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Defence against the intergenerational cost of reproduction in males: oxidative shielding of the germline

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ABSTRACT

Reproduction is expected to carry an oxidative cost, yet in many species breeders appear to sustain lower levels of oxidative damage compared to non-breeders. This paradox may be explained by considering the intergenerational costs of reproduction. Specifically, a reduction in oxidative damage upon transitioning to a reproductive state may represent a pre-emptive shielding strategy to protect the next generation from intergenerational oxidative damage (IOD) – known as the oxidative shielding hypothesis. Males may be particularly likely to transmit IOD, because sperm are highly susceptible to oxidative damage. Yet, the possibility of male-mediated IOD remains largely uninvestigated. Here, we present a conceptual and methodological framework to assess intergenerational costs of reproduction and oxidative shielding of the germline in males. We discuss variance in reproductive costs and expected payoffs of oxidative shielding according to species' life histories, and the expected impact on offspring fitness. Oxidative shielding presents an opportunity to incorporate intergenerational effects into the advancing field of life-history evolution.

Key words: reproductive costs, life history, trade-off, germline, mate competition, oxidative stress, sperm competition.

CONTENTS

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I. INTRODUCTION

A bedrock assumption of life-history theory is that reproduction involves costs to fecundity and/or survival (Harshman & Zera, 2007; Monaghan, Metcalfe & Torres, 2009; Speakman & Garratt, 2014; Zhang & Hood, 2016). At a mechanistic level, trade-offs are expected to occur because resources are finite, and must be shared amongst two or more traits, forcing allocation decisions throughout life (Stearns, 1992). However, the precise nature of the limiting resources has remained a matter of contention for decades. Recently, oxidative stress has been suggested to be a potential proximate mechanism underlying life-history trade-offs (Monaghan *et al.*, 2009; Metcalfe & Alonso-Alvarez, 2010; Isaksson, Sheldon & Uller, 2011; Austad, 2018). Reproduction and other metabolically costly activities such as growth can generate large amounts of reactive oxygen species (ROS) as a by-product of cellular metabolism. At the same time, reproduction can cause the body's antioxidant defence systems to become exhausted by neutralising these ROS produced through reproductive effort (Casagrande & Hau, 2018), and weakened when antioxidants are utilised to enhance sexual ornaments rather than for defence (Blount *et al.*, 2003*a*). ROS production can overwhelm the body's antioxidant defences, resulting in damage to important biomolecules such as DNA, proteins and lipids – a state known as oxidative stress (Speakman & Garratt, 2014; Halliwell & Gutteridge, 2015). The large energetic requirements of reproduction (Hood, Williams & Hill, 2019) may therefore result in oxidative costs, potentially leading to associated fitness costs for survival and future reproduction.

Despite the theoretical expectation that reproduction should incur an oxidative cost, evidence that oxidative damage increases as a consequence of reproductive investment is equivocal and largely restricted to females. Many studies have reported that oxidative damage correlates positively with the level of reproductive effort in terms of offspring number or mass (Speakman & Garratt, 2014; Cram, Blount & Young, 2015b; Blount *et al.*, 2016; Zhang & Hood, 2016; Vitikainen *et al.*, 2016). However, results from various mammals and some bird species often show a reduction in oxidative damage in breeders compared to non-breeders (Garratt *et al.*, 2011; van de Crommenacker, Komdeur & Richardson, 2011; Al Jothery *et al.*, 2016; Vaanholt *et al.*, 2016; Vitikainen *et al.*, 2016; Viblanc *et al.*, 2018), or no or equivocal differences in damage (Xu *et al.*, 2014; Cram, Blount & Young, 2015*a*), although a few studies show the opposite trend (Tomruk, Guler & Dincel, 2010; Fletcher *et al.*, 2013). A meta-analysis confirmed overall that a counter-intuitive reduction in oxidative damage in breeders is a common pattern across species (Blount *et al.*, 2016).

It has been suggested that such puzzling results regarding the oxidative cost of reproduction might make sense if we adopt an intergenerational perspective (Blount *et al.*, 2016). Indeed, classical life-history theory considers only within-generation costs, principally those borne by breeding individuals, while offspring are only considered in terms of optimising their number or size, or the sex ratio of litters and broods (Stearns, 1992; Roff, 2001). Yet, there is increasing recognition that parental condition can profoundly impact individual offspring fitness through a variety of pathways, for example, the quality of parental care that offspring receive (Kölliker, Alonso-Alvarez & Velando, 2013; Bales & Saltzman, 2016), or through inherited patterns of epigenetic gene regulation (Bonduriansky & Day, 2009). Similarly, oxidative damage can be transferred across generations, such as through the resources that mothers provision to offspring *via* the egg (Grune *et al.*, 2001; Blount *et al.*, 2016), placenta (Gupta *et al.*, 2007; Al-Gubory, Fowler & Garrel, 2010), or milk (Bouwstra *et al.*, 2008; Shoji & Shimizu, 2019). We define the intergenerational transfer of oxidative damage from parent to offspring, either in previously damaged biomolecules (DNA, proteins, lipids, etc.), or in subsequent damage due to transfer of oxidative damage products, we define as intergenerational oxidative damage (IOD). Previous work has linked maternal oxidative damage to impacts on offspring survival. Higher maternal oxidative resistance in alpine swifts (*Apus melba*) (Bize *et al.*, 2008) and lower maternal oxidative damage in banded mongooses (*Mungos mungo*) (Vitikainen *et al.*, 2016) and common lizards (*Zootoca vivipara*) (Dupoué *et al.*, 2020) were associated with increased offspring survival, although a follow-up study on banded mongooses (Meniri *et al.*, 2022) showed this result was dependent on the type of maternal damage measured. Lipid oxidative damage was negatively correlated with offspring survival while protein oxidative damage showed the opposite pattern (Meniri *et al.*, 2022). Maternal oxidative damage has also been connected to offspring reproductive fitness: greater oxidative damage was associated with reduced breeding success of daughters in Japanese quail (*Coturnix japonica*) (Romero-Haro, Pérez-Rodríguez & Tschirren, 2022).

Oxidative damage incurred by parents as a consequence of reproduction may therefore put offspring at risk of harm. The oxidative shielding hypothesis suggests that there is selection for mothers to reduce oxidative damage during reproduction to protect themselves, but also their physiologically dependent offspring from IOD caused by the increase in oxidative damage that will ensue over the course of a reproductive bout (Blount *et al.*, 2016). Incorporating this intergenerational perspective may be key to understanding why levels of oxidative damage have been found to become either diminished or remain stable during reproduction (Blount *et al.*, 2016). For example, reductions in oxidative damage in breeding female banded mongooses are associated with enhanced offspring survival (Vitikainen *et al.*, 2016; Meniri *et al.*, 2022), although the same pattern was not found in Columbian ground squirrels (*Urocitellus columbianus*) (Viblanc *et al.*, 2018).

The oxidative cost of reproduction, including intergenerational oxidative costs, has almost exclusively been studied in females (Blount *et al.*, 2016; Vitikainen *et al.*, 2016; Viblanc *et al.*, 2018). This bias towards studies of females is in large

part due to the traditional view that mothers invest more into reproduction than fathers, by producing relatively large gametes which they provision before hatching or birth, and often by investing more than males in postnatal care (Zhang & Hood, 2016). However, males may be particularly likely to transmit IOD to offspring *via* damage to sperm DNA. Indeed, it is estimated that over three-quarters of mutations passed on to the next generation are of paternal origin (Monaghan & Metcalfe, 2019). The risk of paternal transmission of deleterious mutations to offspring may result in strong selection to prevent such harm. Moreover, although oocytes play an important role in repairing both maternal and paternal DNA damage (Fernández-Díez *et al.*, 2016), it is crucial for males to prevent such DNA damage from occurring for two reasons.

First, high levels of DNA damage in sperm cells can prevent fertilisation from occurring (Agarwal & Said, 2003; Kumar *et al.*, 2013; Agarwal *et al.*, 2014; Peña *et al.*, 2019; Xavier *et al.*, 2019). Second, although paternal DNA damage can be repaired by females to some extent after fertilisation, repair is not perfect, and paternal DNA damage still leads to consequences for offspring such as delayed development, increased risk of cancer, pathological epigenetic regulatory changes, birth defects, and developmental disorders and abnormalities such as autism (Velando, Torres & Alonso-Alvarez, 2008; Chabory *et al.*, 2009; Chen *et al.*, 2013; Lane *et al.*, 2014; Feinberg *et al.*, 2015; Menezo, Elder & Dale, 2015; Milekic *et al.*, 2015; Jenkins *et al.*, 2017; Evans *et al.*, 2019). For example, experimentally induced DNA fragmentation in the sperm of laboratory mice (*Mus musculus*) caused offspring to suffer epigenetic alterations, organomegaly, neurodevelopmental and behavioural abnormalities, and tumours in the lungs and the skin (Fernández-Gonzalez *et al.*, 2008), and reduced survival to 1 year of age (Kumar *et al.*, 2013), with similar findings reported in trout (*Oncorhynchus mykiss*) (Fernández-Díez *et al.*, 2016). Therefore, despite maternal mechanisms of repair, males are likely also to attempt to mitigate levels of oxidative damage to prevent intergenerational consequences for offspring.

Yet the extent to which males attempt to mitigate IOD has rarely been considered. A few studies have attributed DNA damage in sperm to the cost of reproductive investment (Silva *et al.*, 2019; Baur & Berger, 2020; Kim & Velando, 2020), including evidence of an intergenerational effect where male competition contributed to the transfer of higher mutation loads in seed beetles (*Callosobruchus maculatus*) (Baur & Berger, 2020). Two studies have evidenced possible mitigation of IOD in breeding males in the form of increases in blood antioxidant levels (Olsson *et al.*, 2012; Vitikainen *et al.*, 2016) (see Section IV.1). However, with the exception of one recent study (Noguera, 2022), none has assessed the extent to which fathers may have mitigated IOD in the germline, and the mechanisms of such mitigation. Here, we address this knowledge gap by detailing the properties of sperm which render them vulnerable to oxidative damage, and showing how the oxidative shielding hypothesis can be tested in males. We also outline predictions of how the expression of oxidative shielding by males may be expected to vary across species according to differences in life history. While our primary focus is on males, to obtain a complete picture any insights into male IOD must be interpreted in the context of the established literature on female intergenerational effects which we reference throughout.

II. SPERM AS VECTORS OF INTERGENERATIONAL DAMAGE

(1) The vulnerability of the male germline

In comparison to other cell types, sperm are particularly vulnerable to oxidative stress. Sperm membranes consist of readily oxidisable polyunsaturated fatty acids (Wagner, Cheng & Ko, 2018), while at the same time sperm are highly metabolically active relative to oocytes, producing far greater quantities of ROS (Monaghan & Metcalfe, 2019). Paradoxically, sperm cells lack antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) that are present in the cytoplasm of somatic cells and oocytes, but which are removed from sperm cells during spermatogenesis (Velando *et al.*, 2008; Aitken & Koppers, 2011; O'Flaherty, 2018; Wagner *et al.*, 2018; Aitken, 2020). Such a reduction in antioxidant defences may be necessary to avoid interference with critical spermatic functions; excess CAT can prevent capacitation by inhibiting local oxidation, and excess SOD and CAT can prevent the acrosome reaction from occurring (O'Flaherty, 2018; Wagner *et al.*, 2018). Nevertheless, diminished antioxidant defence in the face of elevated ROS production makes sperm highly susceptible to the accumulation of oxidative damage. The risk of oxidative damage impairing important sperm functions is exacerbated by the proximity of the nucleus to the mitochondria in sperm, with DNA thus in close proximity to the source of ROS generation (Zhang & Hood, 2016). Furthermore, sperm have truncated DNA repair pathways compared to oocytes or somatic cells, limiting the extent to which damage can be repaired (Velando *et al.*, 2008; Herati *et al.*, 2017; Bui *et al.*, 2018). Repair is further inhibited by the exceptionally condensed form in which DNA is packaged in sperm, which reduces accessibility to repair enzymes (Aitken & Koppers, 2011).

Although sperm cells are vulnerable to oxidative damage during maturation (O'Flaherty, 2019), after ejaculation they are bathed in seminal fluid. Seminal fluid provides protection and nourishment in the form of immune cells, sugars, and epigenetic influencing factors that have important consequences for offspring fitness and development (Chambers & Anderson, 2015; Edwards & Cameron, 2017; Gerlinskaya *et al.*, 2017; Watkins *et al.*, 2018; Evans *et al.*, 2019; Morgan *et al.*, 2020). Critically, seminal fluid provides antioxidant protection that sperm cells lack, such as SOD and GPx (Fitzpatrick & Lüpold, 2014; Martin-Hidalgo *et al.*, 2019). In particular, vitamin E could have an important role due

its presence in sperm cell membranes (Lesser, 2006; Lazzarino *et al.*, 2019; Cilio *et al.*, 2022). There is evidence in male banded mongooses that levels of vitamin E in blood plasma increase during the breeding season, although levels in ejaculates were not assessed (Vitikainen *et al.*, 2016). Endowment of seminal fluid with antioxidants could be an important shielding mechanism by which males mitigate against the risk of IOD, however this remains to be investigated.

(2) Determining male ability to shield

It is expected that males face strong selection to prevent IOD through the (albeit limited) DNA-repair mechanisms present in sperm, and by antioxidant investment into seminal fluid. These mitigations must carry a cost, and the extent to which they can be deployed is likely to be conditionally determined. For example, shielding may vary among individuals depending on their early life history. A range of studies on zebra finches (*Taeniopygia guttata*) have shown long-term consequences of early-life hardship on adult antioxidant defences or oxidative stress status (Blount *et al.*, 2003a; Romero-Haro, Sorci & Alonso-Alvarez, 2016; Noguera, 2017; Monaghan & Metcalfe, 2019; Romero-Haro & Alonso-Alvarez, 2020). Oxidative status can also vary due to carry-over effects from previous reproductive events (Romero-Haro *et al.*, 2016; Merkling *et al.*, 2017). Other exogenous factors such as psychological stress and obesity can further compromise oxidative status through activation of endocrine pathways associated with ROS production (reviewed in Darbandi *et al.*, 2018). Diseased individuals may also be compromised: hyperglycaemia increases ROS production (Wagner *et al.*, 2018) and is associated with high testicular DNA damage in diabetic laboratory rats (*Rattus norvegicus*) (Rato *et al.*, 2014; Oliveira *et al.*, 2015). Similarly, genital tract infections expose the male germline to excessive ROS production through the action of leucocytes (Bui *et al.*, 2018). As males age, senescence may increasingly degrade a male's ability to shield against oxidative damage. Studies on red jungle fowl (*Gallus gallus*) and in humans have linked senescence to higher oxidative DNA damage in sperm cells (Noguera *et al.*, 2012; Monaghan & Metcalfe, 2019). Therefore, although males may be selected to shield offspring from IOD, constraints as a result of a male's past developmental or reproductive history, condition or age could hinder shielding responses.

III. AN OXIDATIVE SHIELDING FRAMEWORK IN MALES

Figure 1 outlines a theoretical framework by which the presence and strength of an oxidative shielding response in males can be assessed. We hypothesise that the presence of a shielding response will vary according to the level of reproductive oxidative cost. Oxidative damage borne by fathers due to expenditure of parental care post-conception (Casagrande & Hau, 2018; Costantini, 2018) could conceivably pass to future, but not current, offspring. However, paternal care is not present in many species (Goldberg *et al.*, 2020). We also recognise that in species where males provide care to offspring, shielding could be extended post-conception in order to maintain male condition so that the quality of care is optimised. That said, if resources are limited, we argue that shielding of the germline should be under stronger selection because it carries greater potential consequences for early development of offspring and thus fitness (Ciereszko, Wolfe & Dabrowski, 2005; Ménézo, Dale & Cohen, 2010; Kumar *et al.*, 2013; Fernández-Díez *et al.*, 2016). Therefore, in this review, we focus on reproductive costs that occur pre-conception, as these are sustained contemporaneously with sperm cell and seminal fluid production, and apply to males across a very wide taxonomic range.

IV. HOW CAN THE OXIDATIVE SHIELDING HYPOTHESIS BE TESTED IN MALES?

Before assessing oxidative shielding, the oxidative cost of reproductive effort must be assessed. This is necessary to judge if any demonstrated oxidative shielding response found is protecting against reproductive oxidative costs in a similar way to previous examples in females (Vitikainen *et al.*, 2016; Viblanc *et al.*, 2018; Meniri *et al.*, 2022). To demonstrate oxidative shielding in breeding males, it will be necessary to establish that there is a decrease in oxidative damage levels in sperm of reproducing males, compared to pre- and post-breeding, and in comparison with non-breeders drawn from the same population or social group. We must then assess whether this decrease in oxidative damage translates into fitness benefits for offspring (Fig. 1).

(1) Sample collection and assessment of oxidative damage

A definitive test of oxidative shielding in breeding males requires the collection of gametes. Ejaculates can be collected from birds, reptiles and fish by rubbing around or stimulating the cloaca (Wolfson, 1952; Zacariotti *et al.*, 2007; Wasden, Roberts & DeLaurier, 2017). In mammals, ejaculates can be obtained by electro-ejaculation (Fasel *et al.*, 2015). Ejaculates can be centrifuged to separate the sperm cells from the seminal fluid (Shekarriz *et al.*, 1995), in order to analyse them independently. A focus for sperm cells is the damage in their DNA and its intergenerational consequences, while vitamin E activity may underlie any cellular oxidative shielding responses (Lesser, 2006; Lazzarino *et al.*, 2019). Oxidative damage in seminal fluid may have less intergenerational relevance. However, SOD and glutathione enzymes – which are largely absent within cells – are important components of antioxidant defence in seminal fluid (Tavilani *et al.*, 2008; Fitzpatrick & Lüpold, 2014; Martin-Hidalgo *et al.*, 2019). Moreover, since sperm cells are formed over a longer time period compared to seminal fluid, comparing oxidative

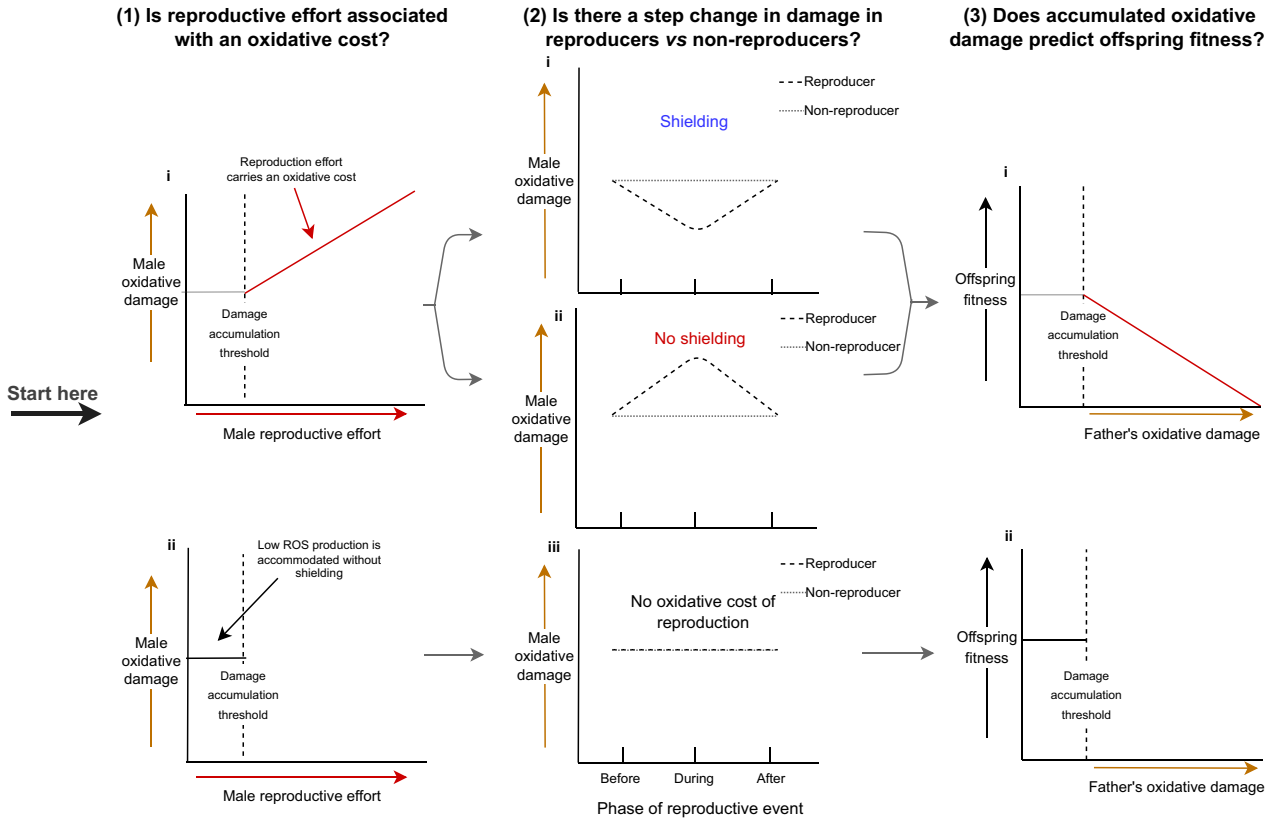

Fig. 1. A framework to test the oxidative shielding hypothesis divided into three assessments: presence or absence of: (1) a positive relationship between reproductive effort and oxidative damage accumulation in males; (2) a step change reduction in damage over the breeding period in reproducing males; and (3) an association between oxidative damage sustained by fathers during breeding and the fitness of their offspring. The framework is presented as a flowchart. For each assessment, separate panel pathways display what would be expected in the presence (1.i, 2.i, 2.ii, 3.i), and absence (1.ii, 2.iii, 3.ii) of an oxidative cost of reproduction. Panel 1.i (red line) highlights cases where reproductive effort is associated with oxidative damage accumulation and 1.ii (black line) cases where reproductive effort does not pass a damage accumulation threshold. Where an oxidative cost of reproduction is present, 2.i represents evidence for oxidative shielding, while 2.ii evidences no oxidative shielding, and 3.i evidences that oxidative damage accumulated by fathers while breeding should have intergenerational consequences for offspring fitness. Where there is no oxidative cost of reproduction (1.ii), there should be no evidence of oxidative shielding (2.iii). Further, with no oxidative damage sustained while breeding any IOD transferred to offspring would not be a result of the oxidative costs of reproduction, and any relationship between oxidative damage in fathers and offspring fitness would not be applicable (3.ii). ROS, reactive oxygen species.

damage and antioxidant activity in such samples could provide an interesting perspective on the kinetics of an oxidative shielding response during the breeding season.

For sperm cells, DNA damage assessment should be a priority as DNA is the main contribution of fathers to the zygote. Other minor contributions of sperm to the zygote include RNA, although in negligible amounts compared to that provided by oocytes, and proteins that are largely important for fertilisation (Immler, 2018). DNA damage can be measured in two ways. First, through assays that specifically detect the level of damage due to oxidation, by quantifying the level of 8-hydroxy-2′-deoxyguanosine (8-OHdG), a by-product of the oxidation of nucleotide bases (Vorilhon *et al.*, 2018). Alternatively, whole-genome DNA fragmentation can be measured using a variety of assays [see Qiu *et al.* (2020) for a detailed review of specific methods].

As sperm cells are largely comprised of lipids, assessing levels of by-products of lipid peroxidation such as malondialdehyde (MDA) and F2-isoprostanes can indicate cellular damage (Monaghan *et al.*, 2009). Sperm cells also contain structural proteins (Lone *et al.*, 2019) and a number of additional proteins that are important for fertilisation (Immler, 2018). Protein oxidative damage can be measured by quantifying the amount of protein carbonylation (Wehr & Levine, 2013) or advanced oxidation protein products (AOPPs) (Taylor *et al.*, 2015; Noguera, 2022). Damage to cellular integrity in the form of lipid and protein damage can prevent fertilisation (Krishnan *et al.*, 2015; Lone *et al.*, 2019). However, even where fertilisation is successful, with the exception of a few specific proteins (Immler, 2018), lipids and proteins in sperm do not pass to the developing zygote and will have less direct relevance to offspring fitness compared to sperm DNA damage.

Diet-derived vitamin E is the principal antioxidant capable of breaking the chain of lipid peroxidation in cell membranes, and is therefore specifically important in decreasing susceptibility to oxidative damage in sperm cell membranes (Lesser, 2006; Lazzarino *et al.*, 2019). Isomers of vitamin E can be readily measured in sperm using high-performance liquid chromatography (HPLC) (Siluk *et al.*, 2007). SOD and glutathione in seminal fluid can be measured using commercially available colorimetric kits (Zengin *et al.*, 2015; Madany *et al.*, 2019).

It is informative to measure oxidative damage and antioxidant defence in somatic tissue alongside the germline to understand if the defence of germline tissue is prioritised over the soma. Understanding this covariance is particularly important where ejaculate samples cannot be collected outside of the breeding season as germline oxidative damage that cannot be measured directly may be inferred indirectly from somatic damage or defence, As multiple samples per individual at different time points are required (see panel 2 in Fig. 1), blood samples may be the most realistic and ethical option to measure somatic oxidative status. Methods for measuring somatic oxidative damage and antioxidant levels have been comprehensively reviewed elsewhere (e.g. Monaghan *et al.*, 2009).

Mothers pass biomolecules to the developing offspring (Haq, Bailey & Chinnah, 1996; Myatt, 2006), providing an avenue for the passage of oxidative damage after fertilisation (Blount *et al.*, 2016). With the mother's somatic and offspring developing tissue being coupled in this way, maternal somatic oxidative damage is of marked importance for IOD. In males, the father's soma and offspring tissue are uncoupled, therefore, with the exception of rare cases of recoupling such as in male pregnancy (Harada *et al.*, 2022), male IOD cannot pass directly from the soma to the developing offspring. Therefore, whereas in females and rare cases in males (Harada *et al.*, 2022) where it is possible to use somatic levels of oxidative stress to test the oxidative shielding hypothesis, in males it is important to have access to germline levels of oxidative stress.

However, it is potentially difficult to sample males outside of the breeding season. Indeed, many species regress their reproductive system to some extent (Chemineau *et al.*, 2007), including examples in mammals (Newell-Fugate, Nöthling & Bertschinger, 2012; Jiménez, Burgos & Barrionuevo, 2015), fish (Fujimoto *et al.*, 2021), squamates (Aldridge *et al.*, 2020), and birds (Gupta, 2014; Srivastava *et al.*, 2015), which can pose problems for sample collection and quality (Chemineau *et al.*, 2007; Newell-Fugate *et al.*, 2012). Oxidative damage in sperm before the breeding season may not be measurable in these species.

Nevertheless, somatic oxidative damage, measurable year round, could help provide a global picture of the within-individual dynamic of oxidative stress levels. However, it is crucial to bear in mind that somatic and germline levels of oxidative stress are likely to vary throughout the year, and so is the relationship between them. First, ROS production is compartmentalised within tissues (Monaghan *et al.*, 2009), therefore reproduction might not cause similar levels of oxidative damage in somatic and germline tissue. Indeed, regulation of oxidative defence between these tissues may differ. In house sparrows (*Passer domesticus*), increased use of the antioxidant glutathione (GSH) in sperm compared to blood plasma was found under a pro-oxidant treatment (Mora *et al.*, 2017), and in reproducing and non-reproducing helper Damaraland molerats (*Fukomys damarensis*) experimentally increased demands of cooperative behaviour favoured oxidative protection of somatic erythrocytes over ejaculates (Mendonça *et al.*, 2020). Moreover, regulation of defence between the soma and the germline may vary throughout the year. Outside of the breeding season, selection is likely to favour somatic oxidative defence over the germline, whereas during the breeding season, germline protection from the oxidative cost of reproduction may be prioritised over somatic tissue.

In the presence of paternal care, preserving the soma may have relatively more importance (than if paternal care is not present) to maintain the father's condition to provision their offspring. However, we reason that germline oxidative status should still be prioritised over the soma as effects of oxidative damage to fathers on their short-term ability to provision will have relatively minor consequences for offspring fitness compared with germline damage inflicted directly during critical early development (Ciereszko *et al.*, 2005; Ménézo *et al.*, 2010; Kumar *et al.*, 2013; Fernández-Díez *et al.*, 2016).

With that in mind, multiple assessments of damage during the breeding season in sperm and somatic tissue can produce a robust assessment of an oxidative shielding response. Over the course of the breeding season, reproductive costs lead to the accumulation of oxidative damage, therefore greater oxidative damage should be identified deeper into the breeding season. A shielding response in the germline should mitigate or reduce this accumulation of damage. A within-individual increase in oxidative damage in the somatic tissue during the breeding season while the levels in the germline remain stable would suggest a prioritisation of germline over somatic oxidative defence, supporting the oxidative shielding hypothesis (Fig. 2).

(2) Is reproductive effort associated with an oxidative cost through damage accumulation?

Pre-conception behaviour and investment in reproduction must be measured in ways that are relevant to each species' biology. For example, in species with active mate competition that gives rise to fighting amongst rival males (Sharick *et al.*, 2015) or territorial defence (Olsson *et al.*, 2012), doubly labelled water (DLW) can be used to measure energy expenditure and thereby quantify the energetic cost of reproductive investment in free-living animals (Speakman, 1993; Westerterp, 2017). The optimal observation interval is one to three times the biological half-life of the isotopes, which depends on the metabolism of the subject (see Westerterp, 2017). Depending on this interval, metabolic

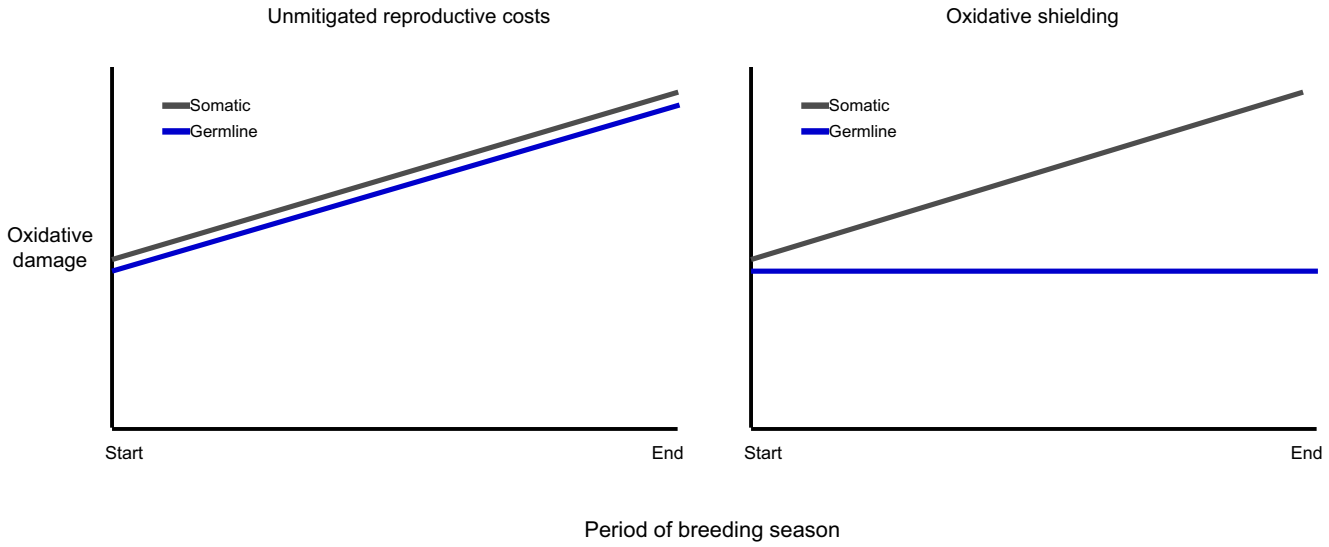

Fig. 2. Expected trends in somatic and germline oxidative damage over the course of the breeding season in the absence (left) and presence (right) of an oxidative shielding response.

rate can be measured over the course of a whole breeding season, or targeted at specific episodes of reproductive activity. The technical requirements for DLW mean it is not always an option for estimating energetic investment over the course of the breeding season. More recently developed methods, such as accelerometery in aquatic species (Metcalfe *et al.*, 2016) and heart rate telemetry (Halsey *et al.*, 2019), make direct energy expenditure measurements possible in a much wider variety of systems than in the past. Where difficulties remain for direct measures of energy expenditure, observations of the intensity and duration of male–male competitive interactions can still provide useful estimates of reproductive effort.

In species with female mate choice, male reproductive effort may include investment in extravagant sexual ornaments (Clifton, Braun & Abrams, 2016). These ornaments often include antioxidant pigments such as carotenoids (Blount *et al.*, 2003*b*; Giraudeau *et al.*, 2013), and reproductive effort can be measured in terms of colouration and size, representing the magnitude of resources diverted away from antioxidant protection (Kopena, López & Martín, 2014; Kim & Velando, 2020). Sperm competition occurs when females mate with multiple males, thus forcing competition between ejaculates to fertilise a given set of eggs (Parker, 1970). In species that engage in sperm competition, sperm quality may be used as a proxy of male reproductive effort. Markers of sperm quality include sperm density, sperm swimming ability, and a variety of morphological traits that contribute to sperm competitive ability (Fitzpatrick & Lüpold, 2014). Midpiece length may be a particularly important trait due to its association with mitochondrial density and ATP production that powers sperm motility, which may trade-off with the stability of the germline due to ROS production (Fitzpatrick & Lüpold, 2014). A given species may have multiple modes of reproductive effort as discussed above, and researchers should consider carefully which proxy or proxies to measure according to reproductive life history.

Increased oxidative damage levels in association with reproductive effort would indicate an oxidative cost to reproduction. However, individuals of higher quality may be able to cope with greater investment in reproduction without showing an increase in oxidative damage [the 'big house, big car' effect (Reznick, Nunney & Tessier, 2000; Metcalfe & Monaghan, 2013)]. This is illustrated in mosquito-fish (*Gambusia affinis*) where reductions in sperm count and velocity due to frequent mating only occurred in senescing males, whereas younger males had the capacity to maintain reproductive effort (Aich *et al.*, 2021). We hypothesise that younger males or those in better condition will have capacity to maintain oxidative shielding responses over the course of frequent reproductive bouts compared to other individuals. To account for such individual differences, experimental manipulation of reproductive effort (Garratt *et al.*, 2012), resource availability (Dupoué *et al.*, 2020) and/or energetic costs (Casagrande & Hau, 2018) will be required to confirm the oxidative cost of reproduction.

Oxidative damage before reproduction is assumed to be a baseline free from any legacy of previous reproductive costs. However, this assumption may rarely be met in iteroparous species due to carry-over effects from previous breeding events (Harrison *et al.*, 2011). Therefore, when possible, targeting virgin individuals in iteroparous species may provide the most powerful test of the oxidative shielding hypothesis.

(3) Is there a step change reduction in damage in reproducers compared to non-reproducers?

The oxidative shielding hypothesis predicts a pre-emptive decrease in oxidative damage levels in breeding

individuals (Blount *et al.*, 2016). To test for a pre-emptive decrease in oxidative damage, it is necessary to sample at three time points: before, during, and after reproduction (see panel 2 in Fig. 1). Indeed, single-time-point measurements of oxidative stress markers during a reproductive event can give a misleading picture of the oxidative costs associated with reproduction (Meniri *et al.*, 2022). Comparison between breeders and non-breeders can validate the expectation that transition to reproduction incurs a decrease in oxidative damage levels; damage levels are expected to remain relatively consistent over time in non-breeders.

Identifying non-breeding males may be challenging in some circumstances. Indeed, other than non-breeding subordinates in social groups, nearly all sexually mature males will attempt to breed to some extent, therefore discrete comparisons between breeders and non-breeders may not always be possible. Where this is the case, individuals with particularly low reproductive effort may be used, since it is possible that the cost of reproduction does not pass the threshold to trigger an oxidative shielding response (see graph 1.ii in Fig. 1). Such individuals may be compared to conspecifics with higher levels of investment – ideally where reproductive opportunity or effort has been experimentally manipulated.

Where all males breed, and the use of males with low reproductive effort in place of non-breeders cannot be justified, a more basic test of the oxidative shielding hypothesis can be made using within-individual comparisons of oxidative damage pre-, during, and post-reproduction (see panel 2 in Fig. 1). As long as there is a non-reproductive period a discrete step-change in reproductive investment should be selected, as maintaining reproductive readiness when there is minimal chance of successfully breeding is a waste of valuable resources (Stearns, 1992). For example in many seasonally breeding birds, male gonads regress outside of the breeding season, as maintaining gonads is costly (McNabb, 2000; Bauchinger, Hof & Biebach, 2007). A within-individual reduction in oxidative damage in breeding males is evidence in support of oxidative shielding (see panel 2 in Fig. 1).

Importantly, at very high levels of male reproductive competition, any decrease in oxidative damage arising from shielding may be overwhelmed by the oxidative costs of high reproductive effort (Fig. 3). For example, in field crickets (*Gryllus bimaculatus*), males exposed to intermediate levels of sperm competition showed a reduction in oxidative damage compared to a no-competition treatment, but there was no such reduction in males exposed to high levels of sperm competition (Noguera, 2022). Levels of antioxidants were not significantly different in males in the high *versus* intermediate sperm competition treatments. These findings suggest that multiple levels of reproductive costs, naturally present or experimentally manipulated, may sometimes be required to identify an oxidative shielding response.

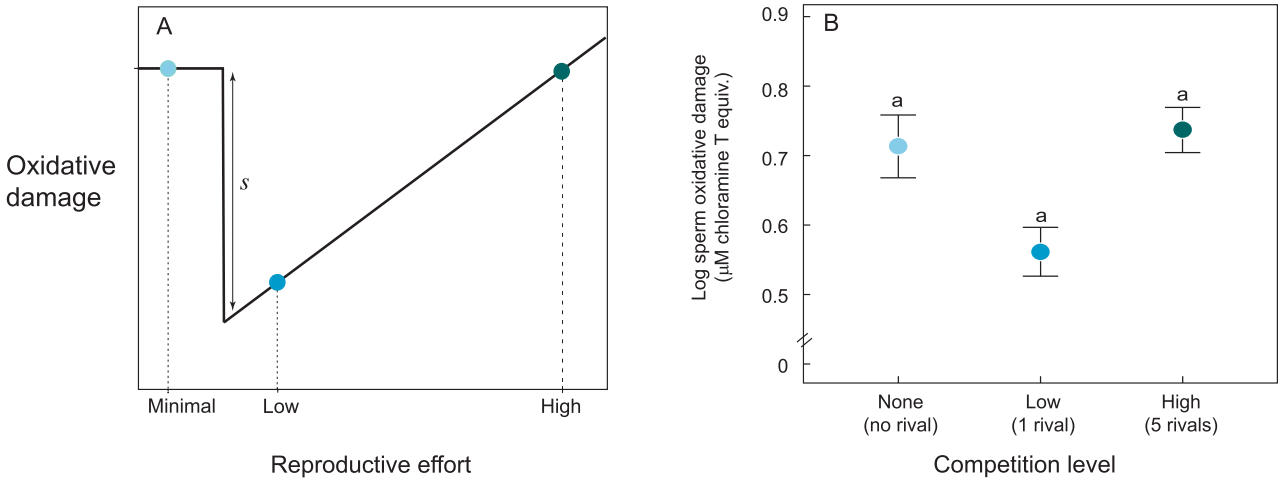

Fig. 3. (A) Hypothesised relationship between reproductive effort and oxidative damage indicative of an oxidative shielding response (modified from Blount *et al.*, 2016). Blount *et al.*'s (2016) figure is modified to illustrate a scenario where high reproductive effort is predicted to overwhelm the shielding response, bringing oxidative damage to levels that equal or exceed cases where there is an absence of shielding or minimal oxidative reproductive costs (such as in non-breeders, or competition-free breeders). *s* indicates the step-change reduction in damage that is hypothesised to be driven by oxidative shielding. (B) Oxidative damage sustained by male field crickets (*Gryllus bimaculatus*) experimentally exposed to three levels of reproductive competition (Noguera, 2022). Error bars show oxidative damage ± SEM and letters indicate significant differences according to *post hoc* tests (Noguera, 2022). Noguera's (2022) results could represent a situation where oxidative shielding can be detected where levels of reproductive competition are low, yet such shielding is overwhelmed when levels of competition are relatively high. In both parts of the figure dot colours correspond to separate groups of males engaging in different levels of reproductive effort (darker denotes greater reproductive effort or higher competition). An individual's transition between minimal effort (or non-breeding) to higher effort levels, such as between breeding seasons, should show the same relationship between effort and damage accumulation.

(4) Does accumulated oxidative damage in males predict their offspring's fitness?

To test whether males shield sperm cells to protect offspring against IOD, it will be necessary to couple data on sperm oxidative damage levels and offspring paternity ascertained either through experimental design or *via* the establishment of a genetic pedigree. Offspring survival and (ideally) the reproductive success of offspring when they themselves reach breeding age should be monitored to assess the connection between paternal oxidative damage and offspring fitness. In the absence of controlled laboratory conditions, it may not be possible to isolate the effect of IOD on offspring fitness from a plethora of correlated environmental effects (Monaghan, 2008; Marshall *et al.*, 2017; Jazwiec & Sloboda, 2019). When studying wild populations, relevant environmental variables such as rainfall, temperature, and food availability should therefore be measured so they can be controlled for in statistical models. Focussing on species without parental care means post-conception condition of parents cannot mask paternal intergenerational effects, providing a more controlled assessment of male oxidative shielding.

A promising avenue for study is provided by egg-laying species as the egg represents a sealed unit of maternal investment, unlike placental mammals where offspring have a physiological interaction with mothers throughout embryogenesis and lactation. The effects of male IOD can therefore be assessed more independently from maternal condition. Cross-fostering studies are similarly advantageous as the effect of parental care on offspring can be disentangled, providing a powerful controlled experimental design to test for evidence of male oxidative shielding.

V. REPRODUCTIVE LIFE HISTORY AND VARIATION IN OXIDATIVE SHIELDING

The above sections provide a guide for testing the oxidative shielding hypothesis in males but we can also make predictions of the types of systems where shielding would be plausible based on a given species' reproductive life history. We predict that the presence of oxidative shielding responses will be influenced by: (*i*) the magnitude of reproductive costs, which corresponds to the level of threat IOD poses to fitness in the next generation; and (*ii*) the extent to which IOD mitigation yields fitness pay offs (Fig. 4).

(1) Magnitude and form of reproductive cost will determine selection on shielding

One source of reproductive oxidative cost is the physical effort required to compete for mating opportunities. In elephant seals (*Mirounga angustirostris*), notable for having particularly costly competition over extensive periods of starvation, males were shown to have higher plasma levels of oxidative damage to lipids and DNA during the breeding season (Sharick *et al.*, 2015). Whether this extends to DNA damage in sperm was not tested, so whether any intergenerational costs or shielding mechanisms are involved is not known. To select for an oxidative shielding response, this physical effort of competing for mates may need to be significant as in many species moderate levels of physical activity have been found to result in no increase in oxidative damage accumulation (Soulsbury & Halsey, 2018), therefore IOD may not manifest at lower levels of reproductive activity. Although oxidative shielding may not benefit males with

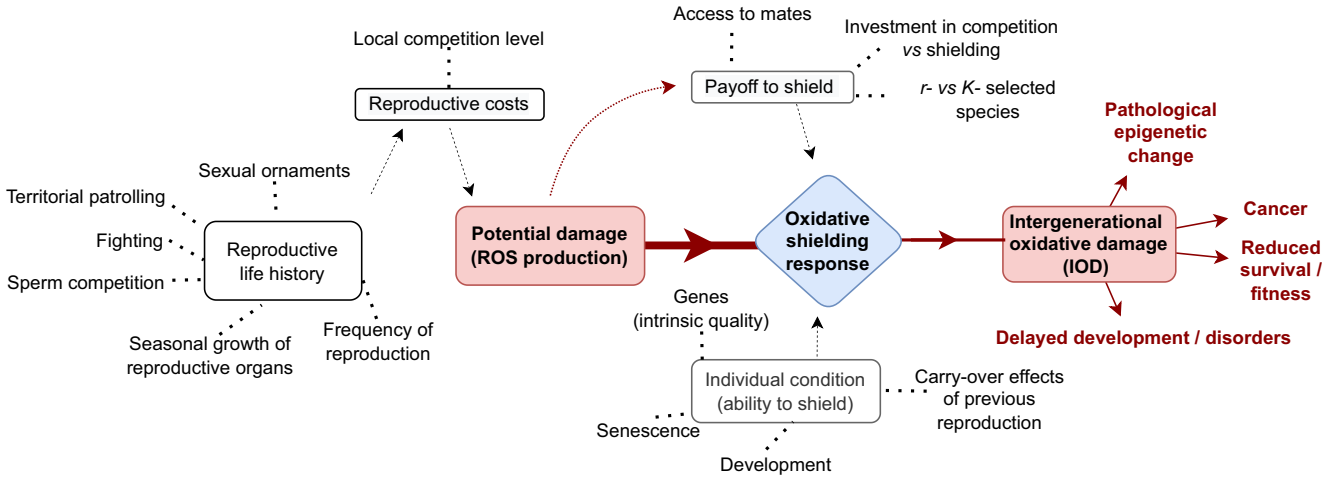

Fig. 4. Summary of factors that could determine the level of oxidative damage generation, the presence or effectiveness of oxidative shielding responses, and ultimately the level of intergenerational oxidative damage (IOD) passed on to the next generation with associated consequences for offspring. Dotted arrows represent causal relationships between factors determining the potential for oxidative damage or the presence/strength of a shielding response. A further level of contributing factors is linked by dotted lines. Red arrows are associated with oxidative damage, with arrow thickness indicating the magnitude of potential oxidative damage pre and post oxidative shielding, culminating in consequences for offspring fitness. Consequences for offspring as a result of IOD are given in red text. ROS, reactive oxygen species.

low levels of reproductive activity in isolated bouts, carry-over effects from the oxidative costs of past reproductive activity can erode oxidative defences (Romero-Haro *et al.*, 2016; Merkling *et al.*, 2017) which may cause IOD in subsequent reproductive bouts. Therefore, as the residual oxidative costs increase with frequency of reproductive events, there should be stronger selection for oxidative shielding.

A second type of reproductive investment is sexual ornamentation. Investment in sexual ornaments can involve antioxidants such as carotenoids, which can trade off against investment in immune defences and health (Blount *et al.*, 2003*b*; Kopena *et al.*, 2014). One of the few known cases of reproductive costs associated with sperm DNA damage has been linked to carotenoid investment into sexual signals in three-spined sticklebacks (*Gasterosteus aculeatus*) (Kim & Velando, 2020). Another type of reproductive investment is seasonal growth linked to reproduction, such as growth of testes in many bird species (McNabb, 2000; Bauchinger *et al.*, 2007). Such growth is likely to lead to increased ROS production, and as such might lead to IOD (Monaghan *et al.*, 2009; Christensen *et al.*, 2016). A shielding response to protect offspring against the oxidative cost of sexual ornaments or seasonal gonadal growth has not been assessed.

Finally, sperm competition is thought to represent a strong evolutionary force, shaping the evolution of ejaculate fertilisation abilities in a competitive environment, for example by selecting for improved sperm quality (Fitzpatrick & Lüpold, 2014; Simmons & Fitzpatrick, 2012). Higher metabolic activity powers more-competitive motile sperm to fertilise the egg, but this consequently increases ROS production, potentially trading off competitive ability against damage to the germline (Monaghan & Metcalfe, 2019; Silva *et al.*, 2019). Species where highly motile sperm are selected for may need oxidative shielding mechanisms to avert such germline damage. Indeed, in zebrafish (*Danio rerio*), higher levels of male–male competition led to the production of sperm with a longer midpiece and flagellum exhibiting greater sperm DNA damage (Silva *et al.*, 2019), likely consequences of higher oxidatively costly metabolic activity caused by this sperm phenotype (Fitzpatrick & Lüpold, 2014). Similarly, a comparative study on rodents found that higher levels of sperm competition were associated with greater DNA fragmentation (Delbarco-Trillo *et al.*, 2016). IOD as a result of competition has been evidenced in seed beetles, where beetles with artificially induced DNA damage to sperm passed on higher mutation loads to the next generation in the presence of social competition (Baur & Berger, 2020). This suggests that seed beetles could mitigate intergenerational damage until additional social reproductive costs materialised.

Oxidative shielding against the reproductive cost of sperm competition was recently shown in field crickets (Fig. 3B; Noguera, 2022). Protein oxidative damage in all crickets predicted offspring hatching success, suggesting that antioxidant investment into the germline shielded offspring from IOD when reproductive costs appeared. Surprisingly, individuals breeding in the absence of sperm competition (i.e. with no rival) did not exhibit an antioxidant-driven shielding response. An absence of male competitors is likely to represent a highly unnatural situation for field crickets, which usually breed within high-density populations (Tregenza & Wedell, 1998). Perhaps sperm competition cues are necessary to trigger an oxidative shielding response in this species (Noguera, 2022). Overall, these results highlight that high levels of sperm competition may lead to increased sperm DNA damage, with potential consequences for transfer of IOD and offspring fitness if preventive mechanisms such as oxidative shielding are deprioritised over competitive ability.

Under conditions of sperm competition, sperm cells can encounter the seminal fluid of rival males. If seminal fluid is important in an oxidative shielding response, the sperm of rival males might take advantage of this investment. Sperm can respond to the seminal fluid of rivals, for example in the grass goby (*Zosterisessor ophiocephalus*) sperm of secondary mating sneaker males in the presence of rival seminal fluid increased their velocity and subsequently fertilisation rate (Locatello, Poli & Rasotto, 2013). Similar dynamics have been found in chinook salmon (*Oncorhynchus tshawytscha*) (Lewis & Pitcher, 2017). The antioxidants invested in the seminal fluid by previous copulators as part of an oxidative shielding response may benefit rivals. Any free protection given through the seminal fluid of previous copulators may allow greater expression of oxidatively costly competitive traits by subsequent copulators without increasing IOD, such as more motile sperm increasing fertilisation success. Hypothetically, to avoid such 'free riding', shielding mechanisms may be selected to become more intracellular, such as through cellular antioxidants like vitamin E (Lesser, 2006; Lazzarino *et al.*, 2019), in cases where sperm competition is common.

Regardless of the form of competition, where a threshold level of energy expenditure is not reached, the body may accommodate additional ROS generated during reproduction through homeostatic mechanisms without a pre-emptive oxidative shielding response being necessary (Alonso-Alvarez, Canelo & Romero-Haro, 2017). Therefore, reproduction may not always be energetically costly enough to pass this damage accumulation threshold (see graph 1.ii in Fig. 1) (Zhang & Hood, 2016). There are plenty of examples, largely identified in females or when assessing cooperative caring effort in both sexes, where reproductive effort had seemingly no effect on future survival or condition of breeders (reviewed in Zhang & Hood, 2016), such as observed in both sexes of wandering albatross (*Diomedea exulans*) (Weimerskirch, 1992), and in female Columbian ground squirrels and male Tengmalm's owls (*Aegolius funereus*) where experimentally increased litter and brood sizes did not affect future reproduction or survival (Skibiel, Speakman & Hood, 2013; Thomson *et al.*, 2014). Similarly, in species where males do not invest significantly into sexually competitive traits, reproductive costs may not pass a threshold where oxidative shielding would be required. An absence of shielding may be expected in species where

male–male competition is rare, such as species with lifetime monogamy (Wittenberger & Tilson, 1980), as significant investment in mate competition may not be necessary to secure paternity.

(2) The payoff from protecting offspring will determine if shielding is selected

The threat of IOD may not always lead to the emergence of a shielding response (graph 2.ii in Fig. 1). There may be a trade-off for shielding with investment in other costly traits. If the payoff is not high enough, an oxidative shielding response may not be selected for, leading to unmitigated IOD transfer (graph 2.ii in Fig. 1). In such cases, we predict that breeders will have greater levels of oxidative damage than non-breeders, and the resulting paternal oxidative damage will strongly predict their offspring's fitness (see graph 3.i in Fig. 1).

The size of the payoffs of shielding may depend on the extent to which a father's reproductive fitness is determined by quality over quantity of offspring. Classical extremes are represented by *r*-selected species, which produce lots of cheap offspring, compared to *K*-selected species that invest more resources into fewer expensive offspring (McLain, 1991). In *r*-selected species, whose offspring may survive and reproduce more through chance than quality, shielding may have much less of a payoff compared to *K*-selected species that have relatively low extrinsic mortality risk.

Additionally, males' reproductive strategies within a species may impact the payoff of shielding. Theoretical models of sperm competition predict that subordinate males should invest proportionally more resources into their germline to make up for a lower access to copulations compared to dominant males, which are predicted to invest more heavily in the soma (Parker, 1990; Parker, Lessells & Simmons, 2013). Such predictions can be extended to our framework, with subordinate males predicted to invest more in oxidative shielding compared to dominant males. In house sparrows, dominant males had more oxidised and inviable sperm compared to middle hierarchy males (Mora *et al.*, 2016) although a recent study on the same system failed to replicate this result (Losdat *et al.*, 2019). Similarly, in three-spined sticklebacks, where large genetic variation in colour has been maintained despite sexual selection, duller morphs are maintained in the population despite reduced attractiveness to females. It was found that males with brighter carotenoid colouration, who are more attractive to females, had increased levels of DNA damage in sperm (Kim & Velando, 2020), a pattern that could be mediated by antioxidant allocation. Therefore, by extension of the predictions of sperm competition models (Parker, 1974), when certain individuals have reduced access to mates, they would be more likely to show an oxidative shielding response in order to maximise their reproductive success.

Targeting comparative analysis on species where the payoff of shielding varies between individuals or populations, such as species exhibiting alternative reproductive tactics, or strong *versus* low levels of mate competition, may be necessary to establish whether oxidative shielding is a ubiquitous mechanism for species at risk of IOD, whether it is only present in species whose life histories make shielding worth the investment, or if there are more flexible patterns of expression.

VI. CONCLUSIONS

(1) Only recently has the idea of life-history trade-offs having intergenerational consequences emerged. Intergenerational oxidative damage (IOD) may be a key factor influencing the fitness of the next generation. IOD may select for an oxidative shielding response, where an individual mitigates reproductive oxidative damage to protect the next generation.
(2) There have been few tests of the oxidative shielding hypothesis. Existing studies have focussed on females, yet males may benefit substantially from shielding as the vulnerability of sperm to oxidative damage makes males particularly prone to passing on oxidative costs of reproduction to the next generation. Additionally, the effectiveness with which males can mitigate IOD may be subject to individual condition.
(3) We present a framework and methods to test the oxidative shielding hypothesis in males. Laboratory studies may be a key first step, allowing the control and manipulation of reproductive costs and individual condition in order to understand the factors that contribute to variation in oxidative shielding.
(4) We make predictions of the plausible appearance of oxidative shielding in different systems based on insights from life-history theory. We propose that variation in the levels of reproductive costs and the payoffs from protecting offspring will determine whether oxidative shielding responses are present in males.
(5) Within-generation life-history trade-offs have been a cornerstone of evolutionary theory for 50 years. Oxidative shielding presents an opportunity to bridge an intergenerational gap that has been largely ignored in males for much of this field's history.

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