Oxidative stress as a cost of reproduction: Beyond the simplistic trade-off model

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The idea that oxidative stress may underpin life history trade-offs has become extremely popular. However, experimental support for the concept has proved equivocal. It has recently been suggested that this might be because of flaws in the design of existing studies. Here, we explore the background to the oxidative stress hypothesis and highlight some of the complexities in testing it. We conclude that the approach recently suggested to be least useful in this context (comparing reproducing to non-reproducing animals) may in fact be the most powerful. Moreover, suggested alternative approaches of limiting food supply or manipulating litter sizes have many complexities and problems. We suggest some useful alternative approaches that have not been previously advocated, particularly the study of individuals reproducing at greater parity later in life. Finally, the measures of oxidative stress and tissues that are analysed influence the experimental outcome. This suggests our conceptual model of the trade-off is currently too simplistic, and that studies based on single or limited numbers of assays, or restricted to single tissues, whether they support or refute the theory, should be interpreted with great caution.

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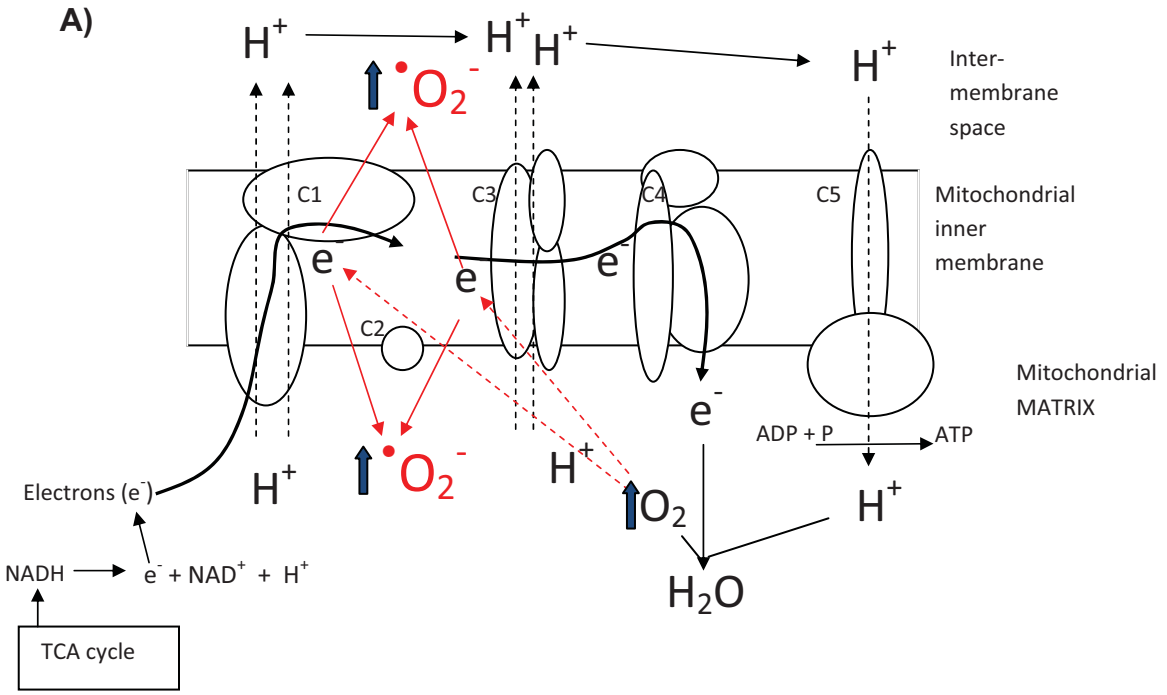
Introduction: Oxidative stress as a theoretical cost of reproduction

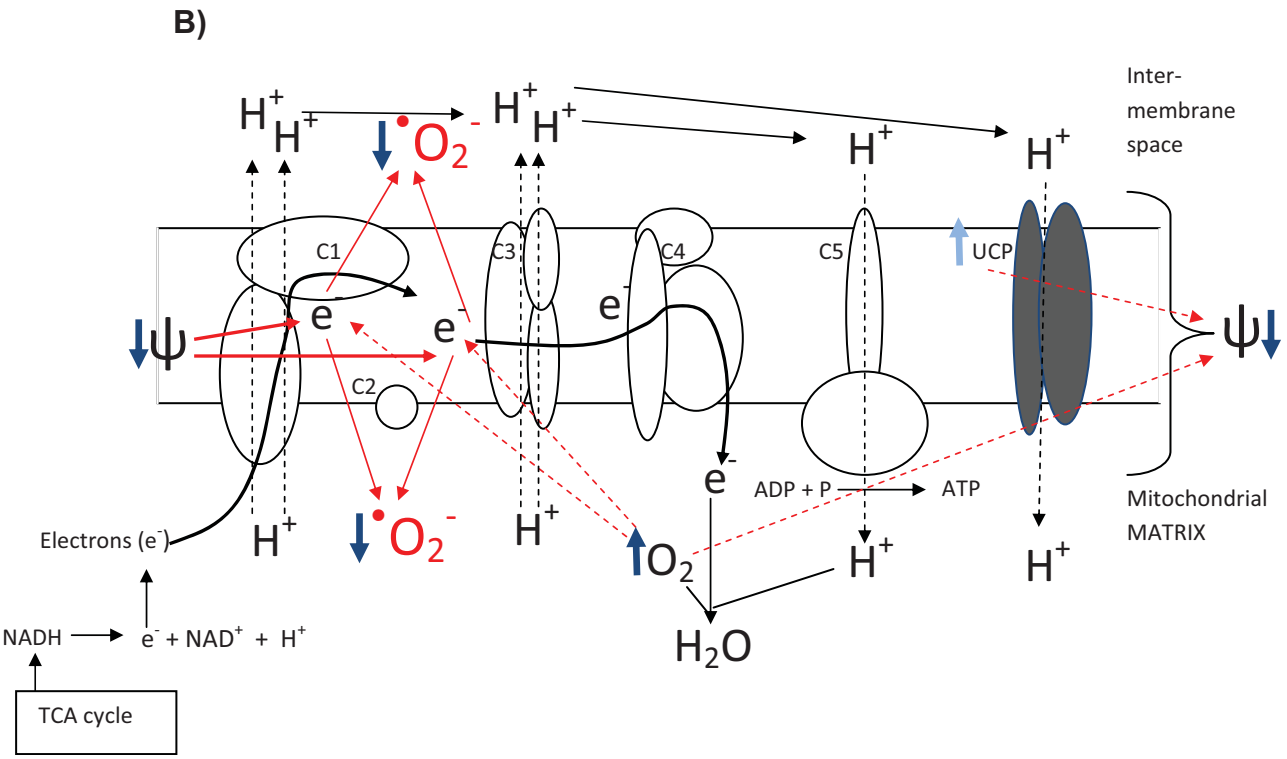
The concept that there are trade-offs in life history parameters is a fundamental aspect of evolutionary ecology [1, 2], and it is widely assumed that there are physiological processes underlying such trade-offs [3, 4]. During the last decade interest in these fundamental physiological mechanisms has intensified. One particular idea, that has captured the imagination of the ecological community, is that the trade-off between reproductive investment and survival may be due to free-radical production and oxidative stress. This idea has its origins over 100 years ago, when Rubner [5] observed that the product of lifespan and metabolic rate across species is almost constant. This observation implied that having a high metabolic rate involves either more rapid depletion of some vital compound, or generation of a toxic by-product that is injurious to health. This notion was subsequently encapsulated in the 'rate of living' (ROL) theory [6], the idea that living fast is inevitably linked to dying young. The 'ROL' theory was given a molecular mechanism in the 1950s when it was noted that during oxidative phosphorylation, the process by which ATP is generated in cells, there is a leak of electrons from the electron transport chain, and that these electrons become involved in promiscuous reactions with free-oxygen, leading to oxygen free-radical production [7, 8]. Such free-radicals are involved in further reactions to form radical oxygen species (ROS) such as the hydroxyl radical ($HO^{\bullet}$) that can be extremely toxic, causing oxidative damage to molecules such as lipids, proteins and DNA. Animals have sophisticated systems to neutralise ROS, and additional mechanisms to repair or mitigate the damage. Nevertheless, some damage always evades these systems and consequently oxidative damage accumulates ultimately leading to dysfunction (ageing) and mortality [9].

The disposable soma theory

The disposable soma theory [10] was formulated in the 1970s around the concept that individuals have limited resources

that they can either devote to reproduction or to somatic maintenance (e.g. protection against ROS). The fundamental basis of the disposable soma theory is the idea of a trade-off between lifespan and reproduction, due to differential allocation of resources between competing demands for reproduction and somatic protection. Although it was not worded in the ecological terminology of life history theory, one potential aspect of the disposable soma theory is





protection from oxidative stress. The 'oxidative stress life-history theory' as adopted by ecologists is effectively a subset of the disposable soma theory. The concept is that free-radicals and ROS are produced in direct proportion to metabolic rate as an inevitable consequence of the molecular functioning of mitochondria and the electron transport chain. Higher rates of metabolism that accompany reproduction therefore inescapably lead to greater free-radical production (Fig. 1A). Because individuals have limited resources to allocate to somatic protection, the resultant damage is the proximal mechanism that leads to increased mortality. There are some obvious attractions of this idea, in particular, it is simple and provides a direct link between physiology and ecology. Unfortunately, in embracing this idea ecologists ignored a large body of work that by 2008 had effectively shown some of the key foundations on which the oxidative stress model is based are critically flawed.

Metabolism as a cause of free radical production and ageing

The notion that oxygen free-radical production is directly and positively related to oxygen consumption (Fig. 1A), and hence inevitably linked to metabolism, as a fixed proportion, is simply incorrect. Oxygen radical production by mitochondria does occur, but it is crucially dependent on the mitochondrial inner membrane potential [11] (Fig. 1B). This membrane potential is lowered when metabolism increases, or directly lowered when uncoupling proteins (or other proteins such as the adenine nucleotide translocase: ANT) embedded in the membrane short circuit the system, allowing protons to travel back to the mitochondrial matrix without generating ATP. Increases in metabolic rate may also lower local levels of oxygen partial pressure, further contributing to reduced ROS production [12]. 'Uncoupling' proteins can further impact ROS production in other ways unrelated to their effects on membrane potential, by for example altering substrate utilisation patterns. In reality then this means higher rates of metabolism often generate lower levels of radical oxygen species (Fig. 1B). Consistent with this latter model, cross sectional studies have shown that individual mice with HIGHER metabolic rate were more uncoupled, and lived longer [13], a finding recently repeated in Glanville fritillary butterflies (*Melitaea cinxia*) [14]. Experimental elevation of metabolism by cold exposure had minimal impact on either oxidative stress or lifespan [15, 16] and experimentally elevating metabolism by activating uncoupling protein 1 chemically [17] or genetically [18] both lead to INCREASED lifespan and metabolic rate. Finally, studies of the link between lifespan and metabolic rate across species, utilising phylogenetically independent contrasts and appropriate corrections for body mass, showed that the original linkage postulated by Rubner [5] between metabolism and lifespan (and reiterated on many subsequent occasions) was simply an artefact of inappropriate statistical analysis [19, 20].

Oxidative stress and ageing

Evidence is also accumulating to suggest that oxidative stress is not the predominant cause of ageing, at least not in the

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Figure 1. Two models for how free-radicals are generated during oxidative phosphorylation in mitochondria. The model in Fig. 1A shows the electron transport chain on the inner-membrane of the mitochondrion comprising complexes 1–5 (C1 to C5). Electrons derived from substrates from the TCA cycle in the mitochondrial matrix enter the electron transport chain at complex 1 (C1). [Note that some electrons derived from other substrates also enter at C2 but are not illustrated here.] Complex 1 is a large protein embedded in the inner membrane of the mitochondria. Energy in the electrons is used to pump protons from the matrix into the inner membrane space at both complex 1 and complex 3 (C3). The electrons then pass to complex 4 (C4) where they pass back to the matrix and combine with molecular oxygen and protons to form water. The protons in the inter-membrane space move back to the matrix via complex 5 (C5): ATP synthase, and their chemiosmotic potential is used to drive synthesis of ATP from ADP and inorganic phosphate. Occasionally, however, oxygen reacts promiscuously with the electrons in the transport chain (red dotted lines) generating superoxide free-radicals ($^{\bullet}O_2^-$). These reactions occur predominantly at C1 and C3 and produce superoxide radicals on both sides of the membrane. ATP is the substrate of most energy consuming reactions in the body. Increases in energy demand are met by elevations in activity of the transport chain. A simple assumption is that the promiscuous oxygen reactions occur in direct relation to the flux of electrons along the transport chain and hence greater levels of energy expenditure lead to greater levels oxygen consumption (upward blue arrow) and increases in radical oxygen production (upward blue arrow). This unfounded assumption forms the basis of the idea that elevated metabolism during expensive life history stages leads to elevated free-radical production, ROS formation and oxidative damage, underpinning the life history trade-off between reproduction and survival. The model in Fig. 1B adds a refinement to the above model that challenges this assumption. Protons may not only pass back to the matrix via C5, but via other proteins embedded in the inner membrane. Some of these are called uncoupling proteins (UCPs) because they uncouple the transport of protons from synthesis of ATP. However, other proteins may translocate protons such as the ANT and confusingly yet other proteins have been called uncoupling proteins based on sequence similarity to the first discovered uncoupling protein (UCP1) but may not actually serve to translocate protons. The accumulation of protons in the inner membrane space creates a potential difference between the inner membrane space and the mitochondrial matrix normally denoted as ψ – the inner-membrane potential. It turns out that the probability of a promiscuous reaction of oxygen and an electron is dependent on ψ – greater probabilities of a reaction and superoxide formation occur at higher values of ψ. This may be simply because higher values of ψ retard the speed at which electrons move along the chain. This effect however has major implications. Anything that increases the flow of protons back through the inner membrane to the matrix will reduce ψ and reduce free-radical production. It is clear from the diagram that the two major things facilitating this movement are C5 and the UCPs/ANT. Thus, greater metabolism and hence greater oxygen consumption (upward blue arrow) are actually more likely to be linked to lower free-radical production via their impact on ψ (downward blue arrows). If the activity of uncoupling proteins (and ANT) increases (upward light blue arrow) this will lower ψ directly, and lead to elevated oxygen consumption for a given level of ATP production. The second model (Fig. 1B) therefore predicts diametrically opposite links between metabolic rate and radical superoxide production from the model in Fig. 1A – indicated by the dark blue arrows adjacent to key components. These complexities in the nature of radical oxygen production by mitochondria have been largely ignored in the development of the life-history oxidative stress hypothesis.

laboratory environment where this has been most intensively studied. Knocking out the key protective enzymes that defend against oxidative stress, either alone or in combination, often leads to greater oxidative stress but has surprisingly little impact on lifespan [21, 22]. Supplementing animals (including humans) with dietary antioxidants, expected to increase lifespan, has led to a variety of different results [23, 24] and can sometimes cause lifespan to be reduced [25] and human mortality to be increased [26]. In several animals with extraordinarily long lifespans, which would be expected to accumulate relatively little oxidative damage through life, such as the naked mole rat (*Heterocephalus glaber*) and subterranean salamander (*Proteus anguinus*), very high oxidative stress and/or unremarkable levels of antioxidant defence have been observed [27, 28]. While oxidative stress might play a greater role in ageing in more stressful environments, this accumulation of evidence has brought the free radical theory of ageing into further doubt.

Oxidative stress as a cost of reproduction

Despite this avalanche of evidence showing that the free-radical theory as a mechanism underpinning life history trade-offs is at best extremely simplistic (see also [28, 29]), several reviews were published in prominent ecological journals at the end of the last decade, suggesting that free-radicals are produced in direct relation to metabolic rate and hence act as a potential mediator of the trade-off between reproduction and survival [30–34]. Publication of these influential reviews led to many 'tests' of the oxidative stress hypothesis. As might be expected in the light of the above information, the results have been equivocal. Some field studies have shown weak positive associations between oxidative damage and reproduction ([35–38], but see [39]). In contrast, some carefully controlled laboratory work has repeatedly concluded that oxidative stress is unchanged or is lower in those individuals that reproduce compared to those that do not [40–43]: while paradoxically showing at the same time that animals raising larger litters had greater damage than those raising smaller ones [42, 44–46] (Tables 1 and 2).

Experimental design considerations

It has recently been suggested [47] that the reason most studies may have failed to support the oxidative stress idea (despite its obvious attractions), is because all the experiments performed to date are fundamentally flawed. This argument is predominantly based on the premise that comparing an experimental group that is forced to reproduce, with a group prevented from doing so, is not an experiment, because the level of reproduction in the experimental group is controlled by the animals themselves. It has instead been proposed that only by manipulating the level of reproductive investment experimentally (instead of its existence compared to non-reproductive controls) is it possible to test the idea [47]. A second criticism of previous studies is that resources have been supplied ad libitum during reproduction, rather than being restricted [47]. We agree that experimental design is a critical issue. The aim of this paper is to highlight some complexities in experimental design that have not been explicitly considered. This analysis leads us to some different conclusions regarding the optimal experimental designs to test these ideas, and suggests we should fundamentally reappraise the simplistic trade-off model.

Comparing experimentally allocated reproductive with non-reproductive individuals is a valid approach

The fundamental prediction of the life history oxidative stress hypothesis, as outlined above, is that during reproduction the elevation in metabolic rate leads to an inevitable increase in free-radical production, which causes lasting oxidative damage that persists after the reproductive event and contributes to ageing. We contend that the best way to test this hypothesis is to randomly assign animals to two groups, one of which is forced to reproduce and another one that is prevented from doing so, and then measure the consequences for oxidative stress (damage to lipids, proteins and DNA). This approach has been criticised because females in the experimental group can choose their own level of reproduction, and hence they can tailor this investment to avoid oxidative damage, dependent on their antioxidant capacities [47]. However, even if individuals can choose their level of reproduction in such an experiment (as would occur in the wild), what they cannot avoid, at any level of reproduction, is an increase in metabolic rate, relative to a non-reproductive control animal. If the fundamental idea outlined above is correct, there should be an increase in free-radical and ROS production, leading to damage that should be detectable during and after the reproductive event. Controlled laboratory studies taking this approach have largely indicated the opposite ([40, 42, 43], but see [44]). If individuals can elevate their defence mechanisms to offset the 'inevitable consequences' of reproduction, then these consequences are not inevitable, and individuals can avoid the physiological mechanism supposed to underlie the trade-off between reproduction and lifespan. Drawing a parallel between these experiments, which have been carefully designed to test whether oxidative stress is a cost of reproduction, and early work that aimed to establish whether there might be a trade-off between reproduction and survival [47] is inappropriate. This is because in those previous life history studies the comparisons between reproduction and survival were generally not made between experimentally allocated animals, in reproductive and non-reproductive groups, but between animals that were high and low investors which chose their own levels of investment. Correlations between naturally occurring levels of reproductive investment and survival observed across a population may be mediated by pleiotropic trade-offs in the functions of genes that vary in their frequency on a population level [48], rather than the accumulation of somatic damage from reproductive investment. Individuals also vary in genetic and phenotypic "quality", which may distort the observed relationships between life history traits when levels of reproduction are not manipulated [49]. Relating these life history studies with recent field studies

Table 1. Studies of the oxidative stress theory of life history evolution in birds and mammals (I)

Species	Sex	L/F	Tissue	Assay	Result	Reference
Birds						
Falco tinnunculus	F	F	Blood	dROMS	Unchanged	[97]
	M	F	Blood	dROMS	Increased	
Acrocephalus sechellensis	F	F	Blood	dROMS	Unchanged[a]	[98]
	M	F	Blood	dROMS	Unchanged[a]	
Aphelocoma coerulescens	F	F	Blood	dROMS	Unchanged	[99]
	M	F	Blood	dROMS	Increased	
Mammals						
Rattus norvegicus	F	L	Lung	TBARS	Increased	[100]
			Uterus	TBARS	Increased	
			Thymus	TBARS	Unchanged	
			Kidney	TBARS	Increased	
Rattus norvegicus	F	L	Kidney	TBARS	Increased	[101]
			Liver	TBARS	Increased	
Soay sheep *(Ovis aeries)*	F	F	Blood	TBARS	Unchanged	[39]
Mus musculus	F	L	Muscle	PT	Unchanged	[42]
				MDA	Decreased	
				OG	Unchanged	
			Liver	PT	Decreased	
				MDA	Decreased	
				OG	Decreased	
			Blood	MDA	Unchanged	
Tamias sciurus	F	F	Blood	TBARS	Increased	[35]
Myodes glareolus	F	L	Liver	TBARS	Unchanged	[102]
			Kidney	TBARS	Unchanged	
			Heart	TBARS	Unchanged	
			Muscle	TBARS	Decreased	
Tamiasciurus hudsonicus	F	F	Blood	PC	Increased	[36]
Mus musculus	M	L	Blood	MDA	Decreased	[40]
			Muscle	MDA	Increased	
			Liver	MDA	Decreased	
				PT	Unchanged	
				OG	Unchanged	
Mus musculus	F	L	Blood	dROMS	Decreased	[44]
Mus musculus	F	L	Liver	OG	Unchanged	[41]
				PT	Decreased	
				PC	Decreased	
			Heart	OG	Unchanged	
				PT	Unchanged	
			Muscle	OG	Unchanged	
				PT	Unchanged	
Lasiopodomys brandtii	F	L	Blood	PC	Increased	[45]
				TBARS	Unchanged	
			Liver	PC	Decreased	
				TBARS	Unchanged	
Meriones unguiculatus	F	L	Blood	PC	Increased	[46]
				TBARS	Unchanged	
			Liver	PC	Decreased	
				TBARS	Unchanged	
Rattus norvegicus	F	L	Kidney	PC	Increased/decreased	[103]
				MDA	Increased/decreased	

This table includes studies where oxidative damage has been compared between reproductive and non-reproductive animals only. Studies exclusively of oxidative protection and repair without damage measurements are not included. M = male and F = female. L/F – laboratory or field. Under assays: D-ROMS is reactive oxygen metabolites, PC is protein carbonyls, PT is protein thiols, TBARS is thiobarbiturate acid reactive substances (mostly MDA), MDA is MDA measured by HPLC, OG is oxidised glutathione. Under result: increased means damage increased, unchanged means damage not significantly different and decreased means damage lower in the reproductive group relative to non-reproductive group. Studies are ordered by date of publication within each section.

[a]Effect interacted with infection status for malaria.

Table 2. Studies of the oxidative stress theory of life history evolution in birds and mammals (II)

Species	Sex	L/F	E/NE	Tissue	Assay	Result	Reference
Birds							
Parus major	F	F	NE	Blood	dROMS	Unchanged	[104]
Sturnus vulgaris	F	F	NE	Blood	dROMS	Negative	[104]
Ficedula albicollis	F	F	NE	Blood	dROMS	Unchanged	[105]
Pygoscelis adeliae	F	F	E	Blood	dROMS	Positive	[106]
	M	F	E	Blood	dROMS	Positive	
Alectoris rufa	F	L	NE	Blood	TBARS	Positive[a]	[87]
					OG	Positive[a]	
Aphelocoma coerulescens	F	F	NE	Blood	dROMS	Unchanged	[99]
	M	F	NE	Blood	dROMS	Positive	
Mammals							
Mus musculus	F	L	NE	Liver	PT	Positive	[42]
					OG	Unchanged	
					MDA	Unchanged	
				Muscle	PT	Positive	
					OG	Unchanged	
					MDA	Unchanged	
				Blood	MDA	Unchanged	
Tamias sciurus	F	F	NE	Blood	TBARS	Positive	[35]
Mus musculus	F	L	NE	Blood	dROMS	Positive[b]	[44]
	F	L	NE	Blood	dROMS	Negative[b]	
Tamiasciurus hudsonicus	F	F	NE	Blood	PC	Positive	[36]
Mus musculus	F	L	E	Liver	PC	Negative	[41]
					PT	Negative	
					OG	Unchanged	
				Muscle	PT	Unchanged	
					OG	Unchanged	
				Heart	PT	Unchanged	
					OG	Unchanged	
Lasiopodomys brandtii	F	L	NE	Blood	PC	Unchanged	[45]
					TBARS	Unchanged	
				Liver	PC	Unchanged	
					TBARS	Positive	
	F	L	E	Blood	PC	Unchanged	
					TBARS	Unchanged	
				Liver	PC	Unchanged	
					TBARS	Unchanged	
Meriones unguiculatus	F	L	NE	Blood	PC	Unchanged	[46]
					TBARS	Unchanged	
				Liver	PC	Positive	
					TBARS	Unchanged	

This table includes studies where oxidative damage has been correlated with reproductive effort (normally clutch or litter size), including studies where litter and clutch size have been experimentally manipulated (E) or not and individuals have raised their natural litter sizes (NE). Studies exclusively of oxidative protection and repair without damage measurements are not included. M = male and F = female. L/F – laboratory or field. Under assays: D-ROMS is reactive oxygen metabolites, PC is protein carbonyls, PT is protein thiols, TBARS is thiobarbiturate acid reactive substances (mostly MDA), MDA is MDA measured by HPLC, OG is oxidised glutathione. Under result: positive means damage increased, unchanged means damage not significantly different and negative means damage decreased in relation to increasing clutch or litter size. Studies are ordered by date of publication within each section.

[a]Age related trends were opposite those of age related trends in egg and chick production.

[b]Relationship positive in lactation but negative in pregnancy.

that have examined the naturally occurring correlation between litter size and oxidative stress is valid, but this argument does not invalidate comparisons of reproducing to non-reproducing animals in the laboratory (as long as animals are randomly assigned to the experimental groups).

If comparisons are to be made between different levels of investment, and their implications for oxidative stress, then such levels ideally should be experimentally manipulated. We note, however, that manipulating reproductive investment of animals is not always easy. One popular way to achieve this is by manipulating litter or clutch sizes. However, changing litter size in mammals does not always lead to a change in the total metabolism (Fig. 2). This is because female mammals may be operating under a physiological constraint that regulates their total investment, independent of litter size and food supply [50–53]. There has been much debate about the

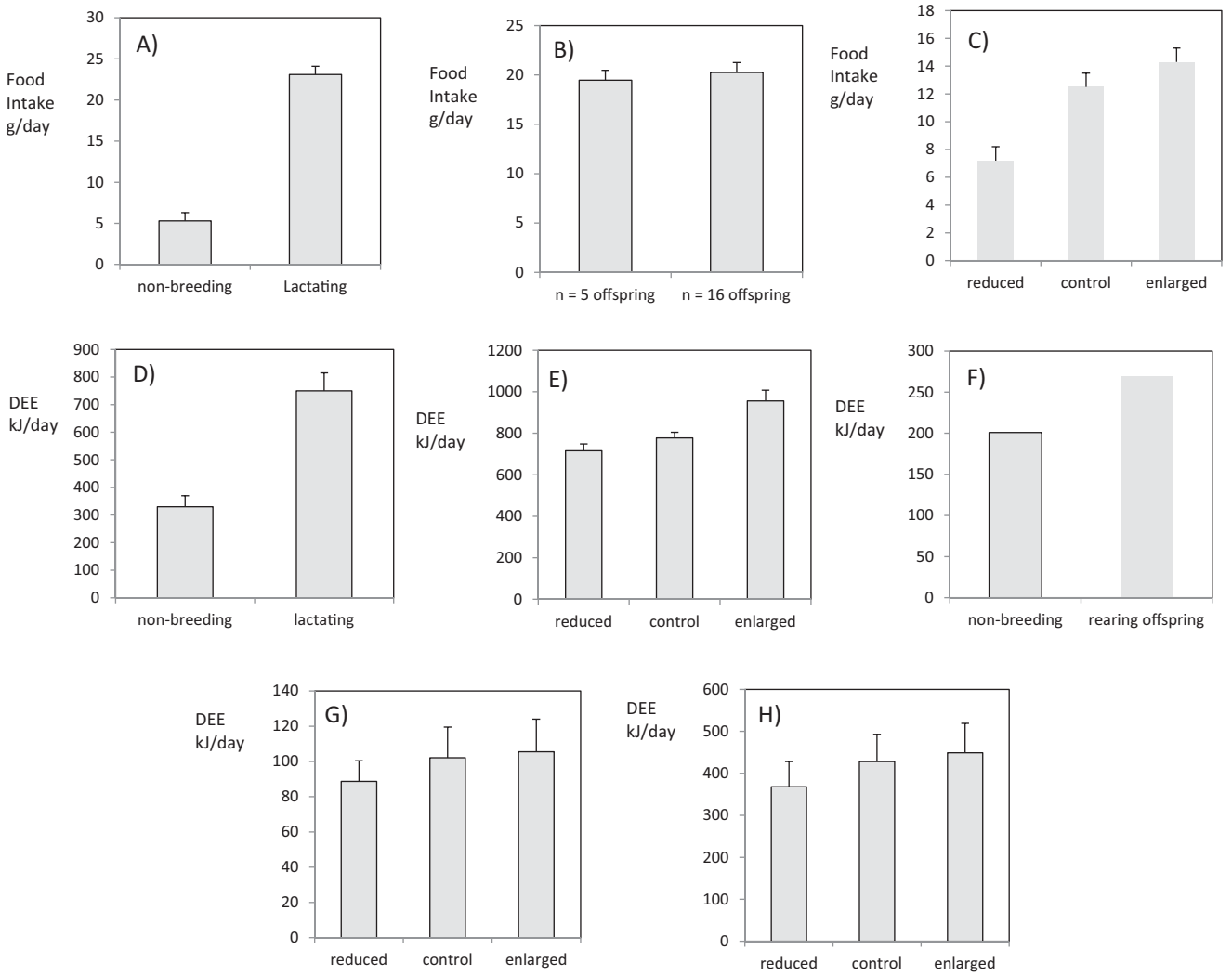

Figure 2. Changes in energy expenditure or food intake, as indices of 'metabolic effort', comparing breeding with non-breeding birds and mammals, and animals where litter or clutch size has been experimentally manipulated. **A:** food intake of the female MF1 mouse (*Mus musculus*) in captivity when non-breeding and at peak lactation (from [51]). **B:** Food intake of the female MF1 mouse in captivity at peak lactation when raising experimentally manipulated litters of 5 or 16 offspring (from [54]). **C:** Food intake at peak lactation of female Brandt's voles (*Lasiopodomys brandtii*) in captivity raising litters that had been experimentally reduced (mean $n = 2.4$ offspring) or enlarged (mean $n = 11.7$ offspring) and control litters (mean $n = 7.5$ offspring) that were not manipulated (from 2012 [89]). **D:** Daily energy expenditure of non-breeding and lactating North American red squirrels (*Tamiasciurus hudsonicus*) in the wild (data restricted to animals both measured at 10˚C to remove temperature effects) (from [36]). **E:** Daily energy expenditure at peak lactation of Columbian ground squirrels (*Urocitellus columbianus*) in the wild raising reduced (natural litter −2), control or enlarged (natural litter +2) litters (from [90]). **F:** Daily energy expenditure of free living female dippers (*Cinculus cincus*) when feeding young in the nest, or non-breeding (from [91]: error not quoted in original paper). **G:** Daily energy expenditure of great tits (*Parus major*) in the wild when feeding reduced (mean $n = 5.3$ offspring), control (mean $n = 9.0$ offspring) or enlarged (mean $n = 14.0$ offspring) clutches (from [92]). **H:** Daily energy expenditure of Eurasian kestrels (*Falco tinnunculus*) in the wild when feeding reduced (mean $n = 2.0$ offspring), control (mean $n = 4.6$ offspring) or enlarged clutches (mean $n = 7.3$ offspring) (from [58]; [65]). These data are presented to emphasise two main points. First, when comparisons are made between breeding and non-breeding animals (plots A, D and F) the difference is always much larger than the differences between individuals raising experimentally manipulated litters (plots B, C, E, G and H). Second, although it is possible to manipulate some species and obtain significant effects on daily energy expenditure or food intake (e.g. plots C, D and H) it is also often observed that there is no effect of litter or clutch size manipulations on expenditure or intake because the individuals seem to be working already at a ceiling (e.g. plots B and the comparison of control to enlarged litters in plots G and H).

nature of such a constraint, but its likely presence means that experimental manipulations of litter size that superficially appear to manipulate investment may not be linked to differences in food intake or metabolism [51, 54]. The comparison of individuals experimentally allocated to different levels of reproduction is probably therefore a poorer test of the hypothesis, when compared to experimentally allocated reproductive and non-reproductive individuals. This is because the extent of the difference in metabolism between groups with manipulated litter sizes is far smaller (Fig. 2). For

example, in the laboratory mouse (MF1 strain) the food intake at peak lactation when raising natural litters amounts to on average about 20–23 g/day [51, 55, 56] depending on the diet, while intake for a non-reproductive mouse is 3–5 g/day (Fig. 2A). In contrast, mice of the same strain that had experimentally manipulated large litters ($n = 16$ offspring) ate on average 20.46 g/day at peak lactation, while those raising experimentally manipulated small litters ($n = 5$ offspring) ate 19.27 g/day: a difference that was not significant (ANOVA: $F_{1.47} = 1.36$, $P = 0.249$) [54] (Fig. 2B). Successful application of such a design therefore requires that the manipulation is large. Ideally the consequences of the manipulation on the actual level of investment should also be quantified, to confirm that a difference in reproductive investment has been achieved (e.g. [41, 57]), otherwise a negative result could simply reflect the fact that an apparent manipulation had no actual impact on investment (as in [54]). In birds the situation appears similar. In some circumstances manipulations of brood size do lead to changes in reproductive effort and metabolism [58]. Increasing evidence, however, suggests that other bird species in the wild may be operating under an intrinsic constraint that is refractory to attempted experimental manipulations of workload [59, 60]. In birds as well as mammals the comparison of breeding to non-breeding individuals generated a much larger impact on metabolism than manipulations of litter or clutch sizes (Fig. 2).

In spite of these design considerations, it should be noted that in two recent studies of wild-derived rodents litter size was experimentally manipulated by a factor of four, to an extent that energy intake was confirmed to increase in the large litters; however, oxidative stress was still independent of the level of reproduction [41, 45]. These results are both at odds with the often reported positive link between litter size and oxidative stress in situations where litter size was NOT manipulated [42, 44], albeit with overall levels lower than in non-reproducing individuals.

Extrinsic resources should not be limited

One potential reason why field studies provide some support, but lab studies have often provided evidence against the hypothesis, is that in the laboratory animals are provided with ad libitum access to food [47]. Such ad libitum access may allow the animals to upregulate their antioxidant defences, while also engaged in reproduction, whereas in the field food resources may be limited, enforcing the hypothetical differential resource allocation. It has therefore been suggested that to correctly test the hypothesis resources should always be supplied in a limited fashion [47]. This argument, however, assumes that the primary factor limiting investment is the extrinsic level of food supply. This is far from clear. In fact many studies have indicated that animals at peak reproduction may be limited by intrinsic physiological factors [50, 52, 53, 61–63]. These can include peripheral limitations in ability of energy consuming machinery, such as the physical properties of muscles to sustain physical exercise for foraging, or central limitations in the way animals can acquire, convert and distribute resources [42]. For example, energy conversion may be limited by heat dissipation in endotherms [41] and there may be limits in the capacity of the alimentary tract to absorb energy [47]. Although the exact nature of these limiting physiological factors remains contentious, their existence does not. Therefore, if animals are working at this physiological capacity, the level of food supply becomes irrelevant to their need to selectively allocate resources. Resources in the field are probably also not limited (see arguments in [52, 64]), but the physiological costs of harvesting these resources could be a major investment constraint [58, 65]. This constraint can apply equally in the laboratory and the field independent of food supply.

If animals are already working at their intrinsic physiological limits, then reducing food resources will not provide a good test of the oxidative stress hypothesis, but will rather produce undesired effects, most prominently reduced investment in reproduction. The effects of limiting resources have been well explored in relation to caloric restriction, where reducing resources by 30–60% causes reduced allocation to reproduction [66–68]. Reducing access to food can also influence the levels of sex specific hormones, such as testosterone [69], which are required for full investment in sex specific reproductive traits and have sometimes been predicted to increase oxidative stress [70]. In rodents reducing food availability during reproduction causes females to reduce their litter sizes (usually by culling pups), or to stop reproducing altogether [71, 72].

In many situations, therefore, food restriction will cause an animal's allocation to reproduction to decrease. The metabolic expenditure that occurs as a consequence of this reproduction will decrease in a similar fashion and will have a minimal impact on any resource allocation trade-offs between reproduction and somatic maintenance. In fact, reducing available food resources can enhance antioxidant defence [73] and theory predicts that, if anything, the trade-off between reproduction and somatic maintenance will shift towards the latter [68, 74]. If oxidative stress is a cost of reproduction it should be observable when animals are reproducing at their physiological capacity regardless of whether extrinsic resources are limited or not. We suggest the largest contrast will be between animals that are reproducing at this physiological limit, compared with individuals that are not reproducing at all (see arguments above).

The role of resource allocation models in the oxidative stress life-history hypothesis

The suggestion that oxidative stress may only be revealed as a cost of reproduction under resource limitation [47] highlights a mechanistic shift of emphasis that is now being relied upon to explain the occurrence of many negative results. One of the first studies suggesting oxidative stress as a cost of reproduction specifically proposed ROS production as a mechanism to explain life history trade-offs that is independent of limitations in resource allocation [75]. In fact, the authors justified the importance of their hypothesis by highlighting that resource allocation models are unable to explain the relationships observed between life history traits ([75]; see further below). In the presence of negative results, predictions for oxidative stress as a cost of reproduction have

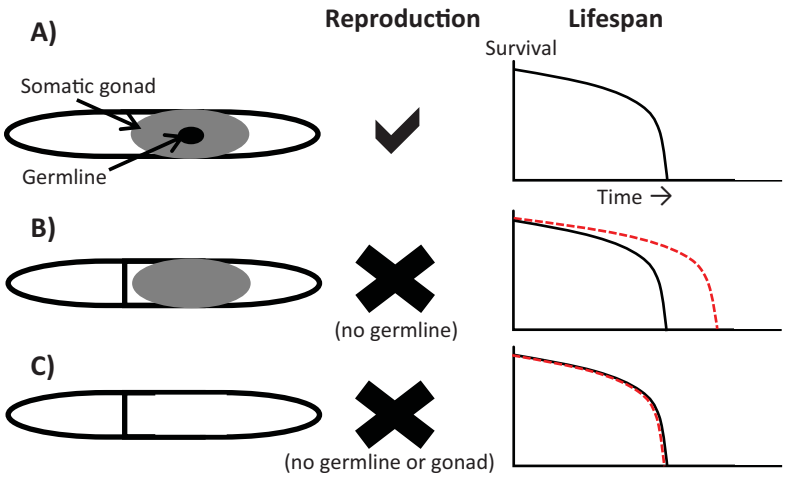

Figure 3. The role of the germline and somatic gonad in modulating the reproduction versus lifespan trade-off. The normal worm (or fly) with a functional germline and somatic gonad can be considered as reproductive and short lived (**A**). Removal of the germline precursor cells causes infertility and increases lifespan (**B**), suggesting a within individual trade-off between reproduction and lifespan [93, 94]. However, when both the somatic gonad and germline are removed (**C**) [93–95], animals show no increase in lifespan even though allocation to reproduction is also reduced. This evidence argues against simplistic resource trade-offs between reproduction and lifespan, and instead suggests that signals emanating from the germline may promote ageing, while the somatic gonad produces opposing signals that repress ageing [95]. Parts of these signalling processes are beginning to be adjudicated, with germline-mediated lifespan extension in *C. elegans* being dependent on the steroid receptor DAF-12/FXR and forkhead transcription factor DAF-16/FOXO, which stimulate various physiological processes that facilitate lifespan extension [96].

gradually turned back to an argument of limited resource allocation, consistent with the disposable soma hypothesis. Animals are forced to choose between allocating resources to either the production of antioxidant defences (or other protective mechanisms) or reproduction ([47], see also [76]). This argument rests on the assumption that defences against oxidative stress are energetically costly to maintain and will therefore trade-off against reproductive expenditure. In spite of the large amount of biomolecular research centring on antioxidant defence carried out in the past two decades, there is little evidence that defences against oxidative stress have a substantial energy cost [22, 52]. Some processes do require energy, such as the NADPH required to maintain glutathione in a reduced form [77]. But some major anti-oxidant enzymes, e.g. superoxide dismutase and catalase, require no energy input for their operation. This contrasts with some other somatic maintenance systems, such as those involved in xenobiotic metabolism, which have substantial energy requirements [22]. One notable exception is the uncoupling proteins, which may reduce mitochondrial ROS production (Fig. 1B), but also lower the efficiency of energy production [11]. However, uncoupling proteins have multiple physiological functions and can also generate heat [78], which may itself reduce investment in reproduction.

Caution should also be used when relying on a mechanistic hypothesis based on constraints in resource allocation. Over the past decade it has become apparent that trade-offs can be mediated by switches in signalling pathways, independent of trade-offs in resource allocation [79, 80]. Some of the best experimental demonstrations of within-individual trade-offs between reduction and lifespan come from laboratory studies in *C. elegans* and *Drosophila*, but evidence suggests that these do not occur as a consequence of simplistic limitations in resource allocation between reproduction and lifespan (Fig. 3). There are now also a variety of examples of single gene mutations that alter the actions of particular signalling systems, such as the insulin signalling and target of rapamycin pathways, that greatly increase lifespan, but have no detectable effects on fertility [81]. There is still much to understand about these processes, but this evidence argues against the importance of simplistic trade-offs in resource allocation, and suggests that theories relying on this assumption should be revisited [82].

Alternative approaches

We highlight that resource availability per se is unlikely to seriously impair antioxidant defence. However, it should be noted that there are other, more established factors that do alter the ability to regulate protective mechanisms, and could cause oxidative stress to increase with reproduction in particular instances. Animals that are in poor body condition, e.g. those that are senesced or diseased, or have pre-existing oxidative stress, are less able to regulate antioxidant defence (reviewed in [83, 84]). It has been suggested these types of individuals may be especially susceptible to oxidative stress when investing in reproductive behaviours that are required to attain mates [85]. Such condition-dependent costs may be applicable to investment in a variety of traits. Under laboratory conditions animals are kept in good condition, are typically free of parasites and pathogens, and have been subjected to fewer stressors that impair redox status in the wild. It could be the general good condition of laboratory animals that allows them to protect themselves against oxidative stress, rather than the fact they have an abundant food supply. It may be more fruitful to instead manipulate the condition of experimental animals, in ways independent of manipulation of their nutritional supplies, for example by increasing parasite loads, or exposing them to other energetic stressors that can influence oxidative stress such as lowered ambient temperature [16, 86]. Studies in ectotherms where the roles of uncoupling proteins may be different and life history patterns more diverse may also provide useful avenues for research to test these ideas.

An additional factor that may be important is that studies of laboratory animals to date have often considered individuals engaged in their first, or early, reproductive attempts [40, 42–44], while field studies have involved females of unknown age or parity ([35, 36], but see [87]). Given the large future reproductive potential of these relatively

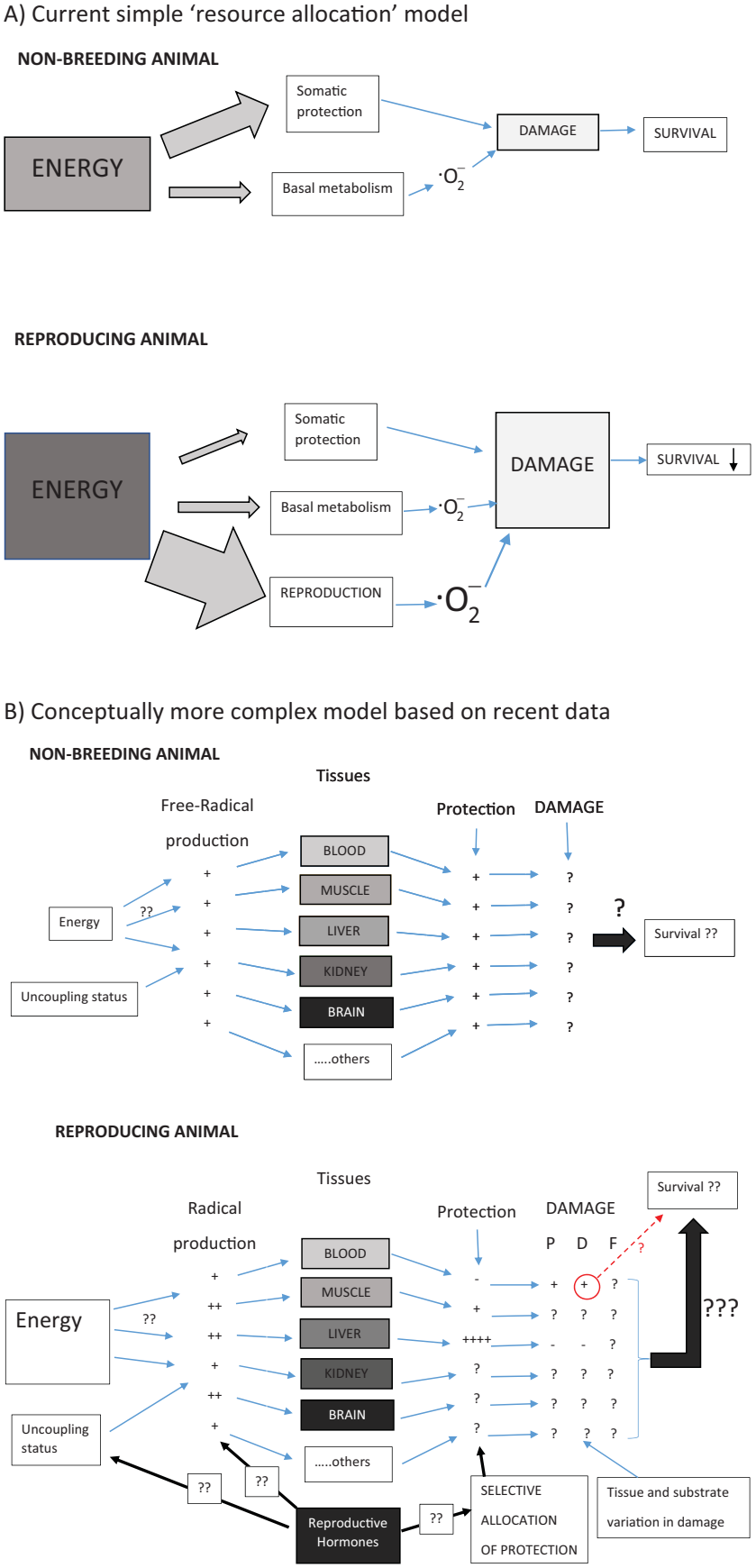

Figure 4. Current simple resource allocation model and a conceptually more complex model based on recent data concerning the links of oxidative stress to life histories. **A:** Current simple resource allocation model. In the non-breeding animal ingested energy is allocated either to somatic protection or to basal energy metabolism. The protection level balances the level of radical production and there is a neutral impact on survival. When an animal starts to reproduce its intake of energy massively increases but most of this energy is allocated to reproduction. In fact energy is diverted away from somatic maintenance. The production of free-radical oxygen species is greatly increased because of the larger energy flux at a time when protection is reduced and the result is an increase in oxidative damage and a decrease in survival: explaining the trade-off between reproduction and survival. **B:** More complex conceptual model based on recent data. In the non-breeding state animals have a basal production of radical oxygen species in all their tissues. This may vary between tissues due in part to differences in uncoupling status. The role of energy flux is less certain. Tissues also have a baseline level of protection and repair mechanisms ongoing that are independent of the energy flow because they are energetically very cheap to operate. Tissues accumulate small amounts of damage at rates dependent on the balance of damage and repair but the implications of such damage for survival and longevity are unclear. When the animal starts to reproduce the overall flow of energy increases. Metabolic rates of different tissues do not respond equally and the implications for free-radical production are uncertain. Of equal importance may be changes in uncoupling status across different tissues and these may be signalled by changes in reproductive hormones. The changes in uncoupling may be related to things like the capacity to dissipate heat and are not necessarily modulated directly to influence free-radical production. These reproductive hormones may similarly drive changes in the allocation of protection to different tissues and the complex balance of free-radical production and protection leads to tissue idiosyncratic responses that vary not only between tissues but also between substrates within tissues (P = protein, D = DNA and F = fats). The impact on survival may depend on the entire matrix of responses (large black arrow), or be crucially dependent on damage to a single substrate in a single tissue (red circle and red dotted line) or a small number of key damage targets. Alternatively there may be no discernable link to survival at all, and the link of reproduction to survival may depend on non-oxidative damage related processes.

young individuals, it may be important for them to invest in protection against oxidative stress resulting from their first reproductive efforts to ensure survival to engage in later reproductive efforts. This could potentially explain the paradoxical result that oxidative stress is on average lower in primiparous individuals during their first reproduction versus virgins [41, 42], but within the primiparous group there is a positive relationship of litter size and damage [42, 44]. That is protection is on average turned up during the first reproduction, but it is not calibrated against reproductive effort. Older multiparous individuals, however, with lower residual reproductive value may optimise their overall reproductive output by investing completely into reproduction at the expense of oxidative protection. The trade-off may therefore be more evident during later reproductive attempts, and studies of multiparous individuals engaged in reproduction later in their lives would be extremely valuable and informative, as would studies of repeated reproductive attempts rather than single events – as the impact may be cumulative.

The assays of oxidative stress used and the tissues analysed affect the outcome

A difference between laboratory and field studies that have been performed to date to address the oxidative stress life history theory is that field studies have almost exclusively focussed on measurements made in blood (see review of studies in Tables 1 and 2). The reasons for this are that it is often difficult to extract and preserve tissues in the field, and removing such organs is only possible if the subject animal is killed. This may conflict with other aims of field studies, many of which include long term monitoring of marked individuals in the wild. In the laboratory, however, the main focus of attention has been on measurements of major organs such as the liver, kidneys and muscle tissue. Two recent studies have examined colonies of wild animals raised in the laboratory (specifically Brandt's voles *Lasiopodomys brandtii* [78] and Mongolian gerbils *Meriones unguiculatus* [79]) and have involved multiple assays of oxidative stress in both blood and other tissues. These results shed some light on the discrepancy between field and laboratory studies. The pattern of damage detected in blood (greater in reproducing animals) broadly corresponded to the studies made previously using blood as the analysis source in wild animals. Yet the analysis of oxidative stress in the liver produced results matching previous laboratory studies, using the same analysis tissue, where damage is reduced in the reproducing animals. However, this result was only found for some assays. In other assays there was no significant impact. This assay effect was also found when greenfinches (*Carduelis chloris*) were fed lethal doses of paraquat to cause oxidative stress [88], with significantly elevated oxidative stress as measured by DNA damage, but no significant effect detected in other assays. These studies demonstrate that the choice of analysis tissue and assay dramatically influences the experimental outcome. The measurements of oxidative stress performed across studies to date have included a wide diversity of techniques assessing different forms of damage, to different biomolecules, in different target tissues (Tables 1 and 2). These forms of damage are not directly comparable. It is notable that as yet no studies of the potential oxidative damage resulting from reproduction have measured damage to DNA (Tables 1 and 2). We should therefore be exceedingly cautious in interpreting the results of experiments that have been based on a limited number of assays in single or limited numbers of tissues. At present we do not know what these differences mean. Is damage detected in the blood more important than damage detected in the liver? Or the reverse? Or could other tissues such as the brain provide more important comparisons? To date no-one has measured the implications of reproduction on oxidative stress in the brain (Tables 1 and 2). Is damage to protein more significant than damage to lipids? Or to DNA?

Interestingly, the patterns of damage detected in Brandt's voles and gerbils were inversely related to the levels of superoxide dismutase [78, 79]. That is, during reproduction superoxide dismutase was upregulated in the liver, paralleling the reduced damage, and downregulated in the blood, paralleling the increased damage. This raises the possibility that during reproduction animals may differentially allocate protection between different tissues, and to different substrates (e.g. DNA may be more protected than proteins). This takes us well beyond the current conceptual models of oxidative stress as a mediator of life history evolution, where the impact of reproduction has been universally presumed to apply to the animal as a whole. That is, animals encounter oxidative stress, or they do not. These recent studies show that this conceptual model is far too simple, and that protection and repair may be strategically targeted between different tissues, according to their varying requirements at different life stages (Fig. 4). Ultimately the significance of these differences between tissues may only be revealed by linking such impacts to functionally relevant endpoints such as survival, as recently suggested [47].

Conclusions

We contend that comparisons of individuals forced to reproduce, with the inevitable enormous increases in energy metabolism compared with control animals prevented from so doing, provides a valid test of the 'oxidative stress' theory. We envisage significantly more difficulties in designs that include manipulation of the level of investment (e.g. litter and clutch size manipulations), simply because animals may work to a physiological limit largely independent of the number of offspring they are provided with. Moreover, because animals may be physiologically constrained at points of peak reproductive investment, rather than limited by food supplies, restricting food during these periods will likely only result in reduced investment in reproduction, rather than enforcing any trade-off against somatic maintenance. We suggest a better approach may be to challenge animals during reproduction with additional energetically costly burdens (as also suggested in [47]), such as exposure to parasites and pathogens, or altered ambient temperature, to magnify the supposed allocation trade-off. A neglected factor in current studies is the parity of reproducing individuals. We suggest that a trade-off with investment into somatic protection may

be more evident among individuals engaged in later as opposed to their first reproductive attempts, or following repeated attempts. In addition, the choice of assay(s) is critical. Recent work has indicated that in the same individual animals different assays of oxidative stress, and measurements made in different tissues, yield fundamentally opposing results (Fig. 4, Tables 1 and 2).

Finally, we need to be more open to the possibility that oxidative stress may not underpin life history trade-offs, despite how superficially attractive such a theory may appear. If oxidative stress is linked to life history diversity, it is not through a simple linear increase with metabolic rate. It is also unlikely that reproductive allocation trades off simplistically against antioxidant defence. Recent observations of significant tissue to tissue diversity in the response to reproduction, suggest that the role of oxidative stress in life history evolution is likely much more complex than currently conceptualised. The most significant future advances will need to move us beyond the simple trade-off model, by including tissue and substrate level diversity in response, the possibility of selective allocation of protection, and the link of these phenomena to ultimate functional outcomes (Fig. 4).

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