidants and antioxidants that occur in cells/tissues. Among-individual variation in oxidative status might be due to different levels of oxidative damage, antioxidants or ROS production. It may also refer more specifically to the status of molecular groups that include both oxidised and non-oxidised forms of the same molecule (e.g. GSH:GSSG). The terms oxidative or redox status are also used to refer to other molecular processes that are not linked to oxidative stress (e.g. redox reactions in cellular respiration).

Reactive oxygen species (ROS)
Collective term that includes the oxygen radicals and some non-radical derivatives of oxygen, such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) (Halliwell and Gutteridge, 2015).

Redox signalling
Subcellular or cellular molecular communication mediated by (1) changes in the level of particular ROS or oxidative damage compounds or (2) the shift in redox status of given molecular groups, such as GSH:GSSG (e.g. Jones, 2006, 2008; D'Autreaux and Toledano, 2007; Collins et al., 2012).

Redox system
Group of interacting atoms or molecules that can either oxidise biomolecules (ROS) or protect biomolecules from ROS-mediated oxidation (antioxidants).

to oxidative stress that would reconcile some of the inconsistencies in the results of experimental manipulations. I also encourage a more integrative approach, where oxidative stress is analysed in relation to other relevant endogenous functions (e.g. inflammation, immunity) for which there is cross-talk with oxidative status. Finally, I highlight key future directions for the field of oxidative stress ecology.

Definitions of oxidative stress: moving towards a biological definition

The first definitions of oxidative stress focused on the biochemical aspects of the phenomenon without explicit consideration of the fitness consequences for the organism (Sies, 1985; Box 1). Implicit in these biochemical definitions is that, at the steady state, pro-oxidants and antioxidants are in balance, meaning no damage occurs. However, some oxidative damage is always generated, and particular molecules are oxidised and recycled back to their reduced form (Costantini and Verhulst, 2009). Thus, seeing oxidative stress as a global imbalance between all pro-oxidants and all antioxidants might be inadequate and conceptually limiting (Jones, 2006). These definitions also do not specify the kind of damage that is being generated. This is a major drawback because an imbalance between pro-oxidants and antioxidants might increase some but not all kinds of damage (Dotan et al., 2004), and the functional consequences might differ if, for example, proteins or nucleic acids are damaged. Furthermore, some damage might be either a form of protection or functionally

Box 1. Definitions of oxidative stress

Oxidative stress can be defined either biochemically or biologically. A number of biochemical definitions focus on the damage caused by oxidative stress. The following have been proposed as damage-centric biochemical definitions of oxidative stress: (1) a disturbance of the pro-oxidant–antioxidant balance in favour of the former, leading to potential damage (Sies, 1985, 1991); (2) the biomolecular damage caused by an attack of reactive species on the constituents of living organisms (Halliwell and Whiteman, 2004; Halliwell and Gutteridge, 2015); and (3) the rate at which oxidative damage is generated (Costantini and Verhulst, 2009). However, biochemical definitions of oxidative stress can also focus on the effects on cellular signalling. Two such signalling-centric biochemical definitions have been proposed: (1) a disruption of redox signalling and control (Jones, 2006); and (2) an imbalance between oxidants and antioxidants in favour of the oxidants, leading to a disruption of redox signalling and control and/or molecular damage (Sies and Jones, 2007). However, I propose that it is important to consider a biological definition of oxidative stress. From this point of view, one may consider biological oxidative stress to be any change in one of the molecular components of the redox system that has an effect on any metric of Darwinian fitness.

irrelevant, such as that to non-coding DNA. Also, the levels of the various antioxidants do not change in the same way in response to increased production of ROS, because antioxidants vary in function, have different substrates and differ in concentration and dynamics (Halliwell and Gutteridge, 2015). Although these issues are well known by those who are familiar with the field, they are very often still raised by people who are new to the area (personal observation).

Damage-centric definitions of oxidative stress (Box 1) also ignore the fact that oxidative stress may result from the disruption of signalling molecules with antioxidant properties [such as thiol molecules including GSH:GSSG (GSH, reduced glutathione; GSSG, oxidised glutathione)] without concomitant oxidative damage (Jones, 2006, 2008; Sies and Jones, 2007; Sohal and Orr, 2012). For example, pro-oxidant peroxides may disrupt signalling without accumulation of oxidative damage in the cell because oxidation of some protein antioxidants stimulates the release of signal-regulating kinase 1, which activates apoptosis (Saitoh et al., 1998; Zhang et al., 2004). Thus, a trade-off between signalling and oxidative stress might underlie the regulation of ROS, and this might have implications for life-history theory [an idea that I refer to as the 'redox signalling hypothesis' of life history (Box 2) to complement the 'oxidative stress hypothesis' of life history (Box 3)]. This trade-off might also be important in other contexts (e.g. tissue development: Love et al., 2013; immunology: Nathan and Cunningham-Bussel, 2013; spermatogenesis: Guerriero et al., 2014).

Biochemical definitions of oxidative stress also have other limitations. Although the term 'stress' is commonly used in the field, it is important to be aware of the effects that its use has on our thinking. The negative meaning given to the word 'stress' is actually misleading, because biochemical definitions of oxidative stress do not make any predictions about its consequences for organismal fitness.

So how should we define biochemical oxidative stress, if not in terms of the underlying biochemistry? Ultimately, oxidative damage, increased ROS production, decreased GSH:GSSG or up-/down-regulation of antioxidant enzymes all reflect a perturbation of the oxidative status, and each of them may have significant fitness consequences for the organism (Table 1). Therefore, it is reasonable to conclude that the biological importance of antioxidant enzymes or of other endogenous antioxidants (e.g. GSH) cannot be neglected in favour of a damage-centric view, particularly in the light of our poor

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Box 2. Redox signalling hypothesis of life history

This hypothesis states that a trade-off between signalling and oxidative damage underlies the regulation of ROS production and the control of ROS concentration, because ROS can both cause damage and regulate important physiological functions (e.g. tissue development and regeneration, egg maturation, immunity, activation of the antioxidant response) through their signalling activity (e.g. Dröge, 2002; D'Autreaux and Toledano, 2007). Thus, the redox signalling hypothesis of life history points to the importance of the cell-regulatory systems and complements the resource- or energy-based models of the oxidative stress hypothesis of life history.

It is difficult to study redox signalling in animals because of technical problems in measuring ROS; furthermore, markers of oxidative damage or antioxidants do not provide adequate insight into the molecular mechanisms underlying oxidative stress. However, redox signalling should be considered in studies of oxidative stress when designing experiments and interpreting results. For example, hydrogen peroxide is a ROS, and its levels are often quantified to give an estimate of oxidative stress, or organisms are experimentally exposed to it in order to increase oxidative stress; however, hydrogen peroxide is also a relevant redox signalling molecule because it can pass easily through cellular and mitochondrial membranes (e.g. Dröge, 2002; D'Autreaux and Toledano, 2007). Thus, quantification of hydrogen peroxide might not always be adequate to infer oxidative costs because it is difficult to disentangle its oxidative and signalling effects.

Often, when functions that rely on ROS are activated (e.g. during an immune response), a variable response involving both up- and down-regulation of antioxidant enzymes is observed. The antioxidant response of an organism has to be coordinated in such a way as to avoid disruption of the signalling activity of ROS, and this may explain such variable responses. This is also the case for the adaptation of skeletal muscle to exercise, which is mediated by ROS, and may explain the fact that antioxidant supplementation may disrupt this adaptation (Niess and Simon, 2007; Merry and Ristow, 2016).

Finally, even increased generation of lipid peroxidation compounds might not necessarily have detrimental consequences, because they can stimulate gene expression and cell survival. Thus, biochemical oxidative stress might not necessarily translate into biological oxidative stress if the signalling activity of ROS stimulates mechanisms that protect the organism. Therefore, ROS may be either positively or negatively associated with the expression of life-history traits.

Box 3. Oxidative stress hypothesis of life history

The current framework used by evolutionary ecologists to understand life-history diversity is based on the concept of trade-offs in the allocation of resources (e.g. energy, nutrients) to the key traits of growth, reproduction and self-maintenance (somatic protection). The oxidative stress hypothesis of life history suggests that the generation of molecular oxidative damage is the mechanism that drives covariation among life-history traits and self-maintenance. For example, the hypothesis predicts that increased reproductive effort causes oxidative damage, which should reduce reproductive or survival prospects (e.g. Alonso-Alvarez et al., 2004; Costantini, 2008). It also predicts that oxidative damage should constrain investment in growth or in reproduction. For bacteria and invertebrates, in comparison to vertebrates, there is better support for the idea that oxidative stress has important effects on fitness, probably because much more research has been carried out, it is easier to perform experimental manipulations and long-term experiments, and it is possible to make whole-organism homogenates, thus overcoming the issue of among-tissue variation in oxidative status and resistance to oxidative stress.

There is scepticism as to whether the oxidative stress hypothesis can be generalised across conspecific populations living in different environmental contexts or across multiple species. Also, the link between oxidative stress and nutrition or energy expenditure is more complex than commonly assumed: there is no direct quantification of how much energy or nutrition is required by, for example, molecular systems that repair damage or produce antioxidants. In addition, biochemical evidence shows that ROS and even some of the products of oxidative damage (e.g. those from lipid peroxidation) may stimulate physiological adaptive responses through signalling, such as activation of enzymes that remove them from cells. Thus, the oxidative stress hypothesis of life history appears to be incomplete; other aspects, such as the mechanisms that regulate ROS production (e.g. mitochondrion, peroxisome, immune cells) and ROS concentration (e.g. antioxidants such as superoxide dismutase, glutathione peroxidase, catalase) require careful consideration.

understanding of oxidative stress in free-living organisms. It is difficult to determine the severity of oxidative stress based on observed changes in components of the redox system. For example, increased cellular necrosis and apoptosis might reflect high oxidative stress at the biochemical level, while upregulation of antioxidant enzymes might reveal a condition of low to intermediate oxidative stress in which enzymes are not being inactivated by ROS or it is physiologically possible for the organism to upregulate them (Lushchak, 2014). Another important distinction to make is between acute (or transient) and chronic oxidative stress, because their consequences for organism fitness might differ dramatically (Dröge, 2002; Lushchak, 2014). It is therefore clear that we need to distinguish between a biochemical and a broader and more biologically oriented definition, which points to the effects of oxidative stress on fitness (Box 1). For example, a study that lacks quantification of fitness outcomes should refer to biochemical oxidative stress, whereas if a cause–effect relationship between biochemical oxidative stress and fitness outcome can be established, then we should refer to it as biological oxidative stress.

Conflicting results are not always truly conflicting

Research on the link between oxidative status and reproduction or lifespan has yielded seemingly conflicting evidence. For example, different studies report that (i) reproducing individuals have either higher or lower oxidative damage in their blood than non-reproducing individuals, (ii) reproducing individuals have higher oxidative damage than non-reproducing individuals in some tissues but not others, (iii) oxidative stress increases with parental effort in some species but not in others, (iv) damage is not always related to future survival or reproductive probability and (v) markers of oxidative status (see Glossary) within an individual correlate only weakly (e.g. Alonso-Alvarez et al., 2004, 2017; Stier et al., 2012; Costantini, 2014; Costantini and Dell'Omo, 2015; Costantini et al., 2016; Beaulieu et al., 2015; Blount et al., 2016; van de Crommenacker et al., 2017). There are, however, mechanistic reasons why these inconsistencies might emerge, which I discuss below.

Variation in oxidative status among tissues: are we comparing apples and oranges?

When conducting research on the oxidative status of free-living animals, blood is the tissue of choice because its collection is not terminal, allowing longitudinal studies that track within-individual changes in oxidative status and link them to the individual's life history. In contrast, research on laboratory animals often relies on a multi-tissue approach, but this means that studies are cross-sectional; thus, individual plasticity or genetic heterogeneity may hide important temporal changes in oxidative status.

Several laboratory studies have found that the response of a given marker to an experimental treatment (e.g. increased reproductive effort, quality of diet) may differ among tissues. For example, experimental manipulations of reproductive effort may increase

Table 1. Examples of experimental manipulations of specific components of the redox system that had a significant effect on fitness-related traits

Manipulation	Species	Effect	Article
Increased ROS	*Caenorhabditis elegans*	Reduced longevity	Larsen, 1993; de Castro et al., 2004
	Passer domesticus	Reduced sperm quality	Rojas Mora et al., 2017
Suppression of GSH synthesis	*Serinus canaria*	Delayed laying date and reduced clutch size	Costantini et al., 2016
	Taeniopygia guttata	Increased reproductive effort	Romero-Haro et al., 2016
	Sturnus vulgaris	Reduced song rate	Messina et al., 2017
Knock out of antioxidant genes	Laboratory mouse	Accelerated pathology and reduced healthy lifespan	Salmon et al., 2010
Knock out of antioxidant enzyme Cu, Zn-SOD	*Salmonella typhimurium*	Reduced survival	De Groote et al., 1997
	Caenorhabditis elegans	Increased production of superoxide and reduced lifespan	Yanase et al., 2009
Increased GSH	*Oryctolagus cuniculus*	Reduction in activity of herpesvirus	Nucci et al., 2000

ROS, reactive oxygen species; GSH, reduced glutathione; SOD, superoxide dismutase.

oxidative damage in one tissue and decrease it in another in female birds or mammals as compared with controls, particularly under captivity conditions (Blount et al., 2016). Implicit in these findings is that results obtained from a single tissue cannot be generalised, because they might not reflect the systemic oxidative status. This also raises the question of whether damage to tissue X or to tissue Y is the more important and, if so, why? Some tissues might be less protected than others if fitness is not affected when they are exposed to biochemical oxidative stress. The speculation that oxidative stress might not be a relevant cost of reproduction or that blood-based markers are neither functionally relevant nor good indicators of oxidative stress neglects at least two important points. First, both the short- and long-term functional or fitness consequences of biochemical oxidative stress are rarely explored. Thus, it is hard to infer anything about whether organisms prioritise protection of those tissues that are more important for fitness. Second, values of any marker of oxidative status measured at a given point in time might not be comparable among tissues.

Tissues differ in properties such as antioxidant machinery, propensity to accumulate or repair damage and cellular turnover (Halliwell and Gutteridge, 2015). However, a large body of literature also shows that a comparison of oxidative damage or antioxidants between experimental groups at a single point in time may be flawed because tissues also differ in the time lag of their response to an experimental treatment. These studies show that, over a certain time frame, all tissues at some point may suffer increased oxidative damage or show changes in the levels of certain antioxidant molecules in the absence of any mortality of individuals. For example, zebrafish (*Danio rerio*) exposed to cold have increased levels of ROS in liver, brain and gills, but the peak level of ROS occurs at different times over the experiment in the various tissues analysed (Wu et al., 2015).

Similarly, the time course of expression of antioxidant genes changes among tissues (Wu et al., 2015). The kinetics of the response of various antioxidants or generation of damage varies even within a single tissue (Regoli and Giuliani, 2014; Dong et al., 2019; Pedro et al., 2019). In humans, after a bout of physical effort leading to exhaustion, the time to the lowest concentration of different markers (e.g. GSH, GSSG, lipid oxidative damage, catalase) varies from 0.5 to 4.4 h on average (Michailidis et al., 2007). The time lag in the response of markers may also depend on the amount of tissue damage generated. For example, after a low-damage exercise, protein carbonyls (a marker of protein oxidative damage) are increased significantly 30 min later, peak at 4 h after exercise and decline thereafter, reaching resting levels by 24 h (Nikolaidis et al., 2012). However, after a high-damage exercise, protein carbonyls are significantly higher than resting levels 1 day after exercise, peak at day 3 after exercise and decline thereafter, reaching resting levels by day 7 (Nikolaidis et al., 2012). Thus, the absence of any changes in markers of oxidative status measured in blood collected immediately after a treatment does not indicate the absence of biochemical oxidative stress. Also, detection of increased oxidative damage in tissue X, but not in tissue Y, does not enable us to infer conclusively that tissue Y is more protected than tissue X, because increased oxidative damage in tissue Y might become detectable later. In other words, these studies show that, in order to detect oxidative stress, the sampling time might be as crucial as the target tissue, and that more research on the kinetics of oxidative status markers is needed. This could explain at least some of the inconsistences in the response of tissues or markers to a given experimental manipulation (e.g. increased reproductive effort).

In addition, the interpretation of results from laboratory studies is not so straightforward for a number of reasons: (i) much laboratory research has been carried out under unnatural and non-standardised conditions; (ii) individual history (e.g. previous reproductive or early-life experience) is typically not considered (Marasco et al., 2013; Zimmer and Spencer, 2015; Romero-Haro et al., 2016), which might be one reason for the large variation in oxidative stress resistance among similar individuals (e.g. Costantini, 2014; Margaritelis et al., 2018); (iii) the basic biology of a species is sometimes ignored; and (iv) studies typically cover a narrow taxonomic breadth and are often carried out on unnatural laboratory strains.

Are we right to use blood as our tissue of choice?

Do basal levels of blood-based markers reflect local or systemic oxidative status? The available evidence suggests that some markers correlate significantly across tissues, suggesting that these markers might be better than others for estimating systemic oxidative status. Argüelles et al. (2004) found that the levels of lipid hydroperoxides (markers of lipid oxidative damage) correlate significantly across five tissues (blood, liver, spleen, heart and kidney). In contrast, the levels of protein carbonyls in blood were correlated with those in liver and heart, but not in spleen and kidney. The non-enzymatic antioxidant capacity was not correlated across all analysed tissues (see also Fig. 1). Veskoukis et al. (2009) also found that the non-enzymatic antioxidant capacity was not correlated across four tissues (blood, muscle, heart and liver), whereas GSSG was significantly correlated across all four tissues. Several significant positive correlations were also found for TBARS (a marker of lipid oxidative damage), protein carbonyls, GSH and catalase. A recent systematic review of 101 articles concluded that TBARS, GSH,

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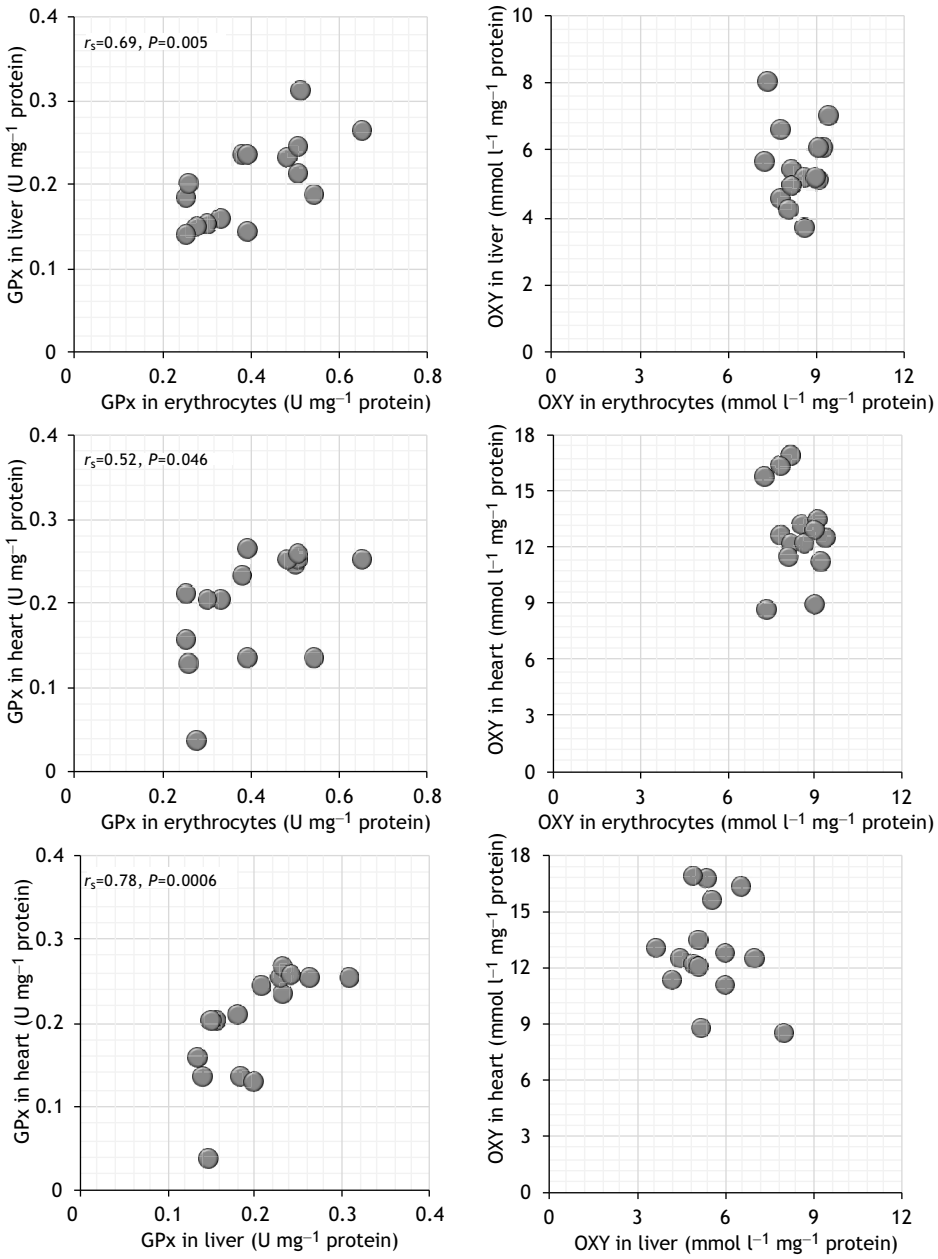

Fig. 1. Correlations among antioxidant markers across different tissues in adult zebra finches (*Taeniopygia guttata*). The activity of the enzymatic antioxidant glutathione peroxidase (GPx; measured using the Ransel test, Randox Laboratories, UK) and the *in vitro* non-enzymatic antioxidant capacity (OXY; measured by the OXY-Adsorbent test, Diacron International, Italy) were measured in blood (using haemolysates obtained from lysis of erythrocytes in buffer) and in homogenates (in buffer) of liver and heart. GPx activity was positively and significantly correlated (Spearman's rank correlation, r_S) among the three tissues. In contrast, the non-enzymatic antioxidant capacity was not correlated across the same three tissues. D.C., unpublished data.

superoxide dismutase, catalase and glutathione peroxidase measured in blood adequately reflect their level in other tissues (Margaritelis et al., 2015). In contrast, this is not true for GSSG and the GSH:GSSG ratio.

The variable agreement of measurements of antioxidant capacity across tissues (Fig. 1) may be explained by the lack of specificity in what assays of non-enzymatic antioxidant capacity actually measure. The concentration of non-enzymatic antioxidants varies across tissues (Halliwell and Gutteridge, 2015), meaning that while these assays may provide relevant information for the tissue being analysed, they do not allow one to infer the non-enzymatic antioxidant status in other tissues. By contrast, the higher consistency in the levels of antioxidant enzymes among tissues (Margaritelis et al., 2015; Fig. 1) may be expected owing to their ubiquitous expression across tissues and transcriptional regulation. Regarding GSSG and GSH:GSSG, the poor agreement among tissues may be due to the difficulties of measuring GSSG in a reliable way – assays may not be sensitive enough and *ex vivo* oxidation of GSH may significantly increase GSSG concentration (Giustarini et al., 2016).

Forgetting the unforgettable: biochemical and biological meaning of markers

A large number of methods are available to quantify markers of oxidative status. These include assays that target single molecules (e.g. GSH) or groups of molecules (e.g. TBARS for the quantification of metabolites from lipid peroxidation). These molecules differ in dynamics, can be formed through different independent biochemical reactions and can be under transcriptional and post-transcriptional control, which may differ by tissue type (Halliwell and Gutteridge, 2015). Molecular substrates also differ in their propensity to be oxidised by ROS. It is therefore clear that correlations among markers are difficult to predict in terms of their strength and consistency across tissues, even when there is *in vitro* evidence that the different molecules measured are known to interact biochemically. This is even true when markers are measured in

whole-body homogenates. For example, in the octocoral *Veretillum cynomorium*, the activities of catalase and glutathione *S*-transferase increase during the emersion phase, whereas superoxide dismutase activity and the lipid oxidative damage remain stable (Teixeira et al., 2013). By contrast, the concentration of lipid oxidative damage and the activity of superoxide dismutase increase significantly during immersion in water, while low activity was found for catalase and glutathione *S*-transferase (Teixeira et al., 2013). This study also highlights that the functional connection (or correlation) in the temporal dynamics between markers might be more informative than calculating simple intra-day or intra-group correlations between them as is usually done. The way this temporal connection is achieved might reflect the physiological strategy used to regulate the oxidative status while coping with demanding conditions.

Markers of oxidative status might also differ in their fitness consequences. For example, nestling magnificent frigatebirds (*Fregata magnificens*) affected by a severe viral pathology have higher lipid oxidative damage than healthy nestlings, while protein oxidative damage or antioxidant enzymes do not differ between sick and healthy nestlings (Sebastiano et al., 2018). It might be argued that this inconsistency makes it hard to determine the link between oxidative stress and the pathological status of the birds. However, as discussed above, there are many reasons to expect a lack of correlation among markers, which should theoretically be more common than significant correlations among them. Most importantly, the amount of lipid oxidative damage is associated with the short-term survival probability of nestlings, indicating that such specific damage might be functionally relevant (Sebastiano et al., 2018).

Oxidative stress and other endogenous functions

When investigating the mechanisms underlying life-history trade-offs, it is often forgotten that the expression of a phenotypic trait is regulated by a complex network of mechanisms. Unfortunately, few studies have attempted to integrate research on oxidative stress with that on other endogenous functions in free-living animals, such as the function of the immune and endocrine systems, metabolism or gene expression (reviewed in Costantini, 2014).

Consider, as an example, the significant cross-talk between oxidative status and innate inflammation-based immunity (Sorci and Faivre, 2009; Costantini, 2014; Sebastiano et al., 2018). Inflammation is triggered when the activity of innate immune cells is increased by infection or tissue injury (e.g. muscle damage due to strong physical effort). Immune cells produce ROS to kill bacteria. Thus, it may be necessary to downregulate antioxidant enzymes at early stages of the immune response, otherwise they would remove ROS, reducing the effectiveness of the immune response. The action of ROS is, however, not specific, and can thus also cause oxidation to the host macromolecules or to the immune cells themselves. This suggests that individuals might show a less than optimal inflammatory response as a consequence of natural selection operating to optimise a compromise between costs and benefits of an immune response.

Inflammation-inducible proteins (e.g. ceruloplasmin, haptoglobin, ferritin) offer another example of how inflammation and oxidative stress may be connected to each other. Apart from their anti-inflammatory role, these proteins contribute to antioxidant protection by binding haemoglobin, haem or metal ions, which may trigger potential radical-generating reactions (e.g. Pacht and Davis, 1988; Wang et al., 2001). Metal chelation is one major way to control lipid peroxidation and DNA damage (Halliwell and Gutteridge, 2015), which is also important because oxidised lipids may promote chronic inflammation (Azzi et al., 2004; Que et al., 2018).

Perspectives and future directions

In this Commentary, I encourage careful consideration of some key points in future research to advance the discipline theoretically and experimentally. The oxidative status of tissues or the response of oxidative status markers to a given challenge might not be directly comparable. Standardisation of marker concentrations across tissues might be needed, although these are not easy to perform. In this context, it might be worth exploring means to control for differential protein turnover or metabolic rates between tissues. Moreover, looking at bivariate correlations between markers of biochemical oxidative stress within and among tissues to assess consistency is over-simplistic. Rather, we should also look at the temporal co-dynamics of the various markers available and carefully consider their different kinetics and biochemical properties. In addition, we need to learn more about the oxidative status physiology of the blood. One way to do so is by the integration of analyses of oxidative status markers with metrics of other important endogenous functions, such as activity of immune cells and inflammation-inducible proteins (Sorci and Faivre, 2009), hormones (Marasco et al., 2013) or mitochondrial function (Stier et al., 2017).

It is important to consider time when assessing the costs and benefits of oxidative stress. We need to measure multiple markers multiple times (avoiding anaemia) before and during an experiment if we aim to determine the dynamics of the individual's oxidative status and to detect biochemical oxidative stress. While baseline values of oxidative status markers provide information about constitutive levels, inducible levels reflect the individual physiological responsiveness to a given stressor (Dotan et al., 2004; Costantini, 2014; Casagrande and Hau, 2018). Furthermore, antioxidant enzyme activities and GSH need to be assessed more frequently in free-living animals because, as compared with some non-enzymatic antioxidants, their functions in the regulation of oxidative status are better known and may have fitness consequences (Norte et al., 2008; Koivula et al., 2011; Casagrande and Hau, 2018).

There is considerable interest in understanding the mechanisms underlying the role of oxidative stress in life-history trade-offs. In this context, we should remember that prior individual experience or phenotypic quality are significantly associated with resistance to oxidative stress (van de Crommenacker et al., 2011; Costantini et al., 2015; Zimmer and Spencer, 2015; Romero-Haro et al., 2016; Messina et al., 2017), thus we need to pay more attention to life-history costs at an individual level. We also need to implement resource- or energy-based models, because the link between oxidative stress and nutrition or energy expenditure is more complex than commonly assumed (e.g. Barja, 2007; Murphy, 2009; Costantini, 2014). We should also focus on other aspects; for example, how does an organism balance the need to use ROS as cellular signals against their pro-oxidant and, potentially, detrimental action (this is the basis of the redox signalling hypothesis of life history, Box 2)? It is worth noting that estimates of within-individual repeatability of markers over time and in different contexts would provide information about the plasticity of and endogenous constraints operating on the markers, but these are rarely done (e.g. Saino et al., 2011; Récapet et al., 2019).

When addressing the interaction of oxidative status with life-history traits, it is important to note that comparison of oxidative status markers among individuals at different life stages (e.g. reproducing and non-reproducing, migrating and non-migrating) or under different environmental conditions is not straightforward, because ecological and endogenous pressures acting on them may differ. Quantifying changes in the expression of genes involved in antioxidant pathways might shed light on the mechanisms that regulate oxidative status and

on how the organism adjusts its oxidative status to environmental stimuli. In addition, it is important to remember that some types of damage (e.g. to DNA or lipids) are reversible because of repair mechanisms. Thus, the functional relevance of this damage might also be dependent on the capacity to repair it (Lamare et al., 2006; Robert and Bronikowski, 2010), but this is often not measured.

Experiments on captive animals can be useful in some contexts; however, captive experiments often lack standardisation of the housing conditions or expose animals to unnatural abiotic conditions (temperature, light and humidity) and social constraints; this may induce measurable physiological changes that might bias experimental results (Beaulieu, 2016; Costantini, 2016). Using laboratory models allows us to delve into mechanisms and identify relevant markers. However, if we seek to understand the role of oxidative stress in determining fitness outcomes, we do not really need to design experiments that disentangle the effects of diverse factors on fitness traits or that describe the molecular pathways.

Conclusions

As a formal discipline, oxidative stress ecology has been growing for just over 10 years. There is now considerable evidence that oxidative stress might play a significant role in the physiological adaptive response to environmental stressors and in generating phenotypic diversity. However, we have yet to clarify the extent to which oxidative damage or changes in given antioxidants affect reproductive strategies or senescence for different circumstances, co-specific populations and species. These issues could be addressed by the types of study outlined here.

In this Commentary, I have highlighted the need to move beyond the classical biochemical definitions of oxidative stress to distinguish regulatory changes of oxidative status (e.g. those involved in immune function or physiological adjustments to photoperiod) from those that have significant effects on Darwinian fitness. Biochemical oxidative stress might not necessarily translate into biological oxidative stress (Box 1). I have also discussed the dangers of comparing tissues or of treating different measures of oxidative damage or antioxidants as conceptually equivalent. Much more work will be needed on the connections among oxidative status and other endogenous functions to further understand the role of biochemical oxidative stress in mediating life-history trade-offs. Such work should be performed while keeping in mind both the redox-signalling and the oxidative-stress hypotheses of life history.

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Competing interests

The author declares no competing or financial interests.

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