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Oxidative stress in sperm competition games: Experimental tests of the soma vs. germline allocation trade-off in wild House Sparrows *Passer domesticus*

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by

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"Oxidative stress in sperm competition games: experimental tests of the soma vs. germline allocation trade-off in wild house sparrows *Passer domesticus*"

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Summary

Female promiscuity leads to the situation where ejaculates of two or more males compete for the fertilization of the ova. Therefore, the reproductive success of a male largely depends on its ejaculate quality, and thus sperm competition exerts strong selection into traits that maximize the fertilizing ability of an ejaculate. Theory predicts that males should progressively increase resource investment into the production of high quality ejaculates as they incur in higher costs to obtain a mate. Yet, the resources being strategically allocated between somatic vs. germline functions remained to be uncovered. Sperm cells are highly vulnerable to oxidative stress (OS), which is known to impair male fertility. Thus, males being able to better protect their ejaculates from oxidative damage should produce higher quality ejaculates. In species where social dominance determines access to fertile females, the oxidation-based soma vs. germline trade-off predicts that subordinate males would strategically allocate more antioxidant resources into their germline, and thus far best in ejaculate quality. In contrast, dominant males should prioritize the protection of their soma over their germline, and thus produce more oxidized and lower quality ejaculates. I tested these predictions using wild House Sparrows *Passer domesticus*, a passerine species where male reproductive behaviors are associated to their social dominance. To approach the oxidation-based soma vs. germline predictions I experimentally (1) manipulated males' social status, (2) increased the amount of oxidative stress, and (3) induced an immune response. For those experiments, I explored how male phenotype (e.g. badge and/or dominance) would correlate to germline traits (e.g. sperm morphology, swimming ability) and to patterns of antioxidant allocation into sperm. I found evidence that males that better protect their ejaculates from oxidative damage produced better quality ejaculates, and thus dominant males produced more oxidized and less motile ejaculates (Chapter 1). For instance, when males face higher levels of oxidative stress, they produce ejaculates that are more oxidized and swim at lower speeds (Chapter 5). Differences in ejaculate quality were not explained by differences in sperm morphology, yet the correlations between sperm morphological design and function across social ranks suggest that ejaculate quality depends on the energetics of the sperm cell (Chapter 2). Further, dominant males also produced ejaculates that have larger morphological variation, suggesting that they invest fewer resources into controlling their ejaculate production (Chapter 3). Remarkably, I showed that males can adjust their ejaculate quality and sperm morphological variation to rapid changes of their social environment (Chapters 1 and 3), and that changes in ejaculate quality are paralleled by changes in antioxidant allocation into the ejaculate (Chapter 3). Further, we did not find evidence that male secondary traits would reflect male fertility, yet it seems to signal the oxidative balance of sperm (Chapter 4). Finally, we observed that males at the lower end of the hierarchy could produce ejaculates similar to those of the dominant males, namely their ejaculates had high levels of morphological variation, high oxidative stress, and low motility (Chapters 1 and 3). We suggest that males at the bottom of the hierarchy cannot invest as much resources as predicted into ejaculate quality without compromising their somatic condition. Thus, when they gain positions within a social hierarchy they proportionally increase ejaculate investment (Chapter 1), and they their ejaculates are the most damaged when males face an oxidative challenge (Chapter 5). Altogether, I suggest that under sperm competition oxidative stress and antioxidant allocation are the physiological mechanism modulating male reproductive tactics.

Keywords: Sperm competition, soma vs. germline, oxidative stress, House sparrow, reproductive strategies

General introduction

Organisms maintain a stable physiological balance (e.g. fluids and energy) that is optimized to the biotic and abiotic conditions, and such homeostatic system depends largely on various physiological and behavioural responses that act in concert to face environmental variation. Stress is any perturbation to homeostasis that will require an individual to expend energy into restoring the original homeostatic state, thus maximizing survival (e.g. fight or flight response) (Nelson 2011). While stress in the literature has become a fuzzy term, an alternative model for homeostasis is allostasis – the process on maintaining stability through both behavioural and physiological responses –. The cumulative costs of allostasis are known as allostatic load, which under optimal circumstances the allostatic load does not exceed the energy available in the environment (McEwen and Wingfield 2003). However, when the energetic costs of allostasis either are maintained over time or exceed the energy available in the environment, the individual will shut down non-essential processes in order to maximize survival (e.g. reproduction, growth) (Wingfield et al. 1998, Nelson 2011).

Box 1. Sperm competition

When females copulate with two or more males, sexual selection continues inside the female reproductive tract (Parker 1998, Birkhead and Pizzari 2002). Strong selection is exerted in traits that enhance the fertilizing ability of an ejaculate (e.g. sperm numbers, sperm velocity, sperm longevity, etc.), and the male with the better ejaculate quality will fertilize the ova (Snook 2005, Fitzpatrick and Lüpold 2014). Various mechanisms for sperm competition have been proposed (reviewed by Parker 1998, Parker and Pizzari 2010), however here I will briefly explain two basic mechanisms that can occur in birds:

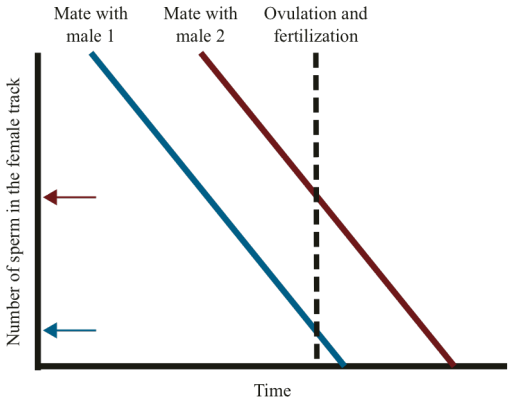

Figure B1. A passive loss raffle where two males copulate with a female and inseminate the same amount of sperm. At the time of ovulation, the sperm of male 1 has been largely lost, and male 2 has higher sperm number in the female tract. Therefore, male 2 is more likely to fertilize the ova. Adapted from Birkhead and Pizzari (2002)

Fair raffle (Parker et al. , Parker 1990)
The fair raffle is the simplest of all mechanisms, where all ejaculates are assumed to have the same fertilizing capacity. Therefore, males gain an advantage by varying the amount of sperm being expended in an ejaculate, and thus sperm competes like in a lottery (e.g. the more tickets one gets, the more likely one is to win). In a two males contest, the probability of male-2 to fertilize the eggs is $P_2 = s_2 / (s_1 + s_2)$, where s_2 is the number of sperm inseminated by male 2. In support of this mechanism, Martin et al. (1974) found that when the ejaculates of genotypes of domestic fowls *Gallus domesticus* were pooled in different proportions and artificially inseminated, paternities were proportional to the proportion of sperm from each phenotype.

Loaded raffle (Parker et al. , Parker 1990)
The loaded raffle is an extension of the fair raffle. The fertilizing capacity of an ejaculate is not assumed to be equal between males, and thus sperm of one male is more likely to fertilize the ova. Therefore, one male will gain a direct advantage expressed as a loading factor (r), which is added to the fair raffle. Thus, if ejaculates from two males are competing, the probability of male-2 to fertilize the ova is $P_2 = s_2 / (rs_2 + s_2)$. A special case for the loaded raffle is the passive loss raffle (Lessells and Birkhead 1990), where sperm is assumed to perish at a constant mortality rate (k) through time (t). Therefore, the loading factor is r=exp(k*t), which reflects a passive loss of sperm through time (Fig. B1). Previous studies in birds had reported that the last male copulating with the female would gain an advantage (Compton et al. 1978, Sims et al. 1987, Birkhead et al. 1989), suggesting that sperm of the last mating male gets stored closer to the ova (e.g. stratification; Lessells and Birkhead 1990). However, such results could be better explained by passive loss of sperm (Birkhead et al. 1995b).

Box 1. Sperm competition (continued)

This doctoral thesis was developed under a recent mathematical model that assumes the situation where males have a resource budget R that can be allocated to either finding mates (c) or producing sperm (s). Thus, a male playing a strategy s is expected to have a number of mates n(s) = R / (s + c), and the fitness of such individual will depend on its reproductive success $v(s, \hat{s})$ per mating. A main assumption of this model is that $v(s, \hat{s})$ follows a fair raffle, and therefore the reproductive success of a male mated with a female that copulate with other k males playing a strategy ŝ is proportional to the amount of resources s that are invested into producing sperm, $s / s + k\hat{s}$. Further, the probability that the female copulates with other k males (p_k) is assumed to follow a Poisson distribution, $\hat{n}^k e^{-\hat{n}} / k!$. Therefore, the reproductive success of a male playing a strategy s will be

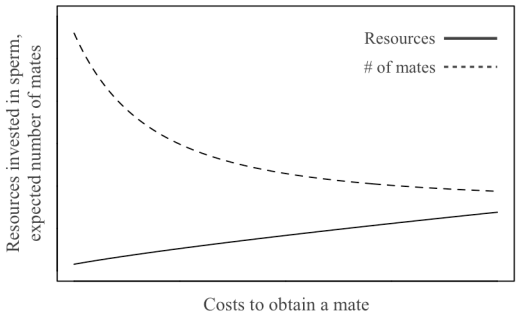

Figure B2. Evolutionary stable strategies of resource allocation into sperm *s(c)* (solid line) and expected number of mates *n(c)* (dashed line) for males paying different costs *c* to obtain a mate. Taken and modified from Tazzyman et al. (2009).

$$v(s, \hat{s}) = \sum_{k=0}^{\infty} p_k \frac{s}{(s + k\hat{s})}$$

Finally, in a population where males have a $\hat{n}$ expected number of mates and a $\hat{s}$ ejaculate investment, the fitness (W) of a male having a resource budge R, mating costs c, and a ejaculate investment invest $s(R, c)$ will be:

$$W(R, s, c|\hat{s}, \hat{n}) = \frac{R}{(s + c)} \sum_{k=0}^{\infty} p_k \frac{s}{(s + k\hat{s})}$$

If the model is iterated for resources, allocation into sperm production remains unchanged (Tazzyman et al. 2009). However, if the costs c to obtain a mate continuously vary, males are predicted to invest more resources into sperm production as the costs c increase (Fig. B2). Such predictions are similar to those of a more recent model (Parker et al. 2013). In species where continuous phenotypic variation is correlated to reproductive success (e.g. dominance), these models could be used to predict the potential physiological mechanisms involved in the trade-off between somatic and reproductive investment (further discussed in Parker and Pizzari 2010).

This later scenario is known as the emergency life history state, and it has been argued to be an adaptative response to challenging environments (Wingfield et al. 1998).

An unavoidable departure from homeostasis is oxidative stress. Reactive oxygen species (ROS) are metabolic by-products that cause random cellular damage, which leads to cell death, ageing, degenerative diseases, or impaired cellular pathways (reviewed in Finkel and Holbrook 2000). Although ROS are needed to control and promote physiological processes (e.g. ATP production, immune response, etc.; Finkel and Holbrook 2000), in many instances antioxidants cannot quench all the ROS and further ROS-induced damage is known as oxidative stress (OS) (Jones 2006). Importantly, regular metabolic processes (Finkel and Holbrook 2000, Zitzmann et al. 2003, Hulbert et al. 2007), immune response (Bedard and Krause 2007, Sorci and Faivre 2009), and pollutants (Banerjee et al. 2001, Li et al. 2003) are unavoidable factors that are responsible for OS generation. Given that all organisms are subject to attacks by ROS, oxidative stress is hypothesized to be a main constraint to life history evolution (Dowling and Simmons 2009, Monaghan et al. 2009, Metcalfe and Alonso-Alvarez 2010).

Reproduction is costly and physiologically demanding (Reznick 1985, Speakman 2008), and as a consequence the costs of reproduction are usually traded-off with other life history traits. For example, increased investment in immediate reproduction is traded-off against survival and thus future reproduction (Stearns 1989). Increasing

number of studies show that oxidative stress might be an important cost of reproduction. For example, egg production in fruit flies *Drosophila melanogaster* was stimulated by supplementing females with extra nutrients (Wang et al. 2001), hormones (Salmon et al. 2001), or mate availability (Rush et al. 2007), and such females producing more eggs were more prone to OS compared to control females. Further evidence that oxidative stress is a resulting cost of reproduction comes from studies in zebra finches *Taenopygia guttata* (Alonso-Alvarez et al. 2004, Alonso-Alvarez et al. 2006, Bertrand et al. 2006). For instance, Alonso-Alvarez et al. (2004) experimentally increased brood size to manipulate reproductive effort, and they found that males in charge of large broods had reduced body mass and higher vulnerability to oxidative damage. Further, in zebra finches the number of breeding events (Alonso-Alvarez et al. 2006) and number of laid eggs (Bertrand et al. 2006) is negatively correlated to the antioxidant capacity of the breeding pairs, and such correlation disappear when breeding pairs are supplemented with antioxidants (Bertrand et al. 2006). Thus, OS might represent a major constrain when sexual selection occurs, and thus male reproductive strategies might be modulated in part by the OS costs in which they incur in.

One important way through which OS constrain male reproductive strategies is the deleterious effects of OS on male fertility. Sperm cells contain a large amount of polyunsaturated fatty acids (PUFA) on their membrane, and the presence of PUFA has been shown to enhance ejaculate traits (reviewed by Wathes et al. 2007). For example, in boars it has been shown that dietary PUFA supplementation results in a higher amount of such fatty acids in sperm, which in turns enhance sperm mobility and velocity (Mitre et al. 2004). However, PUFA can be easily oxidized by ROS (reviewed by Hulbert et al. 2007), and lipid peroxidation largely affects the integrity of sperm membranes (Aitken 1999, Aitken and Baker 2004). Oxidative damage on sperm membranes can cause impaired cell-egg interactions, reduced motility, or cell death (Aitken 1999, Aitken and Baker 2006, Tremellen 2008). Additionally, ROS can also attack DNA causing changes in chromosome arrangements, histones modifications, deletions, base modifications, or changing methylation patterns (Lloyd et al. 1997, Kemal Duru et al. 2000, Wellejus et al. 2000, Aitken and Krausz 2001, Zitzmann et al. 2003), and sperm DNA is easily attacked by ROS leading to male infertility (Aitken 1999). For example, oxidative damage on sperm DNA can cause deleterious mutations that can affect embryo development or reduce offspring fitness (i.e. 8-oxo guanine is ROS-caused based modification that leads to infertility in sons of smokers, Zitzmann et al. 2003). Hence, oxidative damage has been proposed as one important cause of infertility (Aitken 1999, Tremellen 2008), and might be a major constraint to male fitness.

In species where females copulate with several males, sperm of two or more male compete for the fertilization of the ova (Box 1 for some of the mechanisms involved in sperm competition). Under sperm competition, variation in both male's ability to monopolize females and the fertilizing ability of their ejaculate determines their reproductive success (Birkhead and Pizzari 2002), and strong selection acts upon traits that maximize ejaculate quality (Snook 2005, Simmons and Fitzpatrick 2012, Fitzpatrick and Lüpold 2014). Both sperm speed and viability have been shown to give a fertilizing advantage under sperm competition in different taxa (for reviews see Snook 2005, Fitzpatrick and Lüpold 2014). In the Atlantic Salmon (*Salmo salar*) it has been found that faster swimming sperm gets a larger share of the paternities when competing with a slower ejaculate (Gage et al. 2004), while in insects male variation in sperm viability explains variation in paternity share under sperm competition (García-González and Simmons 2005). Interestingly, those traits that confer a higher fertilizing

ability to an ejaculate are also traits that can be affected by oxidative stress and/or antioxidant availability (in the rainbow trout Ciereszko and Dabrowski 1995, e.g. in rats Chitra et al. 2003, in birds Helfenstein et al. 2010a). For example, Helfenstein et al. (2010a) observed on male great tits (*Parus major*) that ejaculates with lower initial speeds were more oxidized. Also, they reported that individuals who underwent through reproductive stress and received carotenoids supplementation had an enhanced proportion of motile sperm in their ejaculate. However, on a following study Losdat et al. (2011) did not find any effect of carotenoid supplementation on sperm quality, though they argue that carotenoids could have been used to increase the antioxidant potential in the soma. Therefore oxidative stress can be hypothesized as a strong constraint on sperm competition games. Further, females might gain both direct and indirect benefits from avoiding males that might have oxidatively damaged sperm (Blount et al. 2001, Velando et al. 2008). For the above-mentioned reasons, I hypothesized that males being able to better protect their ejaculates from oxidative stress will enjoy of better ejaculate quality, and thus be more likely to outcompete other males in sperm competition.

In species where social dominance determines the access to fertile females, theory predicts a negative correlation between male's ability to monopolize resources and ejaculate quality (Parker 1998, Parker and Pizzari 2010). Indeed, the predictions of those models have been previously observed in various taxa (Preston et al. 2001, Rudolfsen et al. 2006, Cornwallis and Birkhead 2007, Thomas and Simmons 2009, Evans 2010, Lemaître et al. 2012). For example, in the Arctic charr (*Salvelinus alpinus*) dominant males produce slower swimming sperm that subordinate males (Rudolfsen et al. 2006), and in feral fowls subordinate males have ejaculates with higher viability (Froman et al. 2002). However and importantly, the physiological proximate mechanisms modulating ejaculate quality are yet to be uncovered. Two recent theoretical models proposed that as the costs to get a mate increase, males are expected to continuously invest more resources in their ejaculate (Box 1) (Tazzyman et al. 2009, Parker et al. 2013). Thus, if continuous male phenotypic variation correlates to variation in access to fertile females, males with the lowest access to females – the typical case of a subordinate male - are expected to increase resource investment into their germline at the expense of their soma (e.g. soma vs. germline trade-off). If males being able to better protect their ejaculates from oxidative stress will enjoy better ejaculate quality, then one could predict that males could vary how much antioxidant resources they would invest into somatic vs. germline functioning (e.g. oxidation-based soma vs. germline trade-off). Therefore, dominant males would be expected to disproportionally invest their antioxidant resources to the somatic rather than the germ-line functioning, and thus maintain their body condition and social status. In contrast, subordinate males should invest more antioxidant resources to the germ-line to increase the competitiveness of their ejaculate.

In this doctoral thesis I tested the predictions of the oxidation-based soma vs. germline trade-off in House Sparrows (*Passer domesticus*), a socially monogamous bird species where females mate with a single male in a reproductive season. In house sparrows around one quarter of the unhatched eggs are due to infertility (Birkhead et al. 1995a). Theory predicts that in socially monogamous species females that engage into extra-pair copulations would benefit form higher fertility rates – even if they know the fertility of their partner – (Hasson and Stone 2009), and for instance promiscuity in this species leads to extra-pair paternity rates ranging from 12-14% (Møller 1987a, Wetton and Parkin 1991, Møller and Birkhead 1994, Wetton et al. 1995). House sparrow males and females have a hierarchical social system (Anderson 2006), where male dominance

correlates to variation in copulation rates (Møller 1988, 1990, 1992, Riters et al. 2004), mate guarding rates (Møller 1990, Anderson 2006), and - for some populations - extra-pair paternity (Václav et al. 2002). Female house sparrows get involved in both forced and non-forced extra-pair copulations (Møller 1987a), but females mated to more dominant males are less likely to be forced into an extra-pair copulation (Møller 1987a, Møller 1992). Thus, while dominant males are able to better avoid sperm competition in their social mate, subordinates are likely facing a higher risk of sperm competition due to their inability to keep other males from copulating with their social partner. For the above-mentioned reasons, house sparrows provide a great animal model to test the predictions from the oxidation-based soma vs. germline hypothesis, and thus I conducted three field experiments with wild-caught House Sparrows housed in outdoor aviaries.

First, I manipulated the social status to test the role of the social environment on sperm quality and antioxidant allocation. Phenotypic plasticity allows individuals to modify traits in other to maximize fitness under different environmental circumstances (Via et al. 1995). Therefore, if ejaculate quality determines the reproductive success of a male under sperm competition (as reviewed above), then it would be expected that males would flexibly respond to changes in their risk of sperm competition by adapting their ejaculate quality. For example, previous studies in birds have shown that males adjust their sperm morphology and ejaculate quality to match changes in the social environment (Cornwallis and Birkhead 2007, Immler et al. 2010), and thus I explored how changes in antioxidant allocation could modulate plastic responses of sperm quality to a changing social environment. In a second experiment, I experimentally manipulated the oxidative balance of males by giving them a potent pro-oxidant. For instance, it has been observed that both the quality and the oxidative balance of an ejaculate are affected by exposure to pro-oxidants (Chitra et al. 2003, Lacoume et al. 2009, Wang et al. 2009, Abarikwu et al. 2010), and thus I explored how induced pro-oxidant production could change patterns of antioxidant allocation into the germ-line functions given the social status of an individual. In a third experiment, I experimentally challenged the immune system by causing a controlled parasite infection (results not included in this document). The immune response is known for causing oxidative stress insults (Bedard and Krause 2007, Costantini and Møller 2009, Sorci and Faivre 2009), giving a perfect natural frame to test whether males would adjust antioxidant allocation into germ-line functions given their social ranks and the oxidative-related costs of the immune response. Finally, ejaculate quality can involve a multidimensional set of traits, including both functional (e.g. motility and swimming speed) and morphological features of sperm (Snook 2005, Fitzpatrick and Lüpold 2014). Therefore, for all of the above-mentioned experiments I explored both traits of ejaculate and morphology, and correlated them to either the social status of an individual or its oxidative balance. Further, the oxidative balance integrates several types of antioxidant defenses and oxidative damage to several molecules (Box 2) (Cohen and McGraw 2009, Monaghan et al. 2009). So, for my doctoral thesis I used markers that would represent both changes in the antioxidant capacity of an individual as well as the oxidative damaged caused to cell membranes in both somatic and germ-line tissues (further details in Box 2). Here in I present evidence that when males face different levels of sperm risks, their reproductive strategies are likely mediated by strategic antioxidant into germline functioning.

Box 2. How to measure oxidative stress

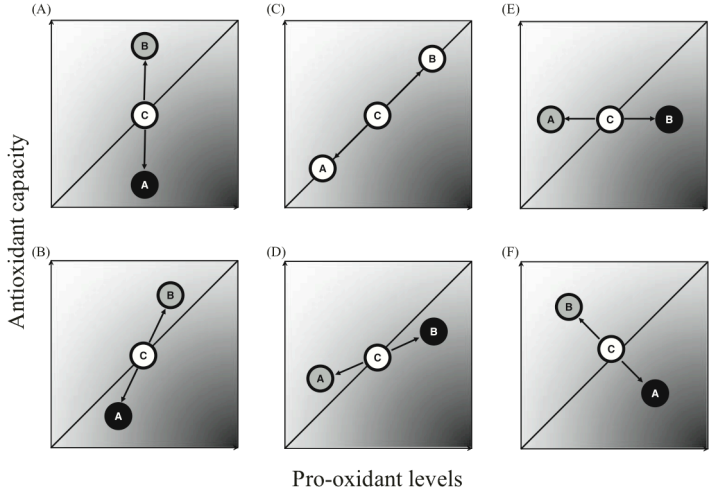

Figure B3. Scenarios for different levels of both antioxidants and proxidants that can lead to low levels of oxidative damage (gray), high levels of oxidative damage (black), or no oxidative damage (white). For further information refer to the text. Taken and adapted from Costantini and Verhulst (2009).

Oxidative stress is the result of an unbalance between antioxidants and prooxidants, in favor of the latest (Jones 2006). However, the oxidative balance is characterised by a multidimensional system integrating several lines of antioxidant defences and oxidative damage to various biomolecules (Cohen and McGraw 2009, Monaghan et al. 2009). Therefore, when evolutionary ecologists use oxidative stress to understand the physiological trade-off of life history traits, it is challenging to distinguish between shifts in antioxidant capacity and oxidative damage (Costantini and Verhulst 2009, Hõrak and Cohen 2010). For instance, Costantini and Verhulst (2009) illustrated several scenarios where the oxidative balance can change leading to different outcomes of oxidative damage and antioxidant capacity (Fig. B3), which I will briefly summarize bellow (for further details and examples refer to Costantini and Verhulst (2009)).

If two individuals A and B do not differ in their production of pro-oxidants, one could conclude that they should bear the same levels of oxidative damage. However, if they differ in their antioxidant capacities, the individual with the lowest antioxidant capacity will suffer the most oxidative damage (Fig. B3 A). However, individuals can overexpress antioxidants in response to an elevated production of pro-oxidants, and yet have lower levels of oxidative damage compare to individuals that produce less pro-oxidants (Fig. B3 B). Thus, individuals can adjust their antioxidant expression in order to match the pro-levels, and differences in either antioxidant capacity or production of pro-oxidants are not necessarily reflected in oxidative damage (Fig. B3 C). However, individuals can suffer higher oxidative damage because the antioxidant response is not sufficient to maintain a low level of oxidative damage (Fig. B3 D). Now, if A and B have the same antioxidant capacity, they might suffer different levels of oxidative damage if their pro-oxidant levels differ (Fig. B3 E). Finally, B can have lower oxidative damage, and yet have higher antioxidant capacity than A (Fig. B3 F).

As evidenced above, it is important to select a battery of biomarkers that represent can provide a general overview of the changes in oxidative balance of an individual. Yet, further methodological problems have been identified with the assessment of oxidative stress markers (for reviews, see Dalle-Donne et al. 2006, Hermans et al. 2007, Knasmüller et al. 2008), and any results coming from unreliable methodologies are difficult to interpret. Therefore, a rigorous set of criteria should be considered when choosing the biomarkers of oxidative stress (Dalle-Donne et al. 2006, Hermans et al. 2007, Hõrak and Cohen 2010):

1. The assay for the biomarker should represent the *in vivo* function of the target molecule: Several assays recreate oxidative environments to measure the peroxidability of a substrate, however they do not represent the same oxidative environments found *in vivo*. Thus, results from such assays are hard to interpret, because the substrate might have different susceptibilities to various potential pro-oxidants (Halliwell and Gutteridge 2007). Among such assays, the most popular one is the total antioxidant capacity assay (TAC), which measures *in vitro* the ability of a tissue to quench a set of pro-oxidants in a solution.

Box 2. How to measure oxidative stress (continued)

2. The biomarker should be stable: Several biomarkers of the oxidative balance are prone to collection and assay conditions (e.g. glutathione oxidation during sample preparation, Rossi et al. 2006). However, this can be easily controlled with if sample repeatability is estimated. Further, some assays can result in an artefactual formation of the target molecule, thus making the results of such assays unreliable. For example, the thiobarbituric acid-reacting (TBARS) assay estimates the levels of MDA by its derivatization with thiobarbituric acid (TBA). However, TBA is also known to react with other compounds like amino acids and sugars (Meagher and Fitzgerald 2000, Abuja and Albertini 2001), thus making any result derived from the TBARS assays unreliable.
3. Validation of the method: When validating an assay, it is important to consider its repeatability, sensitivity, and specificity. If any known confounding factors might affect the assay, they should be considered during the laboratory analyses and/or sample collection. Therefore, samples should be ideally done in duplicate to estimate either repeatability or coefficients of variance. However, if sample volumes are not sufficient, a previous validation of the method using aliquots of samples is essential.
4. Sample collection should not interfere with the normal life of the individuals: The samples taken from animals should adhere to a well practice of animal welfare, which in some cases it means that the biological sample volumes will result small for the implementation of popular laboratory kits for biomarker determination. For example, when repeated sampling on an individual is required, changes in the oxidative balance could be result from either physiological stress (Costantini et al. 2011) or inflammatory responses (Costantini and Møller 2009) coming from any harm caused during the previous sampling session.
5. Not confounded by diet: Several biomarkers of oxidative stress could be ingested and absorbed, thus the diet of the individual could bias any measurement of oxidative stress. For example, aldehydes and lipid hydroperoxides can be absorbed from diet, and thus measurements of MDA could be confounded (Draper et al. 2000, Wilson et al. 2002). However, in experiments where individuals are fed a standardized diet, this is a minor confounding factor (Dalle-Donne et al. 2006, Hõrak and Cohen 2010).

For the above mentioned reasons, in this doctoral thesis I chose to describe the oxidative balance of different either somatic or reproductive tissues through (1) MDA as a marker of oxidative damage, i.e. the levels of end-products of lipid peroxidation, (2) a marker of cellular oxidative stress, i.e. the ratio of oxidized over reduced glutathione, and (3) the activity of the antioxidant enzyme SOD, i.e. an endogenous antioxidant that catalyzes the dismutation of superoxide anions into molecular oxygen or hydrogen peroxide.

Chapter 1

Role of social status in patterns of antioxidant allocation into sperm quality

In preparation for Journal of Animal Ecology

Antioxidant allocation modulates ejaculate quality across changing social environments

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Abstract

In promiscuous species the sperm of several males compete for fertilization, and the fertilizing ability of an ejaculate, i.e. ejaculate quality, determines males' reproductive success. Theory predicts males to progressively increase resource investment into ejaculate quality as they have lesser access to females. Sperm cells are highly vulnerable to oxidative stress (OS), which impairs male fertility. Since social dominance is a major determinant of mating opportunity, we predict that subordinate males should be faring best by investing more antioxidants in their ejaculates. We tested this hypothesis in House Sparrows and found that ejaculate quality correlates with OS level in sperm, while dominant males produce more oxidized and lower quality ejaculates. After experimentally manipulating the social environment, males matched their ejaculate quality to their new rank, while increases in antioxidant investment into ejaculates paralleled increases in ejaculate quality. Our results highlight OS and strategic antioxidant allocation as the proximate physiological mechanism underlying ejaculate quality and male reproductive tactics.

Keywords: Sperm competition, social dominance, oxidative stress, antioxidant allocation, soma vs. germ-line, ejaculate plasticity

Introduction

Under sperm competition risk, ejaculate quality determines the fertilizing ability and the reproductive success of a male (Birkhead and Pizzari 2002, Parker and Pizzari 2010). Thus, selection acts upon various ejaculate traits (e.g. velocity, proportion of motile sperm, ATP production, etc.) to maximize the fertilizing ability of the ejaculate, generally referred to as ejaculate quality (Snook 2005, Simmons and Fitzpatrick 2012, Fitzpatrick and Lüpold 2014). Several theoretical models have explored how much males in different social roles are selected to invest into post-copulatory traits, i.e. ejaculate quality and competitiveness (Parker 1998, Parker and Pizzari 2010). The predictions of those models have been tested in several taxa (Preston et al. 2001, Rudolfsen et al. 2006, Cornwallis and Birkhead 2007, Thomas and Simmons 2009, Evans 2010, Lemaître et al. 2012), though only discrete social roles have been tested (e.g. favoured vs. disfavoured). A recent model predicts that continuously increasing costs to obtain a mate should select for progressive increases of resource investment in the ejaculate (Tazzyman et al. 2009), resulting in a continuous trade-off between

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somatic vs. germ-line functioning. Some evidence exists in support to this model, such as Engqvist's (2011) finding of a negative genetic co-variation between attractiveness and mating investment in the scorpionfly (*Panorpa cognata*). Remarkably, although several studies have investigated the potential outcomes of a soma vs. germ-line allocation trade-off, the actual resources to be strategically allocated to the germ-line are yet to be identified.

Oxidative stress (OS) is the unbalance between pro-oxidants and antioxidants (Finkel and Holbrook 2000), and has been hypothesized to be a major cost of reproduction (Wang et al. 2001, Alonso-Alvarez et al. 2004). Sperm cells are especially prone to OS (Aitken 1999), and OS has been shown to be deleterious to sperm (Jones et al. 1978, Kemal Duru et al. 2000, Chitra et al. 2003, Tremellen 2008, Ribou and Reinhardt 2012). Various studies have shown that ejaculate quality correlates with the level of oxidative stress in the ejaculate (Chitra et al. 2003, Helfenstein et al. 2010a, Losdat et al. 2011), while antioxidant supplementation seems to improve ejaculate quality (Ciereszko and Dabrowski 1995, El-Demerdash et al. 2004, Ateşşahin et al. 2006, Almbro et al. 2011). Consequently, OS has been identified as an important cause of male infertility (Aitken 1999, Aitken and Baker 2004, Tremellen 2008), and may thus act as a selective pressure in shaping various sperm traits. For example, a comparative analysis has shown that across mammals the amount of polyunsaturated fatty acids, which make sperm membranes prone to OS (Hulbert et al. 2007), negatively correlates with the species level of sperm competition (delBarco-Trillo and Roldan 2014). Here, we propose that antioxidants are the resource being traded-off between somatic vs. germ-line functions, and hence strategic antioxidant allocation in the ejaculate should modulate ejaculate quality.

In species where dominance determines access to fertile females we predict that sperm quality positively correlates with antioxidant allocation in the ejaculate, while OS should be negatively correlated with sperm quality. We further predict that males occupying lower social ranks should invest more antioxidant resources to the production of higher quality ejaculates. In contrast, more dominant males should invest less antioxidant resources, and thus produce more oxidatively stressed and lower quality ejaculates. We tested the above predictions in wild House Sparrows (*Passer domesticus*), a socially monogamous passerine bird that forms social hierarchies, where more dominant males have greater access to fertile females (Anderson 2006). We tested our predictions by maintaining 15 groups of four males and four females for four weeks in outdoor aviaries. In order to test whether patterns of antioxidant allocation to the ejaculate were causally related to a males' social dominance, we then experimentally changed the social rank of males by shuffling them across groups and aviaries to test whether changes in antioxidant allocation would match adjustments of ejaculate quality.

Materials and Methods

The experiment

We trapped a total of 60 male and 60 female House Sparrows using mist-nets in western Switzerland in April 2014. After trapping, we measured body mass and tarsus length prior to transferring birds into 15 mixed outdoor aviaries at the Ethological Station Hasli, University of Bern, Switzerland. In House Sparrows, courtship displays, solicitations by females, and copulation rates vary among males in different social ranks (Møller 1988, 1990, 1992, Riters et al. 2004), and thus any differences in ejaculate quality may result from sperm depletion (Preston et al. 2001) or fresh sperm effects ((Siva-Jothy 2000, Pizzari et al. 2008a), but see (Firman et al. 2015)). Hence, after four

weeks of acclimatization we transferred all females into a separate aviary during four days. On the day when females were removed, we took a blood and a sperm sample from each male, followed by a second sperm sample the day after and a third sperm sample after four days of being sexually rested. This procedure allowed us to ensure that any difference in ejaculate quality is the result of intrinsic male characteristics. Then, males were shuffled across groups and aviaries according to their current status, in a way that optimized the expected number of males moving up and down the hierarchy, and females were re-introduced in their initial aviaries. After an 18-day period allowing males to settle down into a new hierarchy and cover at least one full spermatogenesis cycle (Jones and Lin 1993), females were released into their localities of origin. On the day females were released, blood and sperm samples were taken from males, and further sperm samples were taken on the second and the fourth day after female release. Then, all males were also released in their localities of origin.

To establish the hierarchy of the males in each aviary, we recorded a total of 13 hours of observations before the manipulation and 10 hours after the manipulation in each aviary. We removed the feeders for 1.5 hours, and then recorded all the antagonistic interactions at the feeders for one hour after reintroducing the feeders into the aviaries. Within each aviary, we computed each male's David's score as a proxy for their social rank (Gammell et al. 2003).

Ejaculate quality

We obtained ejaculates by massaging male's cloaca, and we immediately video recorded sperm using a camera mounted on a microscope at 100X magnification. A subsample of the ejaculate was diluted in PBS and stored at -80º C for laboratory analyses. From the videos we estimated standard ejaculate quality traits using a Computer Assisted Sperm Analyser plug-in (Wilson-Leedy and Ingermann 2007) for ImageJ (Schneider et al. 2012). We modelled the rate at which initial speed decreases through time (from now on endurance) as well as the rate at which initial proportion of swimming sperm decreases through time (from now on longevity) using mixed linear models with time as a fixed effect and individual identity as a random factor. For further details, refer to the Materials and Methods Appendix.

Oxidative stress and antioxidant defences

We assessed the amount of lipid peroxidation by determining the levels of malondialdehyde (MDA) in plasma, red blood cells (RBCs), and sperm. MDA levels were determined by derivatisation with thiobarbituric acid and further separation by ultra-high pressure liquid chromatography (UHPLC) with fluorescent detection (Agarwal and Chase 2002, Moselhy et al. 2013). We also measured superoxide dismutase (SOD) activity per ml of tissue in sperm and RBCs applying minor modifications to a commercial kit (Cayman Chemical, USA). Finally, we determined the levels of glutathione, an intracellular antioxidant in both its reduced (GSH) and oxidized (GSSG) forms both in sperm and RBCs using UHPLC-MS/MS (Bouligand et al. 2006). More details are available in the Materials and Methods Appendix.

Statistical analyses

For the above-mentioned reasons, the data used for all the analyses corresponds to the last sampling, where males were sexually rested and previously sperm depleted. We used linear mixed models to test our hypotheses. We first predicted that ejaculate quality and antioxidant allocation would vary according to social ranks. In a first set of statistical models we investigated ejaculate quality and OS markers as a function of the initial social rank, including body weight and tarsus length as covariates. The dependent

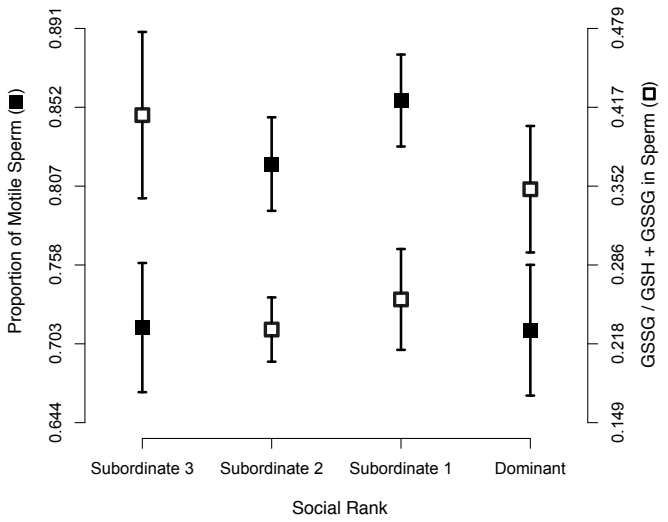

Figure 1. Mean proportion of motile sperm ± SE (■) and proportion of oxidized over total glutathione (GSSG / GSH + GSSG) in sperm ± SE (□) of males with different social status. Ejaculates with higher motility have lower proportions of GSSG, while the greater the proportion of GSSG (e.g. the more oxidatively stressed the sperm cells) the less motile the ejaculate is. The axes for proportion of motile sperm and GSSG / GSH + GSSG in sperm are arcsine scaled.

variables describing sperm quality were the proportion of motile sperm, initial swimming speed, sperm longevity and sperm swimming endurance. The dependent variables describing antioxidant allocation and oxidative stress were the ratio of oxidized glutathione [GSSG/(GSSG+GSH), used as a measure of the oxidative stress endured by the cells in sperm and RBCs], SOD activity in sperm and RBCs, the proportion of SOD activity in the sperm relative to the total SOD activity in sperm and RBCs [SOD_{sperm} /($SOD_{sperm + RBC}$)], used as a measure of relative SOD investment into spermatic vs. somatic functions, MDA levels in the sperm and plasma, and the proportion of MDA in the sperm relative to the total level of MDA in sperm and plasma [MDA_{sperm}/($MDA_{sperm + plasma}$)], used as a measure of relative oxidative stress in the sperm vs. soma.

Second, we predicted that males would adjust sperm quality and antioxidant allocation in accordance to an experimental change in their social status. To test such hypothesis, we ran a set of models that used the same set of dependent variables as mentioned above, but measured after the experimental manipulation of the social ranks. The tested factors were the initial social rank, the final social rank and their interaction, as well as final body mass and tarsus length as covariates.

Proportions were either logit- or arcsin-transformed when necessary, while other dependent variables were log-transformed to match normality. All the models included the aviary and the batch of males as random factors, using the restricted maximum likelihood method for parameter estimation, and Kenward-Roger approximation for the computation of fixed effects degrees of freedom. To avoid inflating the type I error (Whittingham et al. 2006), we did not apply model selection, and therefore always report results for full models. The analyses were performed using SAS v. 9.3.

Results

Ejaculate quality

Before experimentally manipulating the hierarchy

We could not collect any ejaculate from one male having a very small cloacal protuberance, thus we obtained a total of 59 ejaculates. We found that ejaculates

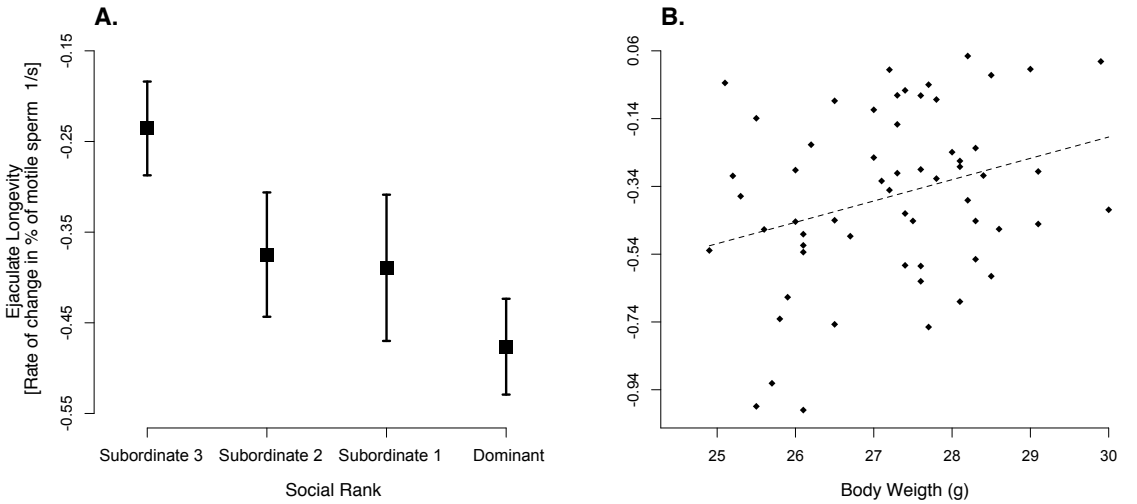

Figure 2. Sperm longevity expressed as the rate of decay in motile sperm (**A**) differs between males at different social status (mean ± SE), (**B**) while heavier males have longer living sperm ($F_{1,49.4}$ = 7.30, p = 0.009, slope = 0.074 ± 0.026). The dotted line represents a linear regression.

produced by males with different social ranks differed in their initial proportion of motile sperm (Fig. 1; $F_{3,40.5}$ = 4.44, p = 0.009), with subordinate males 1 and 2 producing ejaculates with a greater proportion of motile sperm than dominant and subordinate 3 males (pairwise difference between groups: D vs. S1: $t_{40.4}$ = 2.99, p = 0.005; D vs. S2: $t_{41.8}$ = 2.32, p = 0.025; D vs. S3: $t_{40.6}$ = 0.28, p = 0.78; S1 vs. S2: $t_{40.6}$ = 0.58, p = 0.57; S1 vs. S3: $t_{40.2}$ = 2.81, p = 0.008; S2 vs. S3: $t_{39.7}$ = 2.23, p = 0.032). Additionally, we found that sperm longevity also covaried with social rank (Fig. 2A; $F_{3,41}$ = 2.98, p = 0.042), while heavier males produced longer-living sperm (Fig. 2B; $F_{1,49.4}$ = 7.30, p = 0.009, slope = 0.074 ± 0.026). More specifically, we found that subordinate 3 males had longer living sperm compared to that of dominant males (pairwise difference between groups: D vs. S1: $t_{22.7}$ = 0.90, p = 0.38; D vs. S2: $t_{26.3}$ = 1.17, p = 0.25; D vs. S3: $t_{28.0}$ = 3.26, p = 0.003; S1 vs. S2: $t_{26.0}$ = 0.14, p = 0.89; S1 vs. S3: $t_{22.4}$ = 1.60, p = 0.12; S2 vs. S3: $t_{26.0}$ = 1.62, p = 0.12). Finally, we did not find any differences in initial sperm swimming speed (all factors and covariates $F \leq 2.91$, $p \geq 0.094$) and endurance (all factors and covariates $F \leq 0.85$, $p \geq 0.36$) across males with different social ranks.

After experimentally manipulating the hierarchy

After having experimentally changed the social rank of males, we could only obtain ejaculates from 59 males. We found that the proportion of motile sperm differed among males in their new social ranks, and this depended on their initial social rank (Fig. 3A-D; $F_{9,41}$ = 2.59, p = 0.018). More specifically, we found the same bell-shaped patterns for dominant and subordinate-2 males (Fig. 3A, and C), while subordinate-1 males increased or decreased the proportion of motile sperm fully matching Tazzyman's et al. (Tazzyman et al. 2009) predictions (Fig. 3B). Finally, males at the bottom of the hierarchy seemed to consistently increase ejaculate quality as they gained social ranks (Fig. 3D). Initial swimming speed, sperm longevity and sperm swimming endurance were neither affected by the final rank nor the interaction between the initial and final rank (swimming speed: all factors and covariates $F \leq 2.23$, $p \geq 0.10$; sperm longevity all factors and covariates $F \leq 3.70$, $p \geq 0.063$; sperm swimming endurance: all factors and covariates $F \leq 1.50$, $p \geq 0.23$).

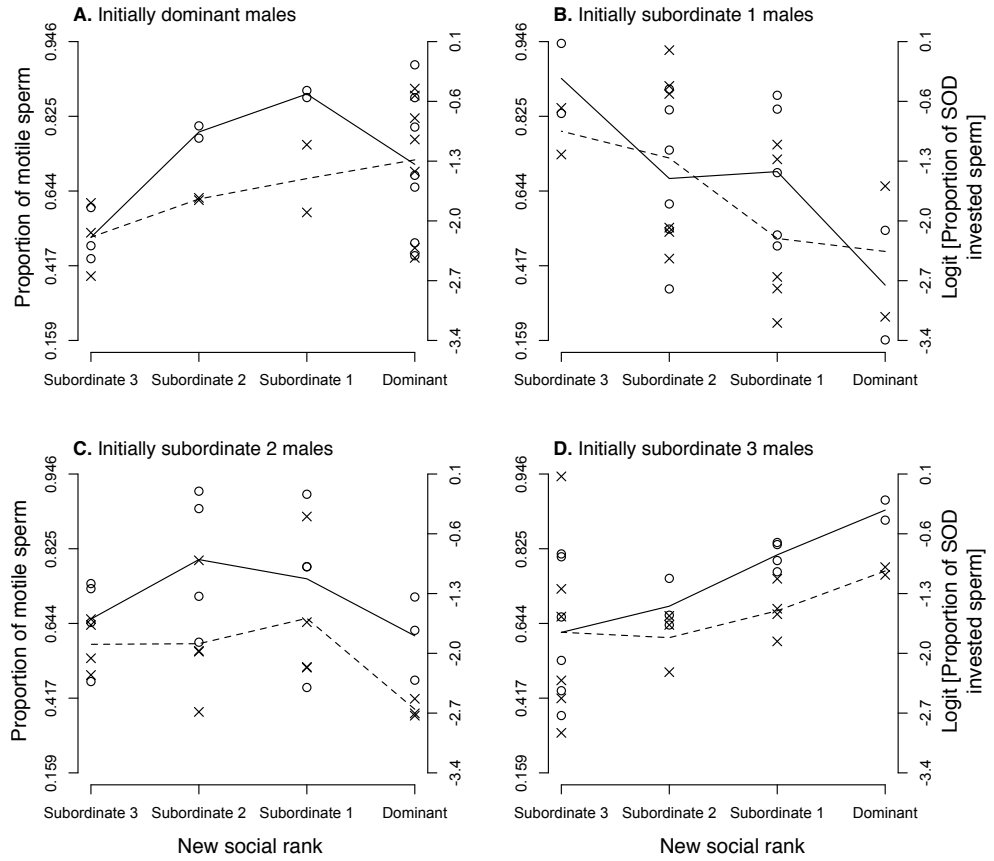

Figure 3. Proportion of motile sperm (—o—) and proportion of SOD activity in sperm relative to the total $SOD_{sperm + RBCs}$ (- -×- -) after shuffling males across aviaries. This relative sperm SOD activity, used as a proxy for the relative investment of antioxidant resources in soma vs. germ-line functions. Initial male social status: (**A**) dominant, (**B**) subordinate-1, (**C**) subordinate-2, and (**D**) subordinate-3 males. The lines connect the mean average of each group, and are only illustrative. The axis for the proportion of motile sperm is arcsine scaled.

Oxidative stress and antioxidant allocation

Before experimentally manipulating the hierarchy

We found that the ratio of oxidized over total concentration of glutathione in the ejaculate varied according to social rank (Fig. 1; $F_{3,48} = 3.85$, $p = 0.015$), with dominant and subordinate 3 males having more oxidatively stressed sperm cells (pairwise difference between groups: D vs. S1: $t_{25.4} = 1.36$, $p = 0.18$; D vs. S2: $t_{20.6} = 1.98$, $p = 0.061$; D vs. S3: $t_{23.2} = -0.71$, $p = 0.48$; S1 vs. S2: $t_{20.4} = 0.50$, $p = 0.62$; S1 vs. S3: $t_{19.8} = -1.89$, $p = 0.073$; S2 vs. S3: $t_{15.5} = -2.40$, $p = 0.029$). Moreover, we found that the higher the ratio of oxidized over total concentration of glutathione in the sperm (the more oxidized the sperm cells) the lower the proportion of motile sperm in the ejaculate (Fig. 4A; $F_{1,42.1} = 5.08$, $p = 0.03$, slope = -0.35 ± 0.16). The ratio between oxidized over total glutathione in the RBCs did not differ across social ranks ($F_{3,54} = 0.74$, $p = 0.53$).

Absolute concentrations of SOD activity and GSH in either sperm or RBC did not differ across social ranks (all $F \leq 3.25$, $p \geq 0.078$). Nor did the proportion of SOD in sperm relative to the $SOD_{sperm + RBCs}$ or the proportion of reduced glutathione GSH in sperm relative to $GSH_{sperm + RBCs}$ (all $F \leq 1.60$, $p \geq 0.21$). The levels of damage to lipids did not differ across males with different social ranks in any of the sampled tissues (all factors and covariates: $F \leq 0.54$, $p \geq 0.46$). The proportion of MDA in sperm relative to plasma or RBC did also not differ across social ranks (all factors and covariates $F \leq 1.23$, $p \geq 0.27$), which indicates that variation in sperm quality is achieved through differential

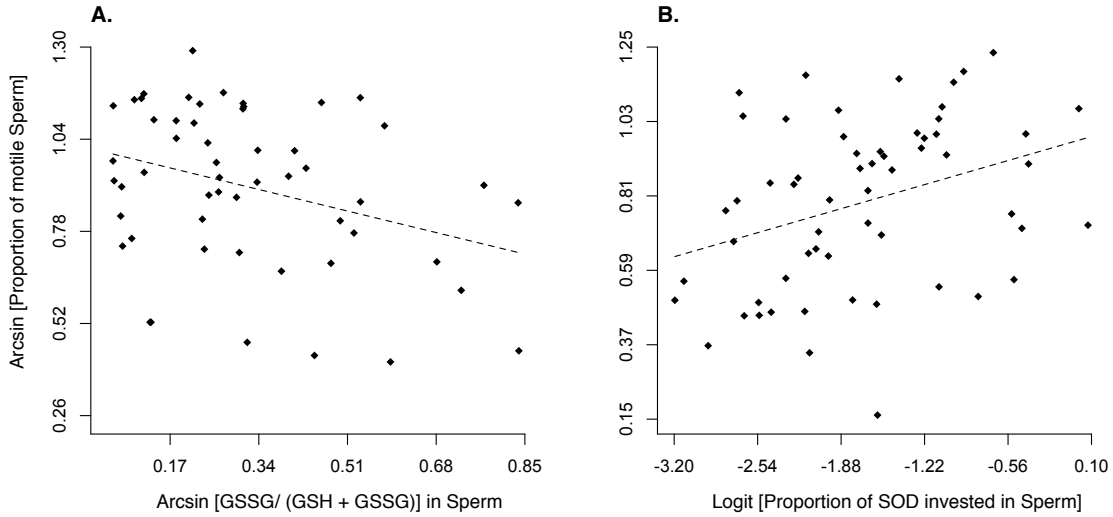

Figure 4. Relationship between the proportion of motile sperm with (**A**) the ratio of oxidized (GSSG) to reduced (GSH) glutathione ($F_{1,42.1} = 5.08$, $p = 0.03$, slope = -0.35 ± 0.16) before manipulating the social status and (**B**) the proportion of SOD invested in the ejaculate ($F_{1,56.9} = 7.78$, $p = 0.007$; slope = 0.43 ± 0.15) after manipulating the social status. The dotted line represents a linear regression.

allocation of antioxidant resources, but at constant levels of oxidative damage both in the soma and the germ-line. Samples sizes and degrees of freedom vary across measures due to technical problems with the different assays.

After experimentally manipulating the hierarchy

Our manipulation of the social status did not affect the levels of SOD activity in the soma ($F_{9,35.1} = 0.99$, $p = 0.47$). However, we found that both the levels of SOD activity in sperm ($F_{9,39.3} = 2.29$, $p = 0.036$) and the proportion of SOD invested in sperm relative to the amount of SOD activity in the soma (Fig. 3A-D; $F_{9,39.4} = 2.40$, $p = 0.028$) depended on the final social rank in function of the initial rank. Moreover, both sperm SOD activity ($F_{1,56.9} = 11.34$, $p = 0.0014$; slope = 0.55 ± 0.16) and the proportion of SOD invested in sperm (Fig. 4B; $F_{1,56.9} = 7.78$, $p = 0.007$; slope = 0.43 ± 0.15) positively correlated with the proportion of motile sperm. No other marker of oxidative stress or antioxidant allocation (absolute or relative levels of MDA or GSH in sperm or blood) responded to our manipulation (all $F \leq 3.62$, $p \geq 0.06$).

Discussion

In the present study, we found a negative correlation between social dominance and the proportion of motile sperm (Fig. 1) and sperm longevity (Fig. 2A). These results match the prediction of previous theoretical models (Parker 1998, Tazzyman et al. 2009, Parker and Pizzari 2010), though not completely. More specifically, subordinate-1 and -2 males produced the highest quality sperm in terms of proportion of motile sperm compared to dominant males (Fig. 1). However and interestingly, subordinate-3 males produced ejaculates with a low proportion of motile sperm, similar to ejaculates of dominant males. While this latter result contradicts the predictions we derived from Tazzyman *et al.*'s (2009) model, it strongly suggests that subordinate-3 males might be constrained by their reduced access to resources. Alternatively, theory predicts that the pay-offs of investing into ejaculate quality decrease when males face a large number of competitors (Parker et al. 1996, Parker et al. 2013), thus males at the bottom of the hierarchy would benefit in lowering their investment into ejaculate quality. However, those models explore how males should strategically expend their sperm according to the number of competitors during a given copulation event, rather than how much

resources they should invest in the production of high quality ejaculates. Moreover, in our study males were not given the chance to adjust their ejaculate expenditure because the experimenter stimulated the ejaculation. Nevertheless, whether our results would be reproduced if males were given the opportunity to copulate with a female under varying levels of risk vs. intensity of sperm competition remains to be tested.

In several species, females store sperm before ovulation (Birkhead and Møller 1993), and sperm have to survive longer to be able to fertilize the ova. For example, Pizzari et al. (Pizzari et al. 2008b) experimentally showed in domestic fowls that spermatozoa stored in the female tract become progressively less numerous with time. Further, when females were inseminated with sperm from two different males, the sperm inseminated in larger numbers had a greater chance to fertilize the eggs than higher-quality sperm when ovulation occurs close to insemination (Pizzari et al. 2008b). Yet, once stored in the female storage tubules, higher quality sperm prevails in the long run, and it is able to fertilize more eggs than low-quality sperm (Pizzari et al. 2008b). Thus, longer-living sperm might increase the likelihood that sperm of subordinate-3 males would be present around the oocyte when the female ovulates, and longer-living sperm could confer a large fertilizing advantage to subordinate-3 males.

We predicted that subordinate males should be better at defending their ejaculate from OS, and thus should produce higher quality ejaculates. In agreement with our prediction, we found that the ratio of oxidized over total amount of glutathione, i.e. an indication of the risk of oxidative stress experienced by the cells, was negatively related to the proportion of motile sperm (Fig. 4A). More importantly, we found that this ratio varied across social ranks in a way that matched the variation in sperm quality (Fig.1). Further, ejaculates from subordinate-3 males were as oxidized as those produced by dominant males (Fig. 1), which again supports the hypothesis that subordinate-3 males might be constrained by resource access.

After changing the social environment for all males, we found that males adjusted both their sperm quality and antioxidant allocation to match their new social status (Fig. 3). Indeed, we found that males that were initially dominant, subordinate-1 or subordinate-2 adjusted the proportion of motile sperm according to our predictions (Fig. 3 A-C). Plasticity in sperm traits given rapid changes in social environments has been reported in previous studies (e.g. Rudolfsen et al. 2006, Cornwallis and Birkhead 2007, Immler et al. 2010), though the physiological proximate mechanism underlying such plasticity remained to be identified. Here, we found that the proportion of motile sperm positively correlated not only with the absolute amount of the endogenous enzymatic antioxidant superoxide dismutase (SOD) activity in the ejaculate, but also with the proportion of SOD activity in their sperm relative to the SOD activity in their blood (Fig. 4B). Such results support our hypothesis that antioxidants are the resource being strategically allocated in the soma vs. germ-line trade-off. Although we did not measure any somatic cost other than oxidative stress, in house sparrows dominant males have been previously shown to have higher mate guarding rates (Møller 1990, Anderson 2006), higher copulation rates (Møller 1990, Anderson 2006), and larger badges (Nakagawa et al. 2007), thus showing a greater investment into pre-copulatory/somatic functions. Finally, subordinate-3 males produced ejaculates with a higher proportion of motile sperm and invested a greater proportion of SOD activity in their ejaculates as they moved up in the hierarchy (Fig. 3D), again supporting the idea that access to resources constrains subordinate-3 males in the development of their reproductive tactic. As they acquire a higher social status, such constraints may be lifted, and they increase their investment into ejaculate quality.

There are a variety of factors causing oxidative stress, e.g. immune response, metabolic processes, pollutants (Finkel and Holbrook 2000). The glucocorticoid stress response has been argued to cause oxidative stress insults (Haussmann and Marchetto 2010, Costantini et al. 2011). Social status has been shown to be linked to stress, and higher glucocorticoid levels are expressed when either dominant or subordinate individuals exhibit larger allostatic loads (Goymann and Wingfield 2004). However, in House Sparrows there is no clear link between dominance and glucocorticoid levels. For example, Gonzalez et al. (Gonzalez et al. 2002) found no relation between social status and glucocorticoid levels, while Buchanan et al. (Buchanan et al. 2010) found subordinate males to have higher glucocorticoid levels during the breeding season. In another study, Lindström et al. (Lindström et al. 2005) found that bigger males incurred higher stress costs when subordinates, while smaller males incurred higher stress costs when dominant. Hence, further studies would be needed to understand how the glucocorticoid stress response and oxidative stress interact in modulating male reproductive strategies.

Oxidative stress is an unavoidable physiological cost (Hulbert et al. 2007), and may thus be a major cost of reproduction (Wang et al. 2001, Alonso-Alvarez et al. 2004) as well as a universal constraint to life history evolution (Monaghan et al. 2009, Metcalfe and Alonso-Alvarez 2010). To our knowledge, we show for the first time in a socially monogamous bird species that males develop their reproductive tactics along a continuum of dominance, and remarkably that oxidative stress is one likely proximate physiological mechanism. Oxidative stress is known to have deleterious effects in sperm of various taxa (e.g. Chitra et al. 2003, Aitken and Baker 2004, Helfenstein et al. 2010a), and thus we predict strategic antioxidant allocation to be a general physiological mechanism to enhance ejaculate quality under sperm competition. Finally, we suggest that future theoretical models should include a minimal cost of bodily functions maintenance that guarantee male condition and survival, which could potentially limit resource allocation to germline functioning as evidenced here with males at the lower end of the hierarchy.

Ethics

This study was conducted under the licenses n° BE41/12 and WTH/g-525/14 for animal experimentation by the Veterinary Office of the Canton Bern, Switzerland.

Acknowledgments

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Authors' contributions

F.H. & A.R.M. designed the study. A.R.M., M.M. & S.C. conducted the experiment, and collected the data. A.R.M. Performed all the laboratory analyses and analysed the sperm videos A.R.M & F.H. performed statistical analyses and wrote the manuscript. G.G& A.V. provided analytical methods to quantify MDA and GSH/GSSG. O.G. contributed reagents, materials, and analysis tools. All authors read and approved the manuscript.

Chapter 2

Relation between sperm morphological design and function

In preparation for Journal of Experimental Biology

Sperm design and function across social hierarchies in common passerine bird

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Abstract

Sexual selection continues after copulation by either sperm competition or female cryptic choice. Hence, selection acts upon sperm traits that maximize the fertilizing ability of an ejaculate. Both sperm swimming velocity and longevity are important determinants of the outcome in sperm competition. Sperm design can influence sperm velocity at least in three different non-exclusive ways: (i) longer sperm to generate more propelling thrust, (ii) bigger midpieces to produce more energy, and/or (iii) larger flagella or midpieces that compensate the drag forces around the head. Theory predicts larger expenditure in ejaculate quality as males face higher sperm competition risks, a situation usually faced by males occupying low ranks in social hierarchies. Here, we investigated how sperm design varied across dominance groups in wild House Sparrows, and we tested the causality of the observed patterns by experimentally changing the social environment. Additionally, we explored whether sperm design functionally correlates to sperm performance. We did not find any differences in sperm design across social ranks. Interestingly, we found that sperm design correlated to ejaculate longevity, and such relation varied across social ranks. After manipulating the social rank, there was a tendency between sperm design and ejaculate longevity as initially observed. Our results suggest that sperm performance might be achieved by strategic allocation of resources, e.g. ATP or antioxidants, that could exploit the functional design of spermatozoa.

Keywords: Sperm competition, social dominance, sperm design, sperm function, resource allocation

Introduction

When females copulate with more than one male, strong selection acts upon traits that maximize the fertilizing ability of sperm (Parker 1998, Birkhead and Pizzari 2002, Parker and Pizzari 2010). Postcopulatory sexual selection can be the result of either female cryptic choice or competition between sperm of different males, known as sperm competition, and it is known to affect sperm morphology (Birkhead and Pizzari 2002, Snook 2005, Fitzpatrick and Lüpold 2014). For instance, in many species sperm is stored in the female reproductive tract, and it has been shown that sperm size coevolves with the female reproductive tract in various taxa (Dybas and Dybas 1981, Presgraves et al. 1999, Miller and Pitnick 2002, Higginson et al. 2012). Moreover, across taxa it has also been observed that sperm morphology correlates to the intensity of sperm competition (Gomendio and Roldan 1991, Gage 1994, Morrow and Gage 2000, Balshine et al. 2001, Anderson and Dixson 2002, Anderson et al. 2005, Immler and

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Birkhead 2007, Tourmente et al. 2011). However, it less understood how differences in sperm morphological traits can translate to better fertilizing ability.

Sperm velocity has been found to be as a major determinant of sperm competitiveness in invertebrates (Kupriyanova and Havenhand 2002), fish (Gage et al. 2004, Gasparini et al. 2010), mammals (Holt et al. 1997, Malo et al. 2005), and birds (Birkhead et al. 1999, Froman et al. 1999). Among the various non-exclusive ways in which sperm design can increase sperm velocity, it has been proposed that (1) longer sperm can benefit from a higher flagellar thrust (Gomendio and Roldan 1991), (2) longer midpiece can produce larger amounts of energy to fuel the flagellar thrust (Cardullo and Baltz 1991), (3) and/or longer flagella or midpiece relative to the head size that compensate with either more energy (i.e. midpiece) or propelling power (i.e. flagellum) the drag forces around the head (Humphries et al. 2008). Evidence that longer sperm has higher swimming velocities have been found in various taxa (Gomendio and Roldan 1991, Lüpold et al. 2009a, Mossman et al. 2009, Fitzpatrick et al. 2010). Similarly, it has been found that midpiece length positively correlates to swimming speed (Cardullo and Baltz 1991, Firman and Simmons 2010). Sperm morphology has been also argued to affect sperm longevity, though the relationship between these two traits is still poorly understood. For instance, as sperm elongates it requires more energy, and thus longer midpiece could prevent the energy reserves to be exhausted. For example, it was found that sperm cells with longer midpiece relative to the head size live longer (Helfenstein et al. 2010b).

When access to fertile females is biased towards males of a given phenotype or engaged in different social roles (e.g. dominant vs. subordinate, territorial vs. sneaker), theory predicts that as males have lower access to females they should increase expenditure into ejaculate traits (Tazzyman et al. 2009, Parker and Pizzari 2010, Parker et al. 2013). For instance, evidence from the predictions of these models has been found in mammals (Preston et al. 2001, Lemaître et al. 2012), fish (Rudolfsen et al. 2006, Evans 2010), insects (Thomas and Simmons 2009), and birds (Cornwallis and Birkhead 2007). Thus, if sperm morphology or design functionally correlates to sperm performance, we predict that male dominance could be associated with differences in sperm morphology. However, predictions on the direction of the differences in sperm morphology across a dominance hierarchy are difficult to state, given that the functional relation between sperm morphology and function is yet unclear.

House sparrows (*Passer domesticus*) live in hierarchical groups where dominance determines reproductive behaviors (Anderson 2006). Further, two previous studies on House Sparrows have found contrasting correlations between sperm design and function (Helfenstein et al. 2010b, Cramer et al. 2015), yet neither of them controlled their results by social hierarchies. For instance, it has been found that male House Sparrows occupying different social ranks differentially invest resources into ejaculate quality (Chapter 1), and thus such differences in ejaculate quality across social ranks might explain the discrepancies between the two studies that explored sperm morphology and function in this species. In the current study, we tested whether males occupying different hierarchical ranks differed in sperm morphology and design. Further, we tested whether male morphology and design functionally correlates to sperm performance, and whether such correlations depend on the hierarchical role that males play. Finally, we tested the causality of the observed patterns by experimentally manipulating the social hierarchies.

Methods

Individuals and sampling

We trapped 60 male and 60 female House Sparrows in western Switzerland between the 8th and 17th of April 2014. Before transferring birds in 15 mixed outdoor aviaries at the Hasli Ethological Station (University of Bern, Switzerland), we measured their body mass and tarsus length. Additionally, birds were scored for their badge size and assigned to their aviaries based both on their badge size score and their body mass, thus maintaining similar group compositions across aviaries. After four weeks of acclimatization, females were transferred into a separate aviary, and we took a sperm sample from each male. We then collected a second sperm sample the day after, and a third sperm sample after 48 hours from the last sample. This procedure ensured that any differences in sperm characteristics would be intrinsic differences in quality rather than differences due to depletion (Preston et al. 2001, Wesseling et al. Accepted) or fresh sperm effects (Siva-Jothy 2000, Pizzari et al. 2008a) (; but see Firman et al. 2015). To maintain sampling time within reasonable limits per day, males were divided in three sampling in groups of 5 aviaries, and each group was processed with 5 days of difference.

Females were then reintroduced to the aviaries and males were shuffled between aviaries according their initial social rank, while maximizing the potential number of positions that males could gain or lose in the social structure. Males were given three weeks to settle their new social ranks, while being time enough for all males to have at least one spermatogenetic cycle (Jones and Lin 1993). Finally, a sperm sampling session was done similarly as above.

Sperm morphology and function

Samples were obtained by gently massaging males cloaca (Wolfson 1952), and ejaculates were collected in 5µl glass capillaries. Right after collection, 0.25 µL of ejaculate were diluted in 40 µL of preheated Dulbecco Modified Eagle Medium at 40º C, and then a 20µm chamber (Leja Products B.V., The Netherlands) was loaded with the diluted ejaculate. Immediately after loading the chamber, a video was done using a Toshiba CMOS HD camera (TOSHIBA Corporation, Japan) camera mounted on a light microscope at 100x and phase contrast 3 annular ring, while keeping a constant temperature of 40ºC with a heating plate mounted on the microscope (Minitube HT200 W, MINITÜB GmbH, Germany). A small droplet from the ejaculate was smeared with 10% formalin on a glass slide for morphology assessments. From each sample, we took photos of ten sperm cells, with coiled midpiece and unbroken heads, using the Nikon ACT-1 v2.70 software (Nikon Corporation, Japan) for a Nikon Digital Eclipse DXM1200 camera (Nikon Corporation, Japan) mounted on a Leica DM R microscope (Leica Microsystems GmbH, Germany) at 400x magnification and phase contrast 2.

From the videos, we estimated the curvilinear velocity and the proportion of motile sperm using a computer automated sperm analyzer plug-in (Wilson-Leedy and Ingermann 2007) for ImageJ (Schneider et al. 2012). Sperm cells having a VSL<5 µm/s, a VCL<15 µm/s, or a VAP<10 µm/s were considered as either moved by drift or immotile. Each video was sampled at 0, 15, 30, 60 and 90 seconds after the beginning of the recording, thus allowing us to estimate sperm longevity and its ability to maintain its initial speed. From each photo, we measured the straight head, midpiece, flagellum, and total length, and S.C. made all the morphological measurements to avoid errors due to observer bias in the measurements. Each photo was independently measure twice blindly from the previous measurements, and the average between the two

measurements was used for further analyses. All samples were processed blind of males identity and social rank.

Statistical analyses

To estimate sperm design, we obtained the ratio between the different morphological traits of each spermatozoon, more specifically we obtain the ratios between head and midpiece, head and flagellum, and midpiece and flagellum. Then, for each male we calculated the average in sperm morphology and design from the 10 spermatozoa, and then we used that data further analyses. To test whether males at different social ranks differed in sperm morphology or design, we made a linear mixed model using sperm morphology or design traits as response variable and social rank as a predictor variable. Additionally, the centered by rank body weight and tarsus length were used as covariates as well as their interactions with social rank, while the aviary and the collection batch were used as random intercepts. Then, we performed linear mixed models to test whether sperm morphology and/or design correlates to sperm function, using the proportion of motile sperm and the curvilinear velocity as response variables. The predictor variables were the social rank, sperm morphology or design trait centered by rank, the time after the video recording started, and the interactions between them, while body weight and tarsus length were used as covariates. The models included the aviary and identity of each individual as random intercepts, and additionally each individual had a random slope given the time elapsed in the video.

After experimentally modifying the social environments, we tested whether males would change sperm morphology to match changes in social status. Thus, we made a linear mixed model that included the sperm morphology or design traits as response variable, and we use the initial social rank, the final social rank, and the interaction between them as predictive variables. Additionally, body weight and tarsus length were used as covariates, while the aviary and the sampling batch were included as random intercepts. Finally, to test whether the sperm morphology and design relations with sperm function were maintained, we constructed a similar model to that one used before manipulating the social status.

Proportion of motile sperm was logit- transformed to match normality. All the models were done using a restricted maximum likelihood method for parameter estimation, and Kenward-Roger approximation of fixed effects degrees of freedom. We did not apply model selection to avoid inflating the type I error probability (Whittingham et al. 2006). All the analyses were performed using SAS v. 9.3.

Results

Sperm morphology and social status

Before manipulating the social status we collected ejaculates from 59 males. We found that neither sperm morphology ($0.96 < F < 1.97$, $p > 0.1$) nor sperm design ($0.21 < F < 2.02$, $p > 0.1$) differed between males occupying different social ranks. However, we found that as males were heavier they had longer sperm ($F_{1,45.4} = 8.33$, $p = 0.006$) and flagella ($F_{1,45.4} = 7.40$, $p = 0.009$), while tarsus length negatively correlated to sperm length ($F_{1,45.4} = 4.24$, $p = 0.045$) and head lengths ($F_{1,45.4} = 4.77$, $p = 0.034$). After manipulating the social status we collected 59 ejaculates from which we found that males occupying different social ranks differed in their midpiece to flagellum ratio ($F_{3,25.8} = 3.12$, $p = 0.043$). Additionally, we found that tarsus length negatively correlated with midpiece lengths ($F_{1,30.5} = 4.05$, $p = 0.053$), while heavier males tended to produced sperm with longer heads ($F_{1,39.0} = 3.91$, $p = 0.055$). We found

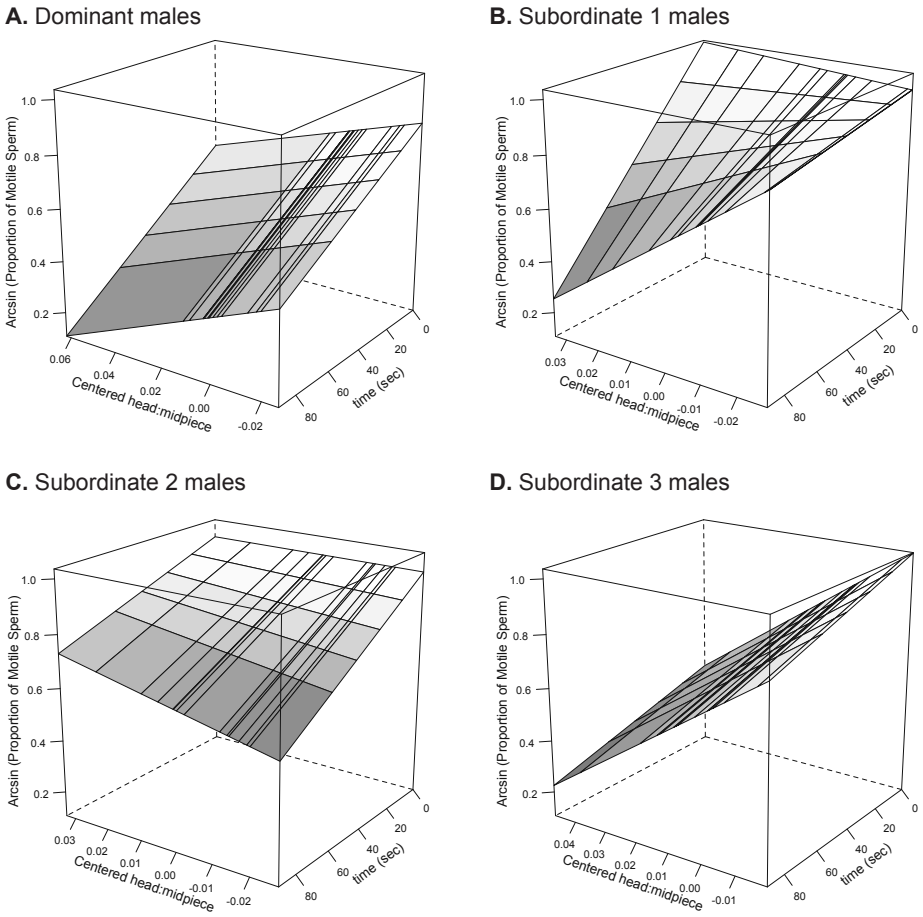

Figure 1. Relation between sperm longevity and the head-to-midpiece ratio across social ranks before manipulating the social environment. The surfaces were contoured based on predicted values obtained from the linear mixed model (see Materials and Methods).

that that neither sperm morphology ($0 < F < 1.85$, $p > 0.1$) nor other measures of sperm design ($0.04 < F < 2.11$, $p > 0.1$) were adjusted to changes in the social environment or differed across the newly acquired social rank.

Sperm design and sperm function

Before manipulating the social status, we found that the relation between sperm design and sperm function depended on the social status of the individuals (Fig. 1). We found that total sperm length correlated to both ejaculate curvilinear velocity (rank x centered total length: $F_{3,47.4} = 5.22$, $p = 0.034$) and proportion of motile sperm (rank x centered total length: $F_{3,44.1} = 3.43$, $p = 0.025$), though the direction and intensity of the correlation depended on the social rank that males occupy. Similarly, social rank determined the intensity and direction of the correlation between midpiece length and ejaculate curvilinear velocity (rank x centered midpiece length: $F_{3,45.5} = 3.37$, $p = 0.026$) or the proportion of motile sperm (rank x centered midpiece length, $F_{3,47.1} = 4.73$, $p = 0.006$). Similar correlations were found for flagellum length (Proportion of motile sperm: rank x centered flagellum length, $F_{3,44.4} = 3.43$, $p = 0.025$) and head length (VCL: rank x centered head length, $F_{3,40.1} = 3.07$, $p = 0.039$). Additionally, we also the relation between the proportion of motile sperm, longevity, and head length depend on the social rank of the individuals (time x rank x centered flagellum length: $F_{3,51.6} = 3.06$, $p = 0.036$).

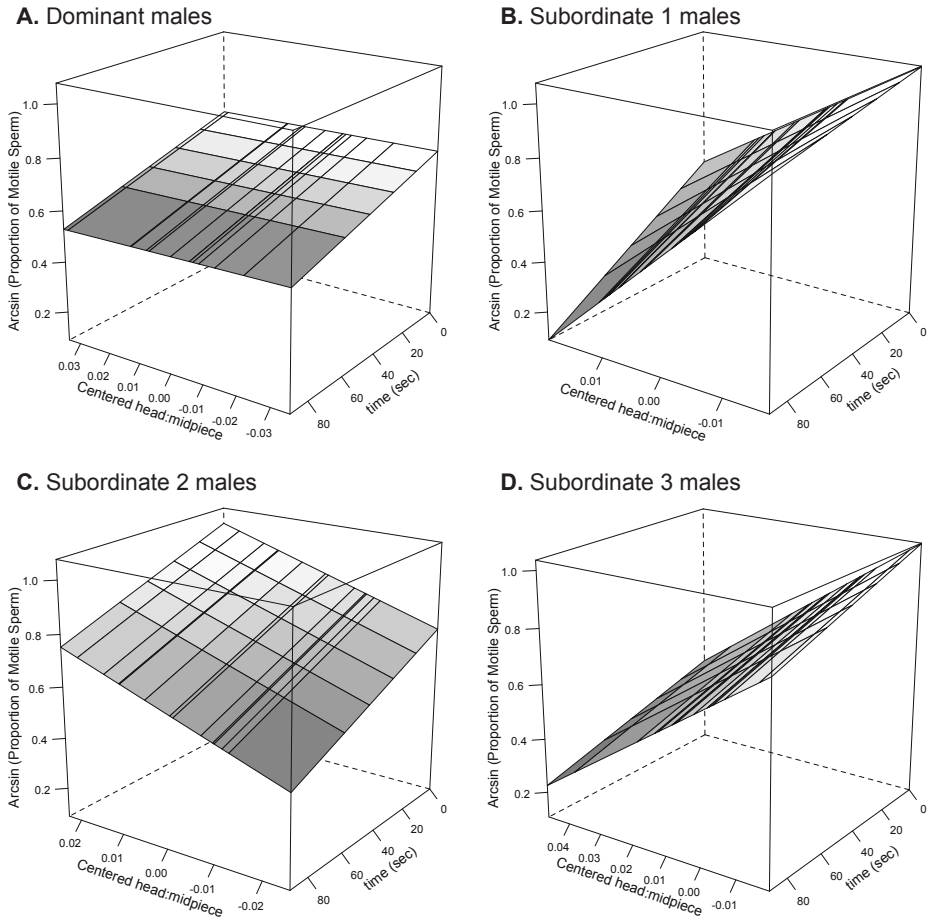

Figure 2. Relation between sperm longevity and the head-to-midpiece ratio across social ranks after manipulating the social environment. The surfaces were contoured based on predicted values obtained from the linear mixed model (see Materials and Methods).

We found that sperm longevity correlated with the head-to-midpiece ratio, and that such correlation varied across social ranks (Fig. 1; time x rank x centered flagellum length: $F_{3,51.8} = 3.62$, $p = 0.019$). We also found similar patterns between the proportion of motile sperm, longevity, and the head to flagellum ratio (time x rank x centered flagellum length, $F_{3,51.7} = 3.59$, $p = 0.020$). Further, we found the body weight positively correlates to the midpiece to flagellum ratio ($F_{1,38.5} = 5.15$, $p = 0.029$). We did not find any correlations between sperm design and sperm velocity ($0 > F > 3.28$, $p > 0.08$ for all fixed effects and covariates) neither any correlation between proportion of motile sperm and the midpiece to flagellum ratio ($0.11 > F > 2.41$, $p > 0.08$ for all covariates excluding proportion of motile sperm). Models showed a significant effect of social rank on the proportion of motile sperm, though that result is discussed elsewhere (Chapter 1). Further, time also affected both sperm velocity and proportion of motile sperm, though it just corresponds to the normal decay in speed and proportion of motile sperm.

After manipulating the social status of the individuals all the previous correlations between sperm morphology or design and sperm function dissolved ($0 > F > 2.53$, $p > 0.1$). However, we found that the correlations across ranks, as well as their directionality, between the proportion of motile sperm, longevity, and the head to

midpiece ratio tended to be maintained (Fig. 2; time x rank x centered midpiece length, $F_{3,50.8} = 2.60$, $p = 0.062$).

Discussion

We tested whether sperm design would vary across a linear dominance hierarchy, and whether the functional correlation between sperm design and sperm performance would depend on the hierarchical position. We did not find any differences in sperm morphology between males at different social ranks, yet we found that the correlation between sperm design and sperm function depended on the social rank that males occupied (Fig. 1). Specifically, we found that in subordinate-1 males ejaculates with sperm having shorter midpiece relative to their head sizes live shorter, while in subordinate-2 males the relation is the opposite. Interestingly, after experimentally manipulating the social environment we did not observed any changes in sperm design that matched the new social ranks, and most of the previously observed correlations between sperm design and function disappeared. However, the correlation between sperm midpiece relative to the head size and ejaculate longevity was weakly maintained (Fig. 2).

Previous studies have found differences in sperm morphology between males facing different risks of sperm competition (LaMunyon and Samuel 1999, Balshine et al. 2001, Immler and Birkhead 2007, Lüpold et al. 2009b). In house sparrows, high between male variation in sperm morphology has been reported (Helfenstein et al. 2010b), yet social dominance does not explain why males differ so much in sperm morphology. These results are striking given that a previous study found that males occupying different ranks differed both in the proportion of motile sperm and longevity, with dominant males producing the lowest quality ejaculates (Chapter 1). Further, plasticity in sperm morphology has been also reported in passerine birds, specifically it was found that the perceived male-male competition given the social environment lead to changes in sperm morphology in Gouldian Finches (Immler et al. 2010). Yet, we did not observe any changes in sperm morphology given the changes in social dominance ranks. It has been reported in birds that sperm size correlates with the length of the sperm storage tubules (SSTs) in the female tract (Briskie and Montgomerie 1992), which is the likely results from female cryptic choice (Birkhead and Pizzari 2002, Andersson and Simmons 2006). Thus, if in House Sparrows sperm morphology is constraint to the SSTs length, selection would favor other means of enhancing ejaculate quality.

In House Sparrows, a previous study found that sperm with bigger heads relative to the flagellum swim at lower speeds, (Helfenstein et al. 2010b), yet a further study found the opposite relation (Cramer et al. 2015). Here, we found that correlations between sperm design and function vary according to the social status of the individual (see results), and thus it could be argued that sampling bias towards more bold vs. shy males might result in the discrepancy between these two studies. Problematically, after experimentally manipulating the social environment, many of the observed correlations between sperm design and function disappeared, while males seem to change their sperm quality to match their new social ranks (Chapter 1) without changes in sperm morphology (this study). Interestingly the only correlation that was maintained after manipulating the social environment was between the head to midpiece ratio and sperm longevity (Fig. 2), likely reflecting a conflict between sperm longevity and energetics. Yet it is difficult to explain why the direction of the correlation between sperm design and function varies across social ranks. The head to midpiece ratio reflects how much energy is need to compensate for the drag caused by the head (Humphries et al. 2008), and mitochondrial energy production can release by-products that harm sperm

membranes (e.g. reactive oxygen species; Aitken and Baker 2004, Hulbert et al. 2007). Thus, the different relationships between sperm design and function might be the result of the differential resource allocation to protect sperm, and for instance a previous study found that the levels of sperm damage due reactive oxygen species varies across social ranks (Chapter 1). Alternatively, the amount of ATP invested in the ejaculate and produced by the mitochondria has been previously shown to affect sperm function (Christen et al. 1987, Lahnsteiner et al. 1996), and thus males at different social ranks might differentially allocate molecules that boost sperm performance (e.g. ATP; Burness et al. 2004, Burness et al. 2005).

Altogether, we suggest that in House Sparrows sperm morphology does not reflect sperm function, but the design between the sperm segments might reflect an energetic problem related to mitochondrial metabolism. Further studies will be required to test whether female cryptic choice might constrain sperm morphology, which could in turn select for other strategies to enhance the fertilizing ability of an ejaculate. Therefore, differences in sperm function between males might be the result of strategic resources allocation into the ejaculates, and thus plasticity in ejaculate quality might rely on changes in resource allocation rather than sperm morphology.

Ethics

This study was conducted under the licences n° BE41/12 and WTH/g-525/14 for animal experimentation and animal detention provided by the Veterinary Office of the Canton Bern, Switzerland.

Acknowledgments

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Authors' contributions

F.H. & A.R.M. designed the study. A.R.M., M.M. & S.C. conducted the experiment, and collected the data. A.R.M. analysed the sperm videos S.C. measured the sperm cells. A.R.M & F.H. performed statistical analyses and wrote the manuscript. All authors read and approved the manuscript.

Chapter 3

Role of social status in patterns of within-ejaculate variation of sperm morphological design

In preparation for Journal of Evolutionary Biology

Social dominance explains within-male variation in sperm design in wild passerine bird

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Abstract

Comparative studies suggest that sperm competition selects for reduced levels of within-male variation in sperm morphology, thus resulting in stabilizing selection towards an optimal sperm design that maximizes male fertility. Yet, these studies also reveal a substantial amount of unexplained within-male variance, and the factors presiding to the maintenance of within-male variation in sperm design at the population level still remain to be identified. Theory predicts that males should progressively invest more resources in their germline as the risk of sperm competition increases, i.e. the soma/germline allocation trade-off hypothesis. When access to fertile females is determined by social dominance, dominance should positively correlate with within-male variance in sperm design. In support of this hypothesis, we found that dominant house sparrow males produce ejaculates with higher levels of variation in sperm design compared to subordinate males. After experimentally manipulating male social status, we observed the same patterns of variation in sperm design. Our results suggest that males are able to control variation in sperm design according to their social status. While energy saving strategies could explain the observed patterns, we discuss how strategic allocation of resources to the somatic vs. germline functions could shape the relationship between within-male variation in sperm design and social status.

Keywords: Sperm competition, social dominance, within-ejaculate variation, sperm design, soma vs. germline

Introduction

Spermatozoa are one of the most morphologically diverse cells across animal taxa (Pitnick et al. 2009), with sizes ranging from 15.5 µm in *Asprotilapia leptura* (Balshine et al. 2001) to 58.29 mm in *Drosophila bifurca* (Pitnick et al. 1995). Such large variation in sperm morphology has presented a conundrum to biologist. Most of the efforts were put into identifying interspecific differences in the mean values of sperm morphology (e.g. Gomendio and Roldan 1991, Briskie and Montgomerie 1992, LaMunyon and Samuel 1999, Breed and Taylor 2000, Morrow and Gage 2000, Balshine et al. 2001, Byrne et al. 2003), though Ward (1998) stressed that there is large intraspecific variation in sperm morphology. Further studies investigated such between-male variation in sperm traits (e.g. Morrow and Gage 2001, Joly et al. 2004, Schulte-Hostedde and Millar 2004, Bernasconi and Hellriegel 2005, Malo et al. 2006, Calhim et al. 2007, Harris et al. 2007, Immler et al. 2008, Kleven et al. 2008), and many of them suggested that post-copulatory selection is an important selective pressure in reducing the levels of between-male variation in sperm morphology. Although within-male

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variation in sperm has also been reported in many studies, what causes and maintains within-male variation in sperm morphology has been seldom explored.

There exist several non-mutually exclusive explanations as to why sperm can vary within ejaculates. A conflict between the diploid (e.g. male phenotype) and the haploid genome (e.g. spermatozoon phenotype) has been predicted to result in morphological variation (Parker 1993, Parker and Begon 1993). However, diploid genes are the main determinants of sperm morphology (Eddy 2002), and the optima for the diploid and haploid phenotype tend to be similar under strong sperm competition (Parker and Begon 1993). Production of large sperm numbers has been correlated with the amount of errors in sperm production (Cohen 1967, 1973), suggesting that inevitable developmental errors can partly maintain within male variation. It has also been suggested that sperm competition could lead to the evolution of different sperm phenotypes that play different roles in the ejaculate (Robin Baker and Bellis 1988, Morrow and Gage 2001), thus resulting in a mixed strategy ejaculate. Alternatively, Birkhead et al. (2005) hypothesized that as the intensity of sperm competition is relaxed, more variation in sperm morphology should be observed. Comparative studies have found support for the latter hypothesis (Calhim et al. 2007, Immler et al. 2008, Kleven et al. 2008, Lüpold et al. 2009b), arguing that sperm competition exerts strong stabilizing selection towards optima in sperm design. While comparative studies stress the role of sperm competition in reducing within male variation across species, intraspecific levels of within-male variation do not seem to follow evident patterns (e.g. within-male variation does not match between-male variation, Hogner et al. 2013). However, while large levels of within-individual variation can be observed, such variation can be filtered by selection in evolutionary time (Uyeda et al. 2011). Thus, different evolutionary processes can operate to shape phenotypic variation across species (e.g. evolutionary time scale) and within species (e.g. short-time scale) (Uyeda et al. 2011).

In species where the access to fertile females differs between males, theory predicts that non-favored males should invest more resources in the production of high quality ejaculates (Parker 1998, Tazzyman et al. 2009, Parker and Pizzari 2010, Parker et al. 2013). In particular, one of these models predicts that a continuous increase in costs to obtain a mate would select for continuously increasing resource investment into the production of high quality ejaculates (Tazzyman et al. 2009). In species were access to fertile females is determined by a linear social hierarchy and male dominance, we propose that the amount of within-male variation in sperm morphology will depend on male social status. If sperm competition exerts stabilizing selection towards an optimum of sperm morphology, we predict that as males are less dominant a reduction in within-male variation in sperm morphology should be observed.

House Sparrows *Passer domesticus* have social hierarchies that determine male reproductive behaviors (Anderson 2006). Moreover, a previous study on House Sparrows reported a large amount of within-male variation that, for some sperm traits, exceeded the between-male variation (Helfenstein et al. 2010b). Further, a more recent study found that, in House Sparrows, dominant males produce ejaculates of lower quality compared to those produced by males in the middle of the hierarchy, while males at the bottom of the hierarchy are apparently unable to invest resources into the production of high quality ejaculates (Chapter 1). To test our hypothesis, we maintained 60 wild male and 60 wild female House Sparrows in outdoor aviaries, and investigated within-male variation in sperm design according to social rank after a 4-week acclimation period. To further test the causality of the observed patterns, we

experimentally manipulated the social status of males in a way that optimized the number of males going up or down the hierarchy.

Methods

Individuals and sampling

We trapped a total of 60 male and 60 female House Sparrows using mist-nets in western Switzerland during the first two weeks of April 2014. From each bird, we measured body mass and tarsus length, and birds were assigned to 15 mixed outdoor aviaries at the Hasli Ethological Station (University of Bern, Switzerland) according to their body weight and an initial score of badge size. Thus, aviaries had on average birds of the same body weight and males with various badge sizes. After four weeks, all the females were transferred into a separate aviary, and we took a sperm sample from each male. We then collected a second sperm sample the day after, and a third sperm sample after 48 hours from the last sample. This procedure ensured that, as copulation rates likely varied across males of different social ranks, any differences in sperm characteristics would be intrinsic differences in quality rather than differences due to depletion (Preston et al. 2001) or fresh sperm effects (Siva-Jothy 2000, Pizzari et al. 2008a). Males were divided in three sampling bouts in groups of 5 aviaries, and each bout was processed 5 days apart.

To test the causality of the observed patterns, females were reintroduced to the aviaries and males were shuffled between aviaries according to their initial social rank. We maximized the number of positions that males could have gained or lost in the hierarchy (details in Materials and Methods Appendix). Males were given three weeks to settle down the new hierarchical positions, and go through at least one spermatogenesis cycle (Jones and Lin 1993). Then, we collected sperm samples following the same procedure as before.

Sperm morphology and function

We gently massaged the males' cloaca to obtain ejaculates that were collected in glass capillaries. A small droplet from the ejaculate was immediately smeared with 10% formalin on a glass slide, while 0.25 µL of ejaculate were diluted in 40 µL of preheated Dulbecco Modified Eagle Medium at 40º C and a video was recorded using a Toshiba CMOS HD camera (Toshiba co., Japan) mounted on a light microscope at 100x magnification. We used an computer automatized sperm analyzer plug-in (Wilson-Leedy and Ingermann 2007) for ImageJ (Schneider et al. 2012) to assess sperm velocity and proportion of swimming sperm at 0, 15, 30, 45, 60, and 90 seconds of recording. Then, we modeled sperm longevity (e.g. rate of decay in motile sperm) and swimming endurance (e.g. rate of decay in swimming speed) by performing a linear mixed model that included the identity of the individuals as a random intercept and slope and time as a fixed effect. From each slide, we took photos of ten intact sperm cells using the Nikon ACT-1 v2.70 software (Nikon Corporation, Japan) with a Nikon Digital Eclipse DXM1200 camera (Nikon Corporation, Japan) mounted on a Leica DM R microscope (Leica Microsystems GmbH, Germany) at 400x magnification and phase contrast 2. From sperm cells, we measured the straight head, midpiece, flagellum, and total length. S.C. did all the measurements blind to the male identity and social status. Additionally, each cell was independently measured twice to assess the measurement error, and the average of these two measurements was used for further analyses.

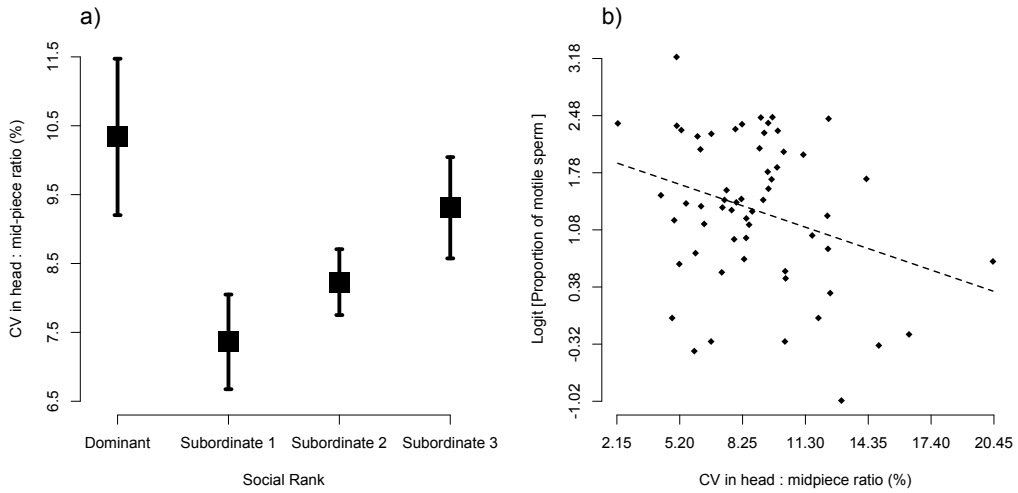

Figure 1. Relationship between the CV (mean + SE) in (**a**) sperm design according to social rank before manipulating the social rank, and (**b**) its relation to the proportion of motile sperm in the ejaculate. The dashed line represents a linear regression.

Statistical analyses

From each male we calculated the coefficient of variation for the different sperm sections ($CV_{segment}$), and then we used linear mixed models to test our hypotheses. In a first set of statistical models we used the $CV_{segment}$ of the different sperm segments as a function of the social rank, while including body mass and tarsus length as covariates. The sperm segments included the head, midpiece, flagellum, and total length. Additionally, the ratios head-to-flagellum, head-to-midpiece, and midpiece-to-flagellum were used as proxies for sperm design (Humphries et al. 2008). We then estimated the CV in sperm design for each male. This allowed us to make a second set of models similar to those using CV in sperm segments (sperm morphology), but with CV in segment ratios (sperm design) as dependent variables.

After we experimentally manipulated the social status of males, we performed similar models on CV in morphology and sperm design. Additionally, we ran a model including both the initial and final social rank, as well as their interaction. This latter model allowed us to account for potential effects of the initial social status on the plasticity in CV. Finally, to test the relationship between within-male variance in sperm morphology and design and sperm function, we modeled sperm velocity, proportion of motile sperm, the rate of decay in speed (swimming endurance), or the rate of decay in the proportion of motile sperm (sperm longevity) as a function of the CV in morphology or sperm design, adding body mass, tarsus length, and their interactions with CV as covariates.

The proportion of motile sperm was logit- transformed to match normality. All models included the aviary and the sampling batch as random factors, and models were estimated using a restricted maximum likelihood method for parameter estimation and a Kenward-Roger approximation of fixed effects degrees of freedom. We did not apply model selection to avoid inflating the type I error (Whittingham et al. 2006). The significance level was set at 0.05. All the analyses were performed using SAS v. 9.3.

Results

Before manipulating the social status

We found that males at different social ranks significantly differed in $CV_{head\ :\ midpiece}$ (Fig. 1a; $F_{3,45.1} = 2.90$, $p = 0.045$). There were tendencies for $CV_{midpiece}$ (Fig. 1a;

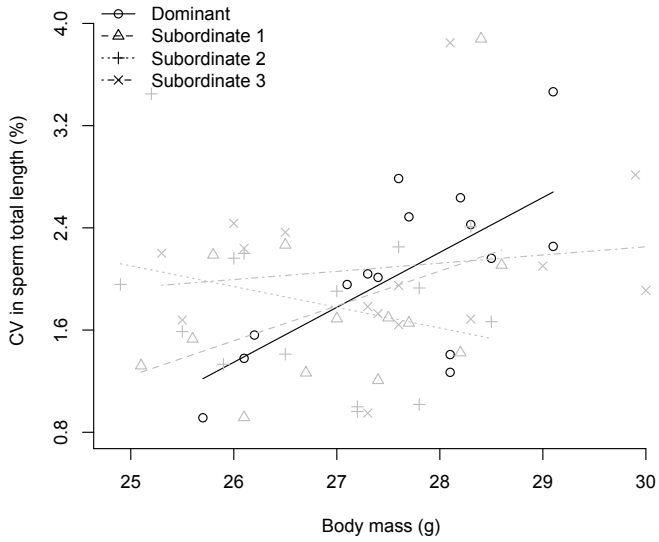

Figure 2. Relationship between body mass and CV in total sperm length for males at different social ranks. The lines represent linear regressions, and black color represents cases where the slope was significantly different from 0

$F_{3,32.5}$ = 2.54, p = 0.074) and $CV_{head\ :\ flagellum}$($F_{3,44.1}$ = 2.90, p = 0.098) ratio to differ across social ranks, with similar U-shaped relationships as found for $CV_{head\ :\ midpiece}$. We also found that CV_{total} varied non-additively according to the social rank and the body weight of the individual (Fig. 2; rank x body weight, $F_{3,43.9}$ = 3.62, p = 0.020). A post-hoc analysis revealed that CV_{total} increased with body mass in dominant males (Fig. 2; body weight slope = 0.004 ± 0.001, $F_{1,10.1}$ = 18.45, p = 0.001), while it was not correlated with body mass in subordinate males 1 to 3 (0.04 < F < 2.61, p > 0.1). We also found that the CVs in head length ($F_{1,44.8}$ = 5.36, p = 0.025; slope = -0.011± 0.004), head-to-flagellum ratio ($F_{1,44.8}$ = 3.44, p = 0.070; slope = -0.011± 0.005), and head-to-midpiece ratio ($F_{1,45.7}$ = 3.30, p = 0.076; slope = -0.011± 0.006) tended to decrease with male body weight. However heavier males tended to have larger $CV_{flagellum}$ ($F_{1,40.7}$ = 3.42, p = 0.072; slope = 0.001± 0.001). Males occupying different social ranks did not differ in the CV of total length, head length, flagellum length, and midpiece-to-flagellum ratio (0.84 < F < 1.77, p > 0.1). Finally, male body mass did not explain CV in sperm total length, midpiece length, and midpiece-to-flagellum ratio (0.09 < F < 2.12, p > 0.1).

After manipulating the social status

Similarly to the results before manipulating the social rank, we found that males at their new social ranks tended to differ in $CV_{midpiece}$ ($F_{3,44.1}$ = 2.63, p = 0.062) and $CV_{head\ :\ midpiece}$ ($F_{3,46.0}$ = 2.44, p = 0.076). Interestingly, we also found a similar U-shaped pattern for $CV_{\ midpiece\ :flagellum}$ according to social status after manipulating the social ranks (Fig. 3a; $F_{3,28.7}$ = 3.22, p = 0.037). Males occupying different social ranks did not differ in CV of total length, head length, flagellum length, and head to flagellum ratio (0.63 < F < 2.20, p > 0.1). Finally, male body mass did not explain CV in sperm total length, midpiece length, flagellum length, head to midpiece ratio, head to flagellum ratio, and midpiece to flagellum ratio (0.27 < F < 1.95, p > 0.1).

Relation between within-male variance in sperm morphology/design and sperm performance

Before manipulating the social status, we found that the proportion of motile sperm was negatively correlated with CV_{total} ($F_{1,50.3}$ = 9.54, p = 0.003; slope = -0.57 ± 0.18),

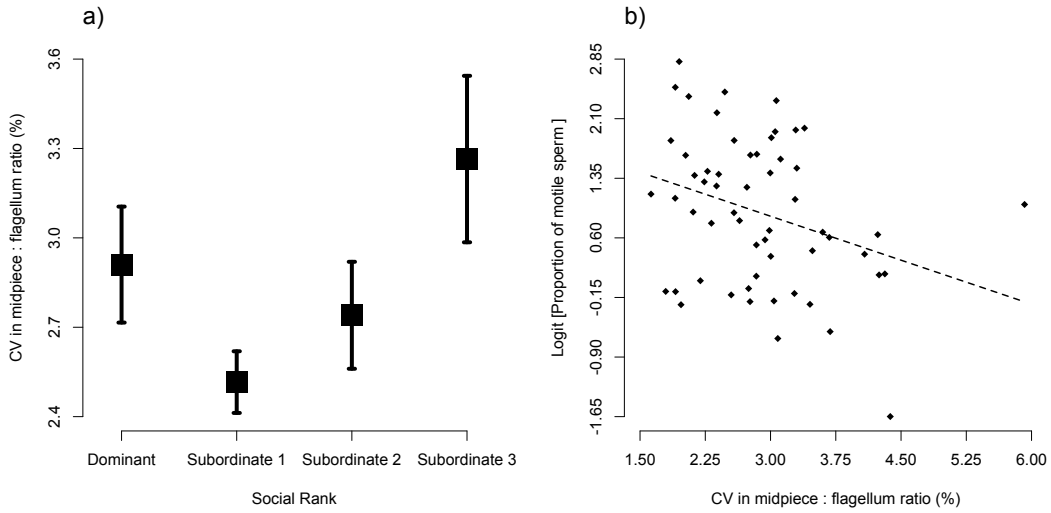

Figure 3. Relationship between the CV (mean + SE) in (**a**) sperm design according to social rank after manipulating the social rank, and (**b**) its relation to the proportion of motile sperm in the ejaculate. The dashed line represents a linear regression. This relation becomes a tendency when the data point with very low motility (see text).

$CV_{flagellum}$ ($F_{1,51.8} = 6.73$, $p = 0.012$; slope = -0.45 ± 0.17), and $CV_{head\ :\ midpiece}$ (Fig. 1b; $F_{1,43.0} = 4.23$, $p = 0.046$; slope = -0.08 ± 0.04). After manipulating the social status we observed that the proportion of motile sperm was negatively correlated with $CV_{midpiece}$ ($F_{1,51.9} = 4.54$, $p = 0.038$; slope = -0.26 ± 0.003) and $CV_{midpiece\ :\ flagellum}$ (Fig. 3b; $F_{1,48.8} = 4.23$, $p = 0.020$; slope = -0.46 ± 0.17). However, after removing one data point with low motility the relationship becomes non-significant ($F_{1,47.7} = 3.32$, $p = 0.07$; slope = -0.30 ± 0.16). No other sperm performance trait did correlate with any other CV in sperm morphology or design ($0.0004 < F < 3.49$, $p > 0.07$). Finally, in most of the models body mass was significantly correlated with longevity. This latter result is discussed in Chapter 1.

Discussion

In the present study we found a U-shaped relationship between social status and within-male variation in sperm morphology and design (Fig. 1 and 3 and results). In addition, we found several negative correlations between sperm motility (percentage of motile sperm) and within-male variation in sperm morphology and design (Fig. 3). Lastly, we found that heavier dominant males produced ejaculates with greater variation in sperm length, while no relationship with body mass was observed for lower ranking males (fig. 2). After manipulating the males' social status we found similar patterns of CV in sperm design in relation to social rank (Fig. 3a).

Birkhead et al. (2005) found high levels of within-male variation in sperm morphology in male zebra finches, and they argued that the low levels of sperm competition in zebra finches may have led to relaxed pressures upon quality control processes in spermatogenesis, and thus the production of ejaculates with substantial variation in sperm morphology. While strong evidence for this hypothesis comes from interspecific comparative studies (Calhim et al. 2007, Immler et al. 2008, Kleven et al. 2008, Lüpold et al. 2009b), the production and maintenance of substantial intraspecific variation in sperm morphology is still not fully understood. Our results are consistent with the hypothesis that dominant males, which are likely to have a privileged access to females, are under relaxed sperm competition pressures, and consequently may loosen the quality control they exert on spermatogenesis, hence producing ejaculates with greater variance in sperm morphology and design.

We also found that, similarly to dominant individuals, fully subordinate males produced ejaculates with larger amounts of variance in sperm morphology and design. Although dominant and fully subordinate males share a common pattern of ejaculates variance in sperm morphology and design we propose that they may be subjected to different constraints or may respond to different selective pressures. Dominant males may produce ejaculates with larger variance in sperm morphology and design as a result of (i) larger sperm production demands to meet higher copulation rates, (ii) energy saving strategies, and/or (iii) strategic allocation of resources between somatic and reproductive tissues. On the other hand, fully subordinate males may rather be under energetic constraints.

In sway rams, it has been shown that dominant males have higher copulation rates throughout the reproductive season, yet they run sperm depleted and lose out in sperm competition at the end of the season (Preston et al. 2001). In house sparrows, dominant males have higher copulation rates (Møller 1988, 1990), and may thus face higher needs for sperm production. However, sperm production is costly (Van Voorhies 1992), and increased sperm production may result in a larger number of errors (Cohen 1967, 1973). A larger number of production errors would be reflected in a lower proportion of motile sperm (Cohen 1973), and we indeed observed a negative correlation between within-male variation in sperm design and proportion of motile sperm (Fig. 1b and 3b; see also results). Thus, increased variation in ejaculate quality may result from a higher sperm demand in dominant, but not subordinate, males.

Alternatively, theory predicts that males should differentially invest in pre- vs. post-copulatory traits depending on the risk of sperm competition they face (Parker 1998, Tazzyman et al. 2009, Parker and Pizzari 2010, Parker et al. 2013). The predictions of these models have been tested in various taxa, and evidence of such a pre- vs. post-copulatory investment trade-off exists (e.g. Preston et al. 2001, Rudolfsen et al. 2006, Thomas and Simmons 2009, Evans 2010). In house sparrows, a recent study showed that dominant males have ejaculates of lower quality compared to ejaculates of subordinate males, while this reflects differences in the resources used to protect ejaculates from oxidative damage (Chapter 1). Thus, the larger levels of within-male variation in sperm design by dominant males might be the result of differential investment in pre- vs. post-copulatory traits. Further, in Chapter 1 it was shown that males at the bottom of the hierarchy had ejaculates of lower quality that are oxidatively stressed, and suggested that males at the bottom may be constrained by resource availability to an extent that they may sacrifice their sperm production and privilege somatic investment to meet requirements of bodily functions and maintenance. The patterns of variation in sperm design that we observe may hence result from strategic allocation of resources between reproductive and somatic tissues (Tazzyman et al. 2009, Parker and Pizzari 2010, Parker et al. 2013) and differential access to resources by males at different social ranks.

Lastly, in house sparrows, body mass affects male dominance (Møller 1987b), and smaller dominant males may face higher levels of male-male competition that may translate into higher risk of sperm competition. Further, smaller males seem to pay higher physiological costs when they are dominant (Lindström et al. 2005). Interestingly, we found that as dominant males were lighter they produced ejaculates with a lower amount of variance in sperm morphology. This supports the idea that among dominant males the less competitive males may increase their investment in ejaculate quality to reduce variation in sperm morphology (Fig. 2), and hence increase the competitive ability of their ejaculates. However, whether slight differences in male

competitiveness among dominant males result in differences in ejaculate quality remains to be experimentally tested.

Different evolutionary processes may explain levels of morphological variation between taxa and within taxa (Uyeda et al. 2011), and sperm competition seems to cause stabilizing selection that leads to lower variation in sperm design across taxa (reviewed in the introduction). At the intraspecific level we suggest that in species where males face different risks of sperm competition, they may adjust their investment in gamete production in a way that results in different levels of morphological variation in their ejaculates. Alternatively, males may invest equal amounts of resources to either producing small volumes of high quality ejaculates or large volumes of lower quality ejaculates. These two alternative hypotheses remain to be tested to better understand how large levels of intraspecific variation in sperm morphology and design are maintained.

Ethics

This study was conducted under licenses n° BE41/12 and WTH/g-525/14 for animal experimentation by the Veterinary Office of the Canton Bern, Switzerland.

Acknowledgements

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Authors' contributions

F.H. & A.R.M. designed the study. A.R.M., M.M. & S.C. conducted the experiment, and collected the data. A.R.M. analysed the sperm videos. S.C. measured all the sperm cells. A.R.M & F.H. performed statistical analyses and wrote the manuscript. All authors read and approved the manuscript.

Chapter 4

Test for the oxidation-based phenotype-linked fertility hypothesis

In review in Frontiers in Ecology and Evolution

Badge size reflects sperm oxidative status in the House Sparrow *Passer domesticus*

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Abstract

The phenotype-linked fertility hypothesis proposes that male ornaments reflect male fertility. Male ornaments could honestly signal sperm quality due to the high susceptibility of sperm to free radicals on the one hand and the negative impact of oxidative stress on ornament elaboration on the other hand. Thus, only males with superior antioxidant defences could bear the cost of more elaborated sexual ornaments without suffering adverse fitness costs. Yet, in species where males experience differential access to fertile females, a trade-off emerges between investing into traits favouring mating opportunities (e.g. secondary sexual ornaments, social dominance, mate-guarding behaviours, etc.) or into traits favouring sperm competitive ability (e.g. sperm numbers and quality). When male sexual ornaments promote greater access to fertile females, a negative relationship can then be predicted between ornamentation and sperm quality. We tested the latter hypothesis and the phenotype-linked fertility hypothesis in wild House Sparrows *Passer domesticus* by exploring the relationships between sperm quality, melanin-based ornamentation, and redox status in blood and sperm. We found no correlation between badge size and sperm swimming performance. However, we found that within a social group, large-badged males better protect their ejaculates from oxidative stress, and thus produce less oxidized ejaculates. Additionally, we found that badge size did not reflect social dominance, and thus the protection of the ejaculate is independent of males' ability to monopolize resources. Our results suggest that badge size might reflect male investment into the antioxidant protection of their sperm relative to a given social environment, and thus females may accrue both direct and indirect benefits by mating with large-badged males producing less oxidized ejaculates.

Keywords: Oxidative stress, phenotype-linked fertility, sperm competition, soma vs. germline

Introduction

Oxidative stress is the imbalance between pro- and anti-oxidants in favour of the former (Jones, 2006). It is pervasive in an animal's life because virtually all activities generate pro-oxidant reactive oxygen species (ROS) and antioxidant defences are not infallible (Benzie, 2000; Jones, 2006; Halliwell and Gutteridge, 2007). An excess of ROS has dramatic physiological consequences given that ROS causes oxidative damage to all biological molecules (proteins, lipids, carbohydrates and DNA) (Halliwell and Gutteridge, 2007), and thus oxidative stress has been identified as a cost of reproduction (Wang et al., 2001; Alonso-Alvarez et al., 2004) and immunity (inflammation; Sorci and Faivre, 2009), as well as a proximate mechanism of senescence and ageing (Harman, 1956; Salmon et al., 2010). Hence, individual ability to circumvent oxidative

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stress may underlie trade-offs between life-history traits (Costantini, 2008; Monaghan et al., 2009; Metcalfe and Alonso-Alvarez, 2010).

Vertebrate spermatozoa are very susceptible to oxidative stress (Tremellen, 2008), and oxidative damage to sperm is known to reduce sperm quality (e.g. percentage of motile sperm, sperm swimming ability) (Blount et al., 2001; Surai et al., 2001; Aitken and Baker, 2004; Helfenstein et al., 2010). Oxidative damage to sperm can have a series of negative effects on male and potentially female fitness, from various degrees of male infertility due to reduced sperm mobility or impaired sperm-egg interaction, to lower offspring quality due to oxidation-induced heritable deleterious mutations to sperm DNA (Velando et al., 2008). Additionally, sperm quality is a key parameter in sperm competition, the circumstance when ejaculates of different males compete to fertilise a common set of ova (Parker, 1970; Birkhead et al., 1999; Gage et al., 2004; Denk et al., 2005). Therefore, oxidative stress may play a pivotal role in species where males face such competition. Thus, oxidation-induced reduction in sperm quality has important ecological and evolutionary implications. Oxidative stress may affect life-history strategies and the resolution of trade-offs such as investing in the antioxidant protection of somatic functions or in the antioxidant protection of gametes (Dowling and Simmons, 2009; Parker et al., 2013). As a consequence, male ability to protect sperm from oxidative stress may determine their reproductive strategies (e.g. territorial, mate-guarding strategies aiming at reducing sperm competition versus copulation-sneaking strategies unavoidably facing sperm competition) (Dowling and Simmons, 2009).

The phenotype-linked fertility hypothesis (Sheldon, 1994) states that male ornaments signal sperm quality and fertilising efficiency. Females choosing to copulate with more ornamented males would accrue direct fitness benefits through fertility insurance (Sheldon, 1994), and/or indirect fitness benefits through the production of better quality offspring (Velando et al., 2008) and/or attractive sons with superior fertilising efficiency and sperm competitive ability (Keller and Reeve, 1995; Yasui, 1997). Male ornaments have been proposed to reflect male antioxidant capacity (von Schantz et al., 1999), and oxidative stress may therefore mediate the link between secondary sexual characters and sperm quality (Blount et al., 2001; Velando et al., 2008b). Evidence exist that carotenoid-based colourful ornaments reflect a male's ability to protect his sperm from oxidative stress and thus signal sperm quality and fertilising efficiency (Helfenstein et al., 2010; Pike et al., 2010). It has recently been suggested that melanin-based ornaments, and particularly eumelanin-based coloured integuments, positively reflect resistance to oxidative stress (McGraw, 2005; Ducrest et al., 2008; Galván and Alonso-Alvarez, 2008; 2009). Such traits would thus have the potential to signal antioxidant protection to sperm and sperm quality, an untested hypothesis so far.

An alternative hypothesis deriving from sperm competition theory also predicts an association between male ornaments and sperm quality. Theoretical models of sperm competition predict that males having reduced access to females such as sub-dominant, low-ranking males, should be selected to invest less in somatic functions involved in acquisition of matings (e.g. secondary sexual characters, mate-guarding, male-male competitive ability) and more in sperm traits (e.g. percentage of motile sperm, sperm swimming ability), which confer greater fertilising efficiency to compensate for a lower number of mating opportunities (Parker, 1998; Tazzyman et al., 2009; Parker and Pizzari, 2010; Parker et al., 2013). Conversely, more attractive males with privileged access to females should be selected to invest more into somatic functions and less into their germline. In species where male ornaments are predictive of a male's ability to monopolize fertile females, this soma/germline allocation trade-off hypothesis predicts

a negative correlation between the degree of elaboration of an ornament and sperm quality. This hypothesis has been substantiated in fish and bird species where more ornamented, resident or dominant males, which can monopolise females and hence face a reduced risk of sperm competition, produce sperm of lower quality than males in disfavoured roles that almost inescapably face sperm competition (Vladić and Järvi, 2001; Froman et al., 2002; Neff et al., 2003; Gage et al., 2004; Rudolfsen et al., 2006; Cornwallis and Birkhead, 2007; Pizzari et al., 2007). Since both somatic functions and sperm cells likely suffer from the deleterious effects of ROS and hence require a tight control of the redox balance via an efficient antioxidant system, it can be hypothesized that males may face a trade-off in the allocation of antioxidant resources to either the antioxidant protection of somatic functions or the antioxidant protection of their germline. Oxidative stress and antioxidant capacity would thus mediate the soma vs. germline allocation trade-off. Under such hypothesis more ornamented males should invest less antioxidant in their testes and semen for the protection of their sperm, leading to lower sperm quality, and more antioxidants to the rest of their body for the protection of their soma, and vice-versa for less ornamented males.

The oxidation-based developments of the phenotype-linked fertility hypothesis and the soma vs. germline hypothesis are not mutually exclusive. If more ornamented males have higher overall antioxidant capacity, they may be able to invest into the antioxidant protection of both their soma and sperm. Males of higher quality may thus have more elaborated sexual ornaments, greater access to females and more mating opportunities, and still produce higher quality sperm. Low-quality, less ornamented males on the other hand, may be more constrained in this trade-off and thus have to sacrifice the antioxidant protection of their soma to the benefit of their sperm to increase their sperm competitive ability. Combining the two hypotheses predicts a non-linear, U-shaped relationship between the degree of elaboration of an ornament and sperm quality where both the most ornamented males and the least ornamented males would produce high quality sperm. We explored this hypothesis in the House Sparrow *Passer domesticus*, a species where males exhibit a badge of black melanin-coloured feathers on their breast. This black badge is known to be associated with social dominance (Nakagawa et al., 2007), and dominance in turn is positively related to sexual displays and copulation rates (Riters et al., 2004). Yet, although some studies found a positive relationship between badge size and copulation success (Møller, 1988, 1990), others failed to do so (See references in Nakagawa et al., 2007), and a non-linear relationship was found in an Austrian population between badge size and paternity losses (Václav et al., 2002). It thus appeared to us that a complex, non-linear relationship between badge size and sperm quality, potentially modulated by the acquisition and allocation of antioxidant resources, might underlie the non-linear relationship between badge size and paternity losses.

Antioxidant defences and an individual's redox balance are characterised by a multidimensional system integrating several lines of antioxidant defences and oxidative damage to various biomolecules (Cohen and McGraw, 2009; Monaghan et al., 2009). Additionally, if we are to identify the resources involved in the maintenance of the redox balance and the honesty of signals, we need to target specific compounds rather than assessing overall antioxidant capacity and oxidative damage. Thus, and as advocated in Hõrak and Cohen (2010), we chose to describe individual redox status through (1) a specific marker of oxidative damage, i.e. the levels of end-products of lipid peroxidation, (2) a marker of cellular oxidative stress, i.e. the ratio of oxidised over reduced glutathione, and (3) the activity of the antioxidant enzyme SOD, i.e. an endogenous antioxidant that catalyses the dismutation of superoxide anions into

molecular oxygen or hydrogen peroxide. We assessed these markers in two somatic tissues, i.e. the plasma and the red blood cells, and in a tissue of the germline function, i.e. the ejaculate.

Materials and Methods

Individuals and sampling

We trapped a total of 60 male and 60 female house sparrows using mist-nets in western Switzerland in April 2014. We measured body mass and tarsus length prior to housing the birds in 15 mixed outdoor aviaries at the Ethological Station, University of Bern, Switzerland. After four weeks of acclimation all the females were transferred into a separate aviary, and we took a blood and a sperm sample from all males. We then collected a second sperm sample the day after, and a third sperm sample 48 hours after the second. This procedure ensured that, as copulation rates likely varied across males of different social ranks, any differences in sperm characteristics would be intrinsic differences in quality rather than differences due to depletion (Preston et al., 2001) or fresh sperm effects (Siva-Jothy, 2000; Pizzari et al., 2008). Males were divided in three sampling groups of 5 aviaries, and groups were 5 days apart.

To establish the hierarchy of the males in each aviary, we recorded a total of 13 hours of observations in each aviary. We removed the feeders for 1.5 hours, and then recorded all the antagonistic interactions at the feeders for one hour after reintroducing the feeders into the aviaries. Within each aviary, we computed each male's David's score as a proxy for their social rank (Gammell et al., 2003).

Ejaculate quality

Ejaculates were collected in glass capillaries after massaging male's cloaca, and immediately 0.25 µL of ejaculate were diluted in 40 µL of preheated Dulbecco Modified Eagle Medium at 40º C. Then the diluted ejaculated was loaded into a 20 µm-deep chamber (Leja, Netherlands) and a video of the moving sperm was recorded using a Toshiba CMOS HD camera (TOSHIBA Corporation, Japan) mounted on a Olympus BX43 microscope (Olympus Co., Japan) at 100X magnification and phase contrast 3 annular ring. A subsample of the ejaculate was diluted in PBS and stored at -80º C for laboratory analyses. Additionally a small drop of sperm was smeared on glass slide using 10% formalin for morphology measurements. From the videos we estimated standard ejaculate quality traits using a Computer Assisted Sperm Analyzer plug-in (Wilson-Leedy and Ingermann, 2007) for ImageJ (Schneider et al., 2012). We modelled the rate at which initial speed decreases through time, referred as ejaculate longevity), as well as the rate at which initial proportion of swimming sperm decreases through time, referred as swimming endurance, using mixed linear models with time as a fixed effect and individual identity as a random slope and intercept.

Oxidative stress and antioxidant defences

Malondialdehyde (MDA) is an end-product of lipid peroxidation that provides a good measurement on the amount of lipid damage due to ROS attack (Halliwell and Gutteridge, 2007; Monaghan et al., 2009), and thus we determined the levels of MDA in plasma, erythrocytes, and sperm. Malondialdehyde levels were determined by derivatization with thiobarbituric acid and further separation by ultra-high pressure liquid chromatography (UHPLC) with fluorescence detection (Agarwal and Chase, 2002; Moselhy et al., 2013; see the Materials and Methods Appendix for details).

Glutathione in its reduced form (GSH) is an endogenous intra-cellular tri-peptide that can quench ROS in a reaction catalysed by the enzyme glutathione peroxidase, thus

being oxidized into glutathione disulphide (GSSG) (Halliwell and Gutteridge, 2007). Hence, the proportion of oxidized glutathione over total glutathione (GSSG/tGSH) is used as an accurate measurement of the oxidative balance of cells (Cnubben et al., 2001). We determined the levels of glutathione in both its reduced and oxidized forms both in sperm and red blood cells using UHPLC-MS/MS (adapted from Bouligand et al., 2006). For details refer to the Materials and Methods Appendix.

Superoxide dismutase is an endogenous enzymatic antioxidant that catalyses dismutation of superoxide anions into molecular oxygen or hydrogen peroxide (Halliwell and Gutteridge, 2007). Thus, we also measured superoxide dismutase activity per ml of tissue in sperm and blood applying minor modifications (1:500 dilution for blood and 1:160 dilution for sperm) to a commercial kit (Cayman Chemical, USA).

Badge size

From each individual we took two independent photos in a dorsal position by holding the bird tarsi and gently pushing down the beak. A millimetre paper was placed bellow the bird, and all the photos were taken at the same focal distance and light conditions. We then used Adobe Photoshop CS5 to measure the badge size using a lasso tool to select the area where the badge is, avoiding the beak and face mask that have also a black coloration. Then, we used the colour range tool to select the black areas of the badge set to 70% of tolerance (hereafter referred as exposed badge). Finally, we used the quick selection tool to manually select the unexposed parts of the badge that were not selected by the automated selection (hereafter referred as total badge). All photos were measured twice blindly from the previous measurements. The measurement repeatability within photos was of r=0.96, while between different photos it was r=0.72.

Statistical analyses

Badge size and social dominance

Several studies have argued that badge size reflects the dominance status of a male (reviewed in Nakagawa et al., 2007). Thus, we tested whether within the social groups badge size would reflect the social rank predicted by the David's score (Gammell et al., 2003). We performed two linear mixed models that included social rank as the response variable as a function of either total or exposed badge size centred by the aviary means. Additionally, body mass and tarsus length were included as covariates, and both aviary and sampling date were used a random factors.

Predictions of the phenotype-linked fertility hypothesis

The PLFH predicts a positive relationship between the sexual signal and sperm quality across the whole population, the signal being an absolute indicator of male fertility. If male fertility and the sexual signal share a common susceptibility to male redox balance, we thus expect a 1) positive relationship between the sexual signal and sperm quality, and 2) negative relationships between the sexual signal on the one hand and the level of oxidative stress endured in the soma and the sperm on the other hand.

Predictions of the oxidation-based soma/germline allocation trade-off hypothesis

This hypothesis does not make predictions at the population level. It is based on differential access to fertile females by males with different sexual attractiveness. Predictions must thus be drawn at the level of the social group within which males and females interact and within which males are assessed by the females. The oxidation-based soma/germline allocation trade-off hypothesis thus predicts that, *within groups*, (1) sexual signal and sperm quality should be either negatively related (simple allocation trade-off) or, if the most preferred males accrue more resources (e.g. the big

house and big car effect; Reznick et al.), follow a U-shaped relationship. There should also be (2) a negative or U-shaped relationship between the intensity of the sexual signal and the amount of antioxidant resources (SOD activity) invested in sperm, and (3) a positive or bell-shaped relationship between the sexual signal and the amount of oxidative damage (MDA) or level of oxidative stress (GSSG/tGSH) in sperm.

We used linear mixed models to test our hypotheses. We modelled both ejaculate traits and antioxidant resources as a function of either the total or exposed badge size, including body mass and tarsus length as covariates. We tested the PLFH using quadratic models with absolute badge size as the explanatory variable. We tested the soma-germline allocation trade-off hypothesis using quadratic models with relative badge size centred on the aviary (social group) mean. We did not include any interactions between badge size terms and body mass or tarsus length, because we had no clear predictions on the direction of such interactions and because our small sample sizes could have produced unreliable surface estimates resulting from interactions between continuous variables.

The dependent variables describing ejaculate quality were the proportion of motile sperm, initial swimming speed, sperm longevity and sperm swimming endurance. The dependent variables describing antioxidant use and oxidative stress in both sperm and red blood cells (RBCs) were the ratio of oxidized glutathione [GSSG/(GSSG+GSH), used as a measure of the oxidative stress endured by the cells], SOD activity, the proportion of SOD activity in the sperm relative to the total SOD activity in sperm and RBCs [SOD sperm/(SOD sperm+SOD RBC), used as a measure of relative SOD investment into spermatic vs. somatic functions], MDA levels, and the proportion of MDA in the sperm relative to the total level of MDA in sperm and plasma [MDA sperm/(MDA sperm+MDA plasma), used as a measure of relative oxidative stress in the sperm vs. soma].

Proportions were either logit- or arcsine-transformed when necessary, while other dependent variables were log-transformed to match normality. All the models included the aviary and the sampling date as random factors and used restricted maximum likelihood method for parameter estimation. A Kenward-Roger approximation was used for the computation of fixed effects degrees of freedom (R package pbkrtest) (Halekoh and Højsgaard, 2014). The modelling was done using the package lme4 (Bates et al., 2015). To avoid inflating the type I error we did not apply model selection (Whittingham et al., 2006), and therefore always report results for full models. The analyses were performed using R v. 3.1.1 (R Core Team, 2014).

Results

Badge size and social dominance

To estimate the dominance score for each aviary, we used an average of 82 dyadic interactions per aviary (min = 31 dyads, max = 235 dyads). Neither total ($F_{1,42.7} = 2.18$, $p = 0.14$) nor exposed ($F_{1,42.4} = 2.51$, $p = 0.12$) badge size predicted the social rank. Interestingly, both models found that tarsus length was significantly positively correlated with social rank ($5.81 < F_{1,54} < 5.85$, $p = 0.019$).

Badge size and ejaculate quality

We did not find any correlations between any sperm performance trait, and either the total ($0.08 < F < 1.30$, $p > 0.1$ for all covariates and models) or the exposed ($0.002 < F < 1.37$, $p > 0.1$ for all covariates and models) badge size. Similarly, neither total ($0.009 < F < 1.45$, $p > 0.1$ for all covariates and model) nor exposed

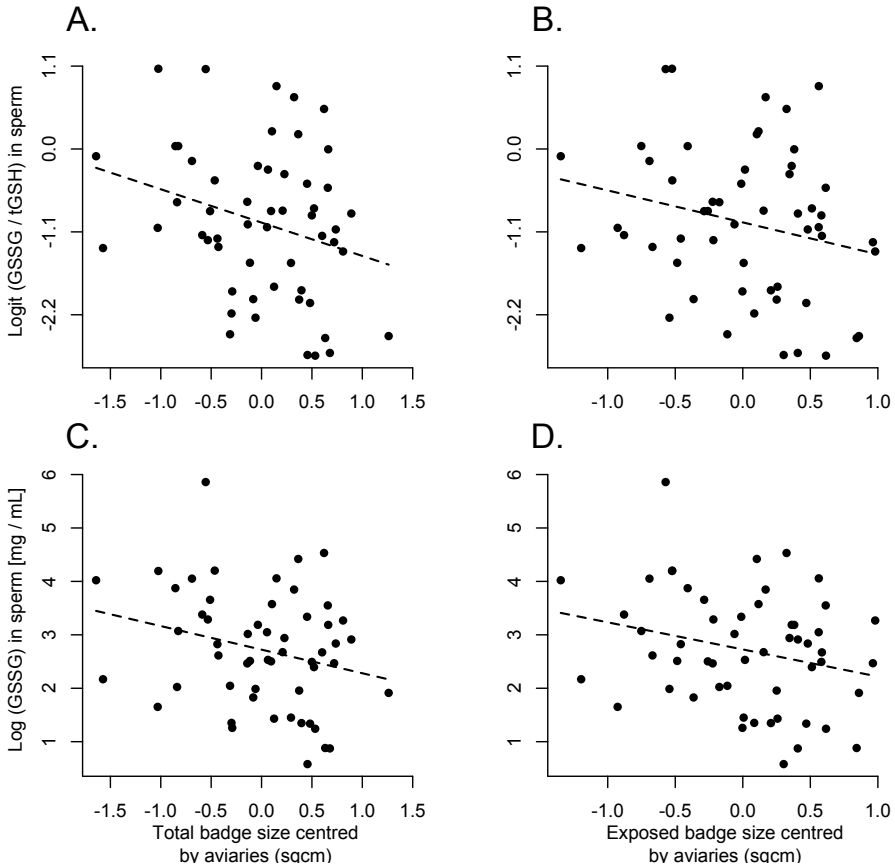

Figure 3. Correlation between the levels of oxidative stress (GSSG / tGSH) in sperm and to the total (**A**) or exposed (**B**) badge size relative to the social group means. The lower panels show the correlation between the amount of oxidized glutathione (GSSG) and the total (**C**) or exposed (**D**) badge size relative to the social group means. The dashed lines represent a linear regression fit.

($0.10 < F < 1.84$, $p > 0.1$ for all covariates and model) relative badge size (within aviary) was related to any sperm performance trait. However, the models for sperm longevity showed a positive and significant correlation between body mass and longevity, but this result is discussed Chapter 1.

Badge size and oxidative stress

We found a quadratic relation between levels of SOD in sperm and absolute total badge size ($F_{1,44} = 4.30$, $p = 0.044$), although this relation was lost after removing an outlier ($F_{1,46.4} = 0.43$, $p = 0.5$). In both sperm and soma, the remaining markers of oxidative stress, antioxidant levels, or relative investment of antioxidants in sperm were neither correlated with the total ($0.0001 < F < 2.39$, $p > 0.1$) nor to the exposed ($0 < F < 2.38$, $p > 0.1$) absolute badge size.

Contrary to the expectations of the soma-germline allocation trade-off hypothesis, we found a negative and significant correlation between the ratio of oxidised glutathione (GSSG) over total glutathione (tGSH) in sperm and the total (Fig. 1A; $F_{1,40.3} = 4.36$, $p = 0.043$) and exposed (Fig. 1B; $F_{1,38} = 4.13$, $p = 0.049$) badge size relative to the aviary mean. Further, we found that the total amount of GSSG was also negatively correlated with the total (Fig. 1C; $F_{1,40.6} = 3.66$, $p = 0.063$) and the exposed (Fig. 1D; $F_{1,38} = 4.38$, $p = 0.043$) relative badge size. Finally, in both sperm and soma we did not find any correlations between the remaining markers of oxidative stress, antioxidant levels, or relative investment of antioxidants in the sperm with the total ($0.004 < F < 2.85$, $p > 0.1$) or exposed ($0.0003 < F < 2.45$, $p > 0.1$) relative badge size.

Discussion

In the present study we tested whether a melanin-based ornament could reflect male fertility (i.e. the phenotype-linked fertility hypothesis) and/or differential investment of antioxidant resources into the sperm vs. soma (i.e. the oxidation-based soma-germline allocation trade-off hypothesis) in male House Sparrows. We found that absolute badge size does not correlate with ejaculate quality, and thus our results do not support the phenotype-linked fertility hypothesis. Nevertheless, we found that males having relatively larger badges compared to other members of the same social group produced less oxidatively stressed sperm. Sperm redox status being an important component of male and female fertility (Aitken, 1999; Tremellen, 2008; Velando et al., 2008;Almbo et al., 2011), females may thus still accrue direct and indirect benefits in choosing males with relatively larger badges.

Our results do not support the hypothesis that male badge size may reflect how males strategically allocate antioxidant resources to their soma vs. their germline. This is surprising given that a recent study in the same model species shows that males occupying different hierarchical ranks differ in their ejaculate quality, and that both dominant males as well as males at the lower end of the hierarchy produce lower quality ejaculates (Chapter 1). Studies on the function of badge size often report contradictory results in House Sparrows (e.g. studies on extra-pair paternity and badge size: Cordero et al., 1999; Whitekiller et al., 2000; Václav et al., 2002). However, a meta-analysis conducted by Nakagawa et al. (2007) showed that the only character significantly correlated with badge size is social status. Yet, in our study badge size and social dominance were unrelated, as previously found in other studies (Riters et al., 2004; Lindström et al., 2005). Thus, the latter result might explain the lack of association between ejaculate quality and relative badge size.

Melanocortins bind to the G-protein-coupled melanocortin 1 receptor (MC1R) to produce darker colorations (Ducrest et al., 2008). However, melanocortins can also bind to other melanocortin receptors (MC2-5R) that have other functions than melanisation of tissues, and thus several traits are predicted to be pleiotropically affected by the production of more melanised ornaments (Ducrest et al., 2008). Further, the production of black ornaments has also been related to lower levels of glutathione, which antagonizes the production of eumelanin (Galván and Alonso-Alvarez, 2008; 2009). Hence, it has been proposed that individuals that have darker ornaments might be more resistant to stress, sexually more active, more aggressive, better at optimizing their energy balance, having better anti-inflammatory responses, and higher antioxidant capacities (Ducrest et al., 2008; Galván and Alonso-Alvarez, 2008; 2009). Such predictions have been observed in other taxa (reviewed by Ducrest et al., 2008), but to our knowledge we are the first to test whether males with larger melanin-based ornaments can better protect their ejaculates from oxidative stress. While we did not find any correlations between redox status in either soma or sperm and absolute badge sizes as predicted by the pleiotropic effects of the melanocortin system, we found that badge size relative to the social group is negatively related to the amount of proportion of oxidised glutathione in the ejaculate (Fig. 1A, B) as well as with the net levels of oxidised glutathione in the ejaculate (Fig. 1C, D). Those results suggest that within a social group large-badged males have the potential to better protect their ejaculate from oxidative insults, and this is unrelated to their ability to monopolize resources (e.g. social dominance). Despite that having larger relative badges did not correlate with enhanced ejaculate quality as measured here, having ejaculates that are more resistant to oxidative insults might provide both direct and indirect benefits to females such as

progeny with higher antioxidant capacity (Keller and Reeve, 1995;Yasui, 1997) or sperm that contain less oxidised DNA (Velando et al., 2008). For instance, a recent study on house sparrows (Chapter 1) found that ejaculate quality is negatively correlated with sperm oxidative stress levels, and that higher antioxidant levels in the ejaculate enhance ejaculate quality. Thus, badge size could be an honest signal of the sperm antioxidant capacity of a male within a social group, and our results indirectly support the PLFH.

Altogether, we found that badge size relative to the social group reflects sperm redox status, and this is unrelated to social dominance, thus providing some support to the phenotype-linked fertility hypothesis. Although, we did not find support for our oxidation development of the soma/germline trade-off hypothesis, we believe that it may still apply in species where male secondary sexual characters are more strongly related to male ability to attract and monopolize females, and that, in such species, it could be revealed by experimentally altering male redox balance, e.g. using an exogenous source of oxidative stress.

Ethics

This study was conducted under the licenses n° BE41/12 and WTH/g-525/14 for animal experimentation and detention by the Veterinary Office of the Canton Bern (Switzerland).

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Authors' contributions

F.H. & A.R.M. designed the study. A.R.M. collected the data. A.R.M. analysed the sperm videos and did all the laboratory analyses. A.R.M. measured all the badge photos. A.R.M & F.H. performed statistical analyses and wrote the manuscript. All authors read and approved the manuscript.

Chapter 5

Effects of increased oxidative stress in patterns of antioxidant allocation into sperm across social ranks

In review in Functional Ecology

Experimentally induced oxidative stress affects sperm performance and ejaculate antioxidants in wild House Sparrows *Passer domesticus*

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Abstract

Oxidative stress (OS) is the result of random cellular damage caused by oxygen radicals that leads to cell death, ageing, or illness. Exposure to pollutants is a major cause of OS, which in turn has been identified as a major cause of infertility. In promiscuous species, the fertilizing ability of the ejaculate partly determines the reproductive success of males. Moreover, when dominance determines access to fertile females, theory predicts that males should invest more resources into enhancing their ejaculate quality as they occupy lower hierarchical ranks. In this study, we hypothesize that subordinate males should be selected to prioritize antioxidant allocation to their ejaculates in order to protect their sperm from OS and thus produce higher quality ejaculates. To test our hypothesis, we chronically dosed wild House Sparrows with diquat, an herbicide used worldwide that cause OS. We found that diquat-treated males produced ejaculates with reduced velocity, and more specifically males at the bottom of the hierarchy suffered the largest reduction in ejaculate speed. This result suggests that resource restrictions hinder an individual's ability to cope with environmental hazards. Additionally, diquat treated males responded to the oxidative threat by increasing their antioxidant investment into their ejaculate. Our results point at OS as a likely physiological mechanism mediating ejaculate quality, while condition may play a role in constraining the amount of resources to be invested in the ejaculate.

Keywords: Oxidative stress, social dominance, soma vs. germ line, sperm competition, diquat

Introduction

Reactive oxygen species (ROS) are metabolic by-products that cause random cellular damage, which leads to cell death, ageing, degenerative diseases, or impaired cellular pathways (reviewed in Finkel and Holbrook 2000). Although ROS are needed to control and promote physiological processes (e.g. ATP production, immune response, etc.), in many instances antioxidants cannot quench all the ROS, and further ROS-induced damage is known as oxidative stress (OS) (Jones 2006). Factors that may be responsible for an increase in OS generation include regular metabolic processes (Finkel and Holbrook 2000), immune response (Bedard and Krause 2007, Sorci and Faivre 2009), and pollutants (Banerjee et al. 2001, Li et al. 2003). Given that all organisms are subject to attacks by ROS, oxidative stress is hypothesized to be a main constraint to life history evolution (Dowling and Simmons 2009, Monaghan et al. 2009, Metcalfe and Alonso-Alvarez 2010).

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Sperm cells contain a large proportion of polyunsaturated fatty acids (PUFA) in their membrane, which has been shown to enhance ejaculate quality (e.g. Mitre et al. 2004, Wathes et al. 2007). However, PUFA can easily be oxidized by ROS (reviewed in Hulbert et al. 2007). The resulting oxidative damage to the sperm membrane leads to impaired cell-egg interactions, reduced ejaculate quality, and sub- or infertility (Aitken 1999, Aitken and Baker 2006, Tremellen 2008). Additionally, ROS attacks on sperm DNA can cause chromosome rearrangements, histone modifications, deletions, base modifications, or changes in methylation patterns (Lloyd et al. 1997, Kemal Duru et al. 2000, Wellejus et al. 2000, Aitken and Krausz 2001, Zitzmann et al. 2003), and these of types damage to DNA may render sperm unable to fertilize the ovum. For the above-mentioned reasons, oxidative stress is considered one of the main causes of male infertility (Aitken and Baker 2004, Tremellen 2008).

In promiscuous species, competition for the fertilization of an ova between ejaculates of two or more males is a strong selective force driving the evolution of ejaculate traits that enhance the fertilization efficiency of an ejaculate and hence a male's reproductive success (Birkhead and Pizzari 2002, Snook 2005, Simmons and Fitzpatrick 2012, Fitzpatrick and Lüpold 2014). Several theoretical models have explored the tradeoffs of engaging in social roles that invest in precopulatory traits to enhance mating success versus postcopulatory traits that enhance the fertilizing ability of the ejaculate (Parker 1998, Parker and Pizzari 2010). The predictions of those models have been tested in several taxa (e.g. Preston et al. 2001, Rudolfsen et al. 2006, Cornwallis and Birkhead 2007, Thomas and Simmons 2009, Evans 2010, Lemaître et al. 2012), although these studies analysed only discontinuous roles (e.g. sneaker vs. territorial, dominant vs. subordinate). A more recent model predicts a continuous increase of resource investment into ejaculate quality as mating costs increase, resulting in a continuum of ejaculate quality (Tazzyman et al. 2009). Some support to this model comes from a study by Engqvist (2011) who found continuous and negative genetic co-variation between attractiveness and mating investment in a Scorpionfly *Panorpa cognata*. Recently, we found direct evidence for such a continuum in ejaculate quality across social ranks in House Sparrows (*Passer domesticus*), with males adjusting their sperm quality via antioxidant allocation to their ejaculates (Chapter 1). Oxidative stress and/or antioxidant availability has also been previously observed to affect ejaculate traits in various other organisms (e.g. Ciereszko and Dabrowski 1995, Bréque et al. 2003, Chitra et al. 2003, Mitre et al. 2004, Helfenstein et al. 2010a, Almbro et al. 2011). Therefore, it could be hypothesized that males better at preventing ROS from attacking their ejaculates will produce ejaculate of higher quality, and as a consequence strategic allocation of antioxidant resources might underlie the reproductive tactics that a male adopts.

In the common house sparrow, the dominance hierarchy is linked to male sexual behaviours, which determines access to fertile females (Anderson 2006). In their natural environment, individuals may be subjected to increased levels of ROS, i.e. through reproductive costs (Wang et al. 2001, Alonso-Alvarez et al. 2004) and/or exposure to pollutants (Banerjee et al. 2001, Li et al. 2003). Here, we tested if exposure to a pro-oxidant contaminant would change antioxidant allocation to favour ejaculate quality in wild house sparrows. Specifically, we chronically dosed males with diquat, a commonly used herbicide that causes an increase in the production of superoxide anions, a strong pro-oxidant free-radical (Bus and Gibson 1984, Saeed et al. 2001). Based on Tazzyman et al. (2009) model predictions, we predicted that, under increased risk of oxidative stress, subordinate males would prioritize the antioxidant protection of their ejaculate at

the expense of their soma, while dominant males would prioritize antioxidant protection of their soma at the expense of their ejaculate.

Materials and Methods

Pilot study

In order to manipulate the levels of oxidative damage in male house sparrows, we introduced two potent pro-oxidants to the study individuals (diquat and paraquat; Bus and Gibson 1984, Saeed et al. 2001). These compounds are non-selective herbicides that enter into a cycling reaction with nicotinamide adenine dinucleotide and oxygen molecules that are broken into singlet oxygen (Bus and Gibson 1984, Saeed et al. 2001). Both paraquat and diquat are broadly used under the brand names Gramoxone® (Paraquat, Syngenta AG, Switzerland) and Reglone® (Diquat 20%, Syngenta AG, Switzerland) respectively. Gramoxone® has been banned in many countries (detailed information on country bans at http://www.paraquat.com). To determine a dose that would disrupt the REDOX balance without harming the birds, we trapped 36 male house sparrows during January 2013 in Ariège, France, and housed them in pairs in 18 indoor aviaries (length: 1.5m, width: 1.5m, height: 2.5m). The birds were then left to acclimatize to the aviary conditions for 1 week. After acclimatization, we dosed the individuals with 0.1 mL of paraquat (60, 120, 180, or 240 mg in 0.1 mL of 0.8% NaCl), diquat (0.012, 0.024, 0.036, or 0.048 mg in 0.1 mL of 0.8% NaCL), or a physiological saline (0.8% NaCl) every second day for 2 weeks. We assigned 2 aviaries to each of the 9 possible solutions. Every fourth day we took a small blood sample (ca. 60 µL), from which plasma was obtained by centrifuging the sample at 7000 RPM for 5 minutes. We determined the levels of malondialdehyde (MDA) from the plasma and erythrocyte samples following standard procedures for derivatization with thiobarbituric acid followed by ultra high-pressure liquid chromatography (UHPLC) coupled to fluorescence detection (adapted from Agarwal and Chase 2002, Moselhy et al. 2013).

Over the course of the diquat treatment, we found that MDA levels in plasma gradually increased (dose x time: $F_{12,41.5} = 1.90$, $p = 0.063$), whereas in the paraquat treatment we found a general increase of MDA levels in plasma (dose: $F_{4,4.8} = 5.22$, $p = 0.052$; dose x time: $F_{12,42.7} = 1.50$, $p = 0.16$). Additionally, the birds experienced very adverse effects when treated with 0.048 mg of diquat (e.g. fibrosis on the crop that caused the death of half the individuals). Based on the fact that diquat is still broadly used (whereas paraquat has been banned in the USA and Europe), that the doses of diquat necessary to cause lipid peroxidation, e.g. OS, were ten-fold less than paraquat, and that a dose of 0.024 mg of diquat caused a 40% increase in plasma MDA at the end of the pilot, we decided to use a 0.024 mg dose of diquat in the main experiment.

Main experiment

Individuals

We trapped a total of 54 male and 54 female house sparrows using mist-nets in Ariège, France, between the 27th of March and 10th of May 2013. After trapping, we measured body mass and tarsus length of each individual, and then transferred the birds into 18 mixed outdoor aviaries (1.5m X 4m X 3.5m) at the CRNS Experimental Ecology Station (Moulis, France). Individuals were also given colour rings and metal rings with unique combinations. A total of 3 males and 3 females were held in each aviary for a 4-week acclimation period. During this period they received a controlled amount of food determined in a pilot study (120 gr per aviary per day; germinated barley seeds 25 %, food supplement Quicko 15%, and a mix of seeds for canaries 60%), and water was provided ad libitum. Body mass and overall condition were monitored during

acclimation to ensure that all individuals were in good health before starting the experiment.

Experimental design

After four weeks of acclimation, we split males and females into 27 unisex aviaries (18 male aviaries and 11 female aviaries). We arranged the aviaries so that males had visual and acoustic contacts with the females (i.e. each male aviary was surrounded by two adjacent female aviaries, one on each side), and males stayed in the aviaries where they acclimated. We maintained only 33 females during the experiment, and the other twenty-one females were released. Birds were given a controlled amount of food (60 g per aviary per day; germinated barley seeds 25 %, food supplement Quicko 15%, and a mix of seeds for canaries 60%) and water ad libitum. On the first day of the experiment we dosed males in nine of the aviaries with 0.1 mL of physiological saline (0.8% NaCl) containing 0.024 mg of diquat (~1g/kg), while the males of the other 9 aviaries were given 0.1 mL of a physiological saline (0.8% NaCl). Every second day, we maintained the 0.1 mL dose of either diquat or physiological saline for a total of 18 days. Additionally, body weight was taken every two days and photographs of the males' badge every six days. After the 18-day experiment, we released all the individuals to their original locality.

Behavioural observations

To establish the male hierarchy in each aviary, we recorded antagonistic interactions between individuals. During the acclimation period, we observed each aviary for one hour three times a week. Additionally, every second morning we recorded each aviary for one hour right after feeding the birds (at ca. 6:00 am GMT +1). From both the behavioural observations and the videos, we counted the number of encounters between individuals (including females during the acclimation period) and determined who was the winner. Using this information, we estimated a David's Score (Gammell et al. 2003) to determine the hierarchical position of each individual in the aviary.

Blood samples

We obtained blood samples every six days by puncturing the alar vein with a 27-gauge needle, and approximately 80 µL of blood was collected in a heparinized capillary (Microvette CB300). Blood was centrifuged for 5 min at 7000 RPM and 4°C, and plasma and red blood cells were stored separately at -80°C.

Sperm samples

Ejaculates (ca. 2 µL) were obtained by gently massaging male's cloaca (Wolfson 1952). Following the collection of ejaculates, 0.2 µL of sperm was dissolved into 40 µL of pre-warmed DMEM (Dulbecco's modified Eagle medium, 4'500 mg glucose/L, 110 mg/L sodium pyruvate and L-glutamine), and then transferred to a 20 µm deep chamber slide (Leja Products B.V., The Netherlands). We video recorded sperm for 75 seconds at 16 frames per second using an Olympus SC100 camera (Olympus Co., Japan) fitted on an Olympus BX43 microscope (Olympus Co., Japan) at 100x magnification under dark field condition generated by a phase 3 annular ring. Temperature was maintained at 40°C using a heating plate on the microscope (Minitube HT 200, Germany). A subsample of 1 µl of sperm was collected into 9 µL of PBS and stored at -80°C for later assessment of malondialdehyde (MDA) concentrations, activity of superoxide dismutase (SOD), and concentrations of reduced and oxidized glutathione (GSH and GSSG).

From each sample, we estimated curvilinear velocity (VCL: total point to point distance

Table 1. Summary from the linear mixed model and the ANOVA on sperm swimming velocity. AIC for the model including body weight and tarsus length was 3002.7 vs. 3001.8 for the model without those covariates. Analisys of deviance: $p_{chi\text{-}sq} = 0.13$

Fixed effects	Slope ±SE	F_{df}	p-value
Intercept	49.77 ± 1.62	-	-
Treatment	3.60 ± 1.62	$4.89_{1,17.3}$	**0.041**
Social Rank		$3.17_{2,25.4}$	***0.059***
- Rank: S1	1.11 ± 1.52		
- Rank: S2	-3.66 ± 1.47		
Day	0.26 ± 0.09	$7.46_{1,316.2}$	**0.007**
Day^2	-0.07 ± 0.02	$12.17_{1,34.8}$	**0.001**
Treatment * Rank		$2.93_{2,25.4}$	***0.072***
- Treatment * Rank:S1	-3.53 ± 1.52		
- Treatment * Rank:S2	0.61 ±1.47		
Treatment * Day	-0.06 ± 0.09	$0.45_{1,316.2}$	0.50
Treatment * Day^2	-0.02 ± 0.02	$1.22_{1,34.8}$	0.28
Day * Rank		$0.01_{2,316.6}$	0.99
- Day * Rank: S1	0.02 ± 0.13		
- Day * Rank: S2	-0.005 ± 0.13		
Rank * Day^2		$0.21_{2,34.7}$	0.81
- Day^2 * Rank: S1	0.02 ± 0.03		
- Day^2 * Rank: S2	-0.003 ± 0.03		
Treatment * Rank * Day		$1.40_{2,316.6}$	0.25
- Treatment * Day * Rank: S1	-0.22 ± 0.13		
- Treatment * Day * Rank: S2	0.05 ± 0.13		
Treatment * Rank * Day^2		$0.93_{2,34.7}$	0.40
- Treatment * Day^2 * Rank: S1	0.04 ± 0.03		
- Treatment * Day^2 * Rank: S2	-0.03 ± 0.03		

travelled by the sperm over the time period analysed averaged to a per second value, μm/s) and proportion of swimming sperm from 4 video segments of 2.5 seconds (at 0, 20, 40, and 60 seconds after recording started) using a Computer Assisted Sperm Analyzer plug-in (Wilson-Leedy and Ingermann 2007) for ImageJ (Schneider et al. 2012). It allowed us to determine the initial sperm speed, the stamina (rate at which initial speed decreases through time), and the longevity (rate at which initial proportion of swimming sperm decreases through time) of sperm in each ejaculate.

Biomarkers of oxidative stress and antioxidant defences

Malondialdehydes are end products of lipid peroxidation that reflect the amount of lipid damage due to ROS attack (Halliwell and Gutteridge 2007, Monaghan et al. 2009). We assessed the amount of MDA in plasma and sperm. MDA levels were assessed by derivatization with thiobarbituric acid (TBA) followed by ultra high-pressure liquid chromatography (UHPLC) coupled to fluorescence detection as described in previous studies with some modifications (Agarwal and Chase 2002, Moselhy et al. 2013); see supplementary material for more details. Superoxide dismutase is a endogenous enzymatic antioxidant, which catalyses the dismutation of superoxide anions into hydrogen peroxide (Halliwell and Gutteridge 2007). We assessed the SOD activity per unit of tissue following standard procedures for a commercial colorimetric assay (Cayman Chemical, USA) with minor changes to the sample concentrations (1:500 for erythrocytes and 1:160 for sperm). Reduced glutathione is an endogenous intra-cellular tri-peptide, which can be oxidized into glutathione disulphide (GSSG) to reduce ROS via a reaction catalysed by the enzyme glutathione peroxidase (Halliwell and Gutteridge 2007). The proportion of oxidized glutathione over total glutathione (GSSG/tGSH) provides accurate information on the oxidative balance of cells (Cnubben et al. 2001). We determined the levels of GSH and GSSG following standard water phase extraction followed by UHPLC tandem mass spectrometry (UHPLC-MS/MS) (Bouligand et al. 2006); see the Materials and Methods Appendix for more details.

Table 2. Model summary and ANOVA results from linear mixed models for antioxidants and oxidative stress in the ejaculate.

Fixed effects	Relative GSH investment*			GSSG**			GSH***		
	Slope ±SE	F_{df}	p	Slope ±SE	F_{df}	p	Slope ±SE	F_{df}	p
Intercept	1.23 ± 0.02			3.41 ± 0.12			3.13 ± 0.07	-	-
Treatment	-0.05 ± 0.02	$8.23_{1,22.9}$	**0.009**	-0.38 ± 0.12	$8.99_{1,22.8}$	**0.01**	-0.19 ± 0.07	$7.07_{1,20.6}$	**0.02**
Social Rank		$0.13_{2,26.3}$	0.87		$0.76_{2,29.2}$	0.48		$0.03_{2,26.2}$	0.97
Rank: S1	-0.006 ± 0.03			0.22 ± 0.18			0.008 ± 0.10		
Rank: S2	0.01 ± 0.03			-0.14 ± 0.17			-0.02 ± 0.10		
Day	-0.009 ± 0.001	$52.67_{1,59.2}$	**<0.001**	0.004 ± 0.01	$0.15_{1,248.7}$	0.7	-0.002 ± 0.008	$0.05_{1,263.8}$	0.83
Day^2	0.001 ± 0.0003	$23.95_{1,31.2}$	**<0.001**	-0.0008 ± 0.002	$0.14_{1,31.1}$	0.71	0.002 ± 0.002	$2.49_{1,33.4}$	0.12
Treatment * Rank		$0.80_{2,26.3}$	0.46		$0.31_{2,29.2}$	0.74		$1.81_{2,26.2}$	0.18
Treatment * Rank:S1	0.02 ± 0.03			-0.13 ± 0.18			-0.05 ± 0.10		
Treatment * Rank:S2	-0.03 ± 0.03			0.10 ± 0.17			-0.14 ± 0.10		
Treatment * Day	0.00002 ± 0.001	$0_{1,59.2}$	0.99	0.01 ± 0.01	$1.02_{1,248.7}$	0.31	0.004 ± 0.008	$0.24_{1,263.8}$	0.62
Treatment * Day^2	0.0005 ± 0.0003	$3.89_{1,31.2}$	***0.057***	0.002 ± 0.002	$1.43_{1,31.1}$	0.24	0.001 ± 0.002	$0.43_{1,33.4}$	0.52
Day * Rank		$2.09_{2,59.1}$	0.13		$1.12_{2,248.1}$	0.33		$5.49_{2,263.4}$	**0.01**
Day * Rank: S1	0.002 ± 0.002			0.01 ± 0.01			0.02 ± 0.01		
Day * Rank: S2	-0.004 ± 0.002			-0.02 ± 0.01			-0.04 ± 0.01		
Rank * Day^2		$0.14_{2,31.1}$	0.87		$4.13_{2,31.1}$	**0.03**		$3.65_{2,33.3}$	**0.04**
Day^2 * Rank: S1	0.00003 ± 0.0004			-0.005 ± 0.003			-0.002 ± 0.002		
Day^2 * Rank: S2	0.0001 ± 0.0004			0.008 ± 0.003			0.006 ± 0.002		
Treatment * Rank * Day		$0.11_{2,59.1}$	0.89		$0.14_{2,248.1}$	0.87		$0.16_{2,263.4}$	0.85
Treatment * Day * Rank: S1	0.0007 ± 0.002			0.005 ± 0.01			-0.006 ± 0.01		
Treatment * Day * Rank: S2	-0.0008 ± 0.002			0.003 ± 0.01			0.005 ± 0.01		
Treatment * Rank * Day^2		$0.12_{2,31.1}$	0.88		$1.81_{2,31.1}$	0.18		$0.20_{2,33.3}$	0.82
Treatment * Day^2 * Rank S1	-0.0002 ± 0.0004			0.004 ± 0.003			0.001 ± 0.002		
Treatment * Day^2 * Rank S2	0.0001 ± 0.0004			-0.005 ± 0.003			-0.0003 ± 0.002		

*AIC for the model including body weight and tarsus length was -176.8 vs. -180.2 for the model without those covariates. Analisys of deviance: $p_{chi\text{-}sq} = 0.76$

**AIC for the model including body weight and tarsus length was 902.8 vs. 898.9 for the model without those covariates. Analisys of deviance: $p_{chi\text{-}sq} = 0.98$

***AIC for the model including body weight and tarsus length was 778.0 vs. 774.9 for the model without those covariates. Analisys of deviance: $p_{chi\text{-}sq} = 0.64$

Statistical analyses

We performed all the analyses using R v. 3.1.1 (R Core Team 2014). We ran linear mixed models with restricted maximum likelihood for parameter estimation using the R package lme4 (Bates et al. 2014), and analyses of variance were assessed using a Kenward-Roger approximation for the degrees of freedom using the R package lmerTest (Kuznetsova et al. 2014) with α (type I error) set at 0.05. The identity of each individual and the aviary in which each individual was housed included as random intercepts, while a random slope was included to account for individuals differing in

their time-related response to the treatment. We modelled time using a linear and a quadratic term following centring on the mean. When required to meet normality of the residuals, some of the response variables as well as fixed effects were $\log_{10}$, arc-sinus, or logit transformed. All models initially included body mass and tarsus length as covariates. However, in most of the models, analyses of deviance showed that the model fit was not improved by the addition of these two covariates, which were then removed from these models (Tables 1, 2). Besides removing these two covariates, we did not perform further model selection on the main effects to reduce the risk of type I errors (Whittingham et al. 2006).

The reliability of results coming from p-values bellow 0.05 has been largely questioned (Ioannidis 2005, Simmons et al. 2011, Nuzzo 2014), and the 0.05 p-value cut-off of significance has caused a bias in the distribution of p-values reported across studies towards values just bellow such cut-off (Masicampo and Lalande 2012). This phenomenon has been argued to harm scientific progress (Cohen 1994), hence we adopted a relaxed view on the p-value cut-off of 0.05. We cannot rule out type I errors with either p-values bellow or just above 0.05, therefore we cautiously present and discuss p-values above such a cut-off. Bonferroni corrections on the *post-hoc* analyses have been deemed to increase the type-II error rate (Nakagawa 2004), and thus we chose not to apply them here.

Results

Ejaculate quality

In total, we recovered 387 ejaculates from 54 males in 18 days. We found that when male social rank was disregarded, ejaculate speed was reduced in birds chronically dosed with diquat (Table 1; means ± sd: Control = 51.17 μm/s ± 12.57, Diquat = 45.76 μm/s ± 12.77). We did not find any effect of the diquat treatment on sperm motility, longevity, or endurance ($0.01 < F < 1.46$, $p > 0.1$). Interestingly, males positioned at different hierarchical ranks did not differ in their proportion of motile sperm, endurance, or longevity ($0.07 < F < 1.82$, $p > 0.1$). Nevertheless, we observed that males at different ranks tended to differ in their ejaculate velocity (Fig. 1) (Table 1; means ± sd: Dominant = 51.4 ± 13.2, Subordinate 1 = 45.3 ± 12.2 μm/s, Subordinate 2 = 49.4 ± 12.7 μm/s). In addition, males at different social ranks tended to suffer differently from diquat intoxication in terms of their sperm velocity (Fig. 1) (Table 1). To better understand how much this interaction contributes to the model fit, we compared models with and without the interaction, and found that the more complex model explained the data better (AIC full model = 3002.7; AIC without treatment x rank = 3004.3; analysis of deviance, $p = 0.062$). In order to further understand how treatment affected sperm velocity according to male dominance, we then ran separate linear mixed models for dominant, subordinate 1 and subordinate 2 males with sperm velocity as the response variable and treatment as the independent variable, while using the identity of each individual as a random intercept with random slope for differences in the time-related response of each individual. We found that subordinate 2 males produced significantly slower sperm when exposed to diquat (pairwise comparisons ANOVAs: Dominant treatment vs. control, $F_{1,12.8} = 0.22$, $p > 0.1$, control = 52.4 ± 11.7 μm/s, diquat = 50.4 ± 14.6 μm/s; Subordinate 1 treatment vs. control, $F_{1,13.7} = 1.13$, $p > 0.1$, control = 45.3 ± 12.2 μm/s, diquat = 41.8 ± 10.4 μm/s; Subordinate 2 treatment vs. control, $F_{1,11.7} = 7.41$, $p = 0.019$, control = 53.5 ± 12.4 μm/s, diquat = 45.2 ± 11.7 μm/s). Finally, given that the interactions between treatment and time were not significant we performed a *post-hoc* analysis to test whether the differences between groups were

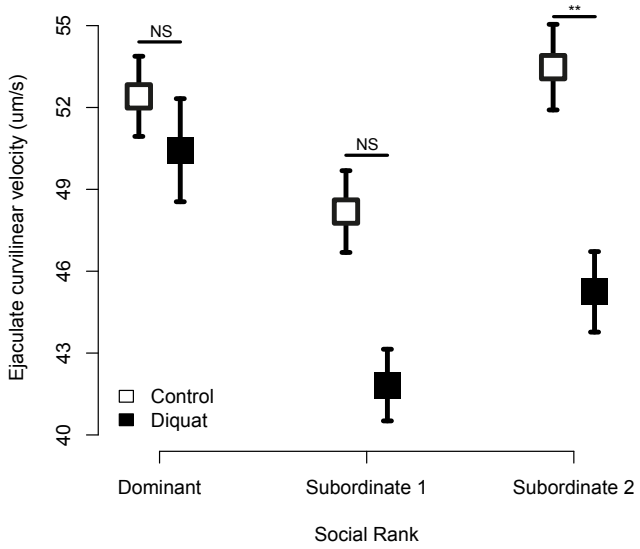

Figure 1. Effects of chronic diquat intoxication on sperm velocity in ejaculates of males occupying different social ranks. The points represent the mean, while the bars are the standard error of the mean. *Post-hoc* pairwise comparisons: NS: non-significant, $p > 0.05$; **: $p < 0.05$.

present before the treatment, and confirmed that none of the traits differed before the treatment was applied ($0.25 < F < 1.54$, $p > 0.1$).

Oxidative stress and antioxidant allocation

Due to technical problems or small ejaculate size, we could only determine the levels of GSH, GSSG, SOD, and MDA from 310 ejaculates and 213 blood samples. The males who received diquat had an increased amount of GSH (Fig. 2b) (Table 2) and GSSH (Fig. 2c) (Table 2) in their ejaculates. Interestingly, the increase of GSH levels in the ejaculates of diquat-treated birds was not the result of a general up-regulation of GSH in the body, but rather the result of a change in the allocation of GSH in their ejaculates relative to their soma (blood cells). Evidence of this is seen in the ratio of GSH in ejaculate over GSH in red blood cells, used as a proxy for relative investment in germ-line functions, increasing in diquat-treated males (Fig. 2a) (Table 2). Moreover, the proportion of oxidized glutathione (GSSG/tGSH) in ejaculates remained unchanged in diquat treated males ($F_{1,26.96} = 2.4626$, $p > 0.1$). The SOD activity ($F_{1,14.7} = 0.28$, $p = 0.6$) and the levels of MDA ($F_{1,20.3} = 0.94$, $p>0.1$) in the ejaculates were unaffected by the diquat treatment, however diquat treated males tended to reduce the levels of MDA in the ejaculate as the experiment progressed (S1; $F_{1,236.7} = 3.54$, $p = 0.061$).

The redox equilibrium in the soma (red blood cells and plasma) appeared unaffected by the diquat treatment with none of the redox biomarkers (MDA, GSH, GSSG, or SOD activity) differing between the groups ($0.23 < F < 0.89$, $p > 0.1$). Male rank in the hierarchy did not covary with any of the redox biomarkers, neither in interaction with the treatment nor alone, and neither in sperm ($0.03 < F < 0.79$, $p > 0.1$) nor in the blood ($0.29 < F < 0.68$, $p > 0.1$). Finally, given that the interactions between treatment and time were not significant, we tested whether the differences between groups were present before the treatment, and confirmed that none of the traits differed between control and treatment groups on the onset of the experiment ($0.005 < F < 1.25$, $p > 0.1$).

Discussion

In the present study we chronically dosed wild House Sparrows with a commonly used herbicide for 18 days, a period during which we obtained 387 ejaculates and 213 blood samples. We found that males chronically dosed with diquat produced slower sperm

(Fig. 1), allocated more antioxidants to their ejaculates (Fig. 2), and suffered a higher risk of oxidation as reflected in the increased levels of reduced glutathione (Fig. 1d). Additionally, we found indications that diquat affected ejaculates differently across the social hierarchy, with subordinate males apparently suffering a greater decrease in sperm velocity when intoxicated with diquat (Fig. 1).

We can see at least three, non-exclusive explanations to the observed reduction in sperm velocity. Reduced sperm velocity could be driven by (1) increases in oxidative damage to the sperm membranes, (2) changes in sperm membrane composition to avoid oxidative damage, and/or (3) impaired mitochondrial respiration due to increased ROS production. It has been previously observed in Great Tits *Parus major* that higher levels of MDA in the ejaculate are negatively correlated with ejaculate velocity (Helfenstein et al. 2010a, Losdat et al. 2011). However, ejaculates of diquat treated males did not have higher levels of MDA compared to control males, nor did MDA levels correlate with sperm velocity. Nevertheless, we found increases in the levels of GSSG in ejaculates of males dosed with diquat (Fig. 2c), which is an indication that the cells faced a greater oxidative challenge and had to stifle greater amounts of ROS.

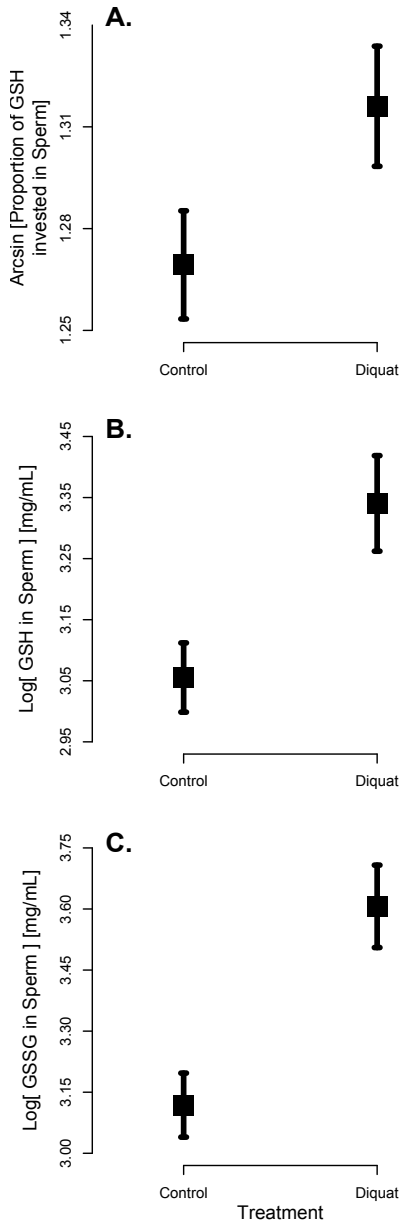

Figure 2. Effects of chronic diquat intoxication on (**A**) proportion of GSH allocated to sperm, and ejaculate levels of (**B**) GSH, and (**C**) GSSG. The points represent the mean ± SE.

If there is an increase in ROS production in the sperm of diquat treated males, a reduction in the proportion of poly-unsaturated fatty acids (PUFA) in the sperm membrane would lower the risk of oxidative damage to the spermatozoa (Wathes et al. 2007, delBarco-Trillo and Roldan 2014). However, a lower proportion of PUFA in the sperm membrane would lead to lower sperm performance (Asturiano et al. 2001, Mitre et al. 2004). Hence, changes in the proportion of membrane PUFA in diquat treated males could explain the observed reductions in ejaculate velocity. For instance, the decreasing trend in MDA levels in the ejaculate of diquat treated males as the experiment progressed (Table 2, Fig. S1) is compatible with a progressive change in sperm membrane lipid composition, i.e. lower poly-unsaturated vs. mono- and saturated fatty acids. Alternatively, the disruption of membrane proteins in the mitochondria as well as the activation of uncoupling proteins due to increased ROS production induced by diquat could reduce ATP production (Hulbert et al. 2007), hence reducing the energy budget available to sperm for swimming. However, we did not determine membrane composition or measure metabolic rates in the ejaculates. Further investigations are

necessary to determine which of those potential mechanisms are responsible for the observed reduction in sperm velocity under diquat treatment.

Based on Tazzyman et al. (2009) model, we predicted that under higher risk of oxidation, subordinate males should prioritize the antioxidant protection of their ejaculate at the expense of their somatic balance. However, we observed that the sperm velocity of males at the bottom of the hierarchy (subordinate-2) appeared impacted by an exposure to diquat, while the sperm velocity of dominant and subordinate-1 males did not seem to be affected by the treatment (Fig. 1). This result is opposite to our predictions, and it could be hypothesized that dominant males having more access to resources can afford to spare resources for the maintenance of good ejaculate quality under a mild oxidative challenge. In contrast, subordinate males may be constrained in terms of resources and may avoid reducing their investment into somatic functions (body maintenance and attractiveness to females) bellow a threshold under which their survival and reproductive success would be dramatically impaired. Such a resource allocation strategy would come at the expense of their ejaculate quality. In another study (Chapter 1), we found evidence for strategic antioxidant allocation in the House Sparrow following predictions of Tazzyman et al. (2009) model. Notably, we also found that males at the bottom of the hierarchy produced low quality sperm and, as in the present study, seemed constrained in the amount of resources to be allocated to bodily maintenance vs. ejaculate quality. This constraint was apparently released when males at the bottom of the hierarchy moved up the hierarchy and could then produce higher quality sperm (Chapter 1). Together, the results of these two studies strongly suggest that sperm production is condition-dependent and highlight resource availability as an important constraint in the development of status-dependent reproductive tactics.

Previous studies on other animals, including humans, have found that pollutants can affect ejaculate quality (e.g. Dawson et al. 1998, Moorman et al. 1998, Dauwe et al. 2004, Lacoume et al. 2009, Abarikwu et al. 2010). In birds, pollutants have been shown to affect reproductive success through increased physiological stress, embryo mortality, disrupted reproductive behaviours, and impaired physiological processes (Fry 1995, Sewalk et al. 2000, Ottinger et al. 2002). Here, we show that ingesting diquat in small quantities (0.024 mg / second day) causes increased oxidative stress to the sperm (Fig. 2) accompanied by a reduction in sperm quality (Fig. 1). In agricultural matrices after a single application of diquat (560 g/ha), residues of 1.5 ppm in seed have been reported, with a maximum of 6 ppm (US Environmental Protection Agency). A house sparrow with an average body mass of 26 g has a daily energy expenditure of 99.31 kJ (Crocker et al. 2002). Such energy requirement is covered by the ingestion of 6.71 to 8.57 g of weed seeds or cereals per day. Therefore, a wild house sparrow would ingest between 0.010 - 0.013 mg of diquat per day (maximum 0.040 - 0.051 mg/day). It should be noted that under such scenario wild house sparrows would absorb a daily dose of diquat that is similar or even higher than the one used in this experiment (i.e. ~ 0.012 mg/day). Moreover, house sparrows also feed on insects during the reproductive season (Anderson 2006), and insects have been reported to have higher residues of diquat than seeds, with 17 ppm of diquat on average and a maximum value of 29 ppm (US Environmental Protection Agency). Diquat may thus be a substantial cause of oxidative stress in the wild with potentially substantial effects on male fertility and bird health in general, and negative consequences on wild bird populations.

Ethics

The experiment was conducted under the permit nº 2012-07 granted by the Directions régionales de l'environnement, de l'aménagement et du logement, France. Birds were

trapped and ringed under the permit nº 13619 granted by the Centre de Recherches sur la Biologie des Populations d'Oiseaux, France.

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Authors' contributions

All authors have read and agreed on this manuscript. F.H. and A.R.M. designed the experiment and wrote the article. A.R.M., A.F., and S.B. conducted the experiments and collected all the behavioural data. A.R.M. conducted all the laboratory analyses on the samples, analysed the sperm videos, and performed the statistical analyses. A.R.M. and F.H. wrote the manuscript.

General discussion

In this dissertation I started with the hypothesis that males being able to better protect their ejaculates from oxidative stress will enjoy of better ejaculate quality. For instance, I showed that the proportion of motile sperm negatively correlates to the amount of oxidative stress (Chapter 1 Fig. 4A), while it positively correlates to the amounts of antioxidants in the ejaculate (Chapter 1 Fig. 4B). These results confirm previous studies that have also found that the levels of oxidative damage negatively correlate to ejaculate traits (Chitra et al. 2003, Lacoume et al. 2009, Helfenstein et al. 2010a, Losdat et al. 2011, Noguera et al. 2012). Further, when I challenged males with a pro-oxidant (diquat, a commonly used herbicide), I observed that sperm velocity decreased in diquat treated males (Chapter 5 Fig. 1 and Table 1), and this was paralleled with increases in antioxidant allocation into the ejaculate and higher levels of oxidation of such antioxidants (Chapter 5 Fig. 2). Those results are also in agreement with other studies that have dosed males with pro-oxidant substances or antioxidant supplements, observing that ejaculate traits are either affected (pro-oxidants) or enhanced (antioxidants) by the experimental treatments (Chitra et al. 2003, Lacoume et al. 2009, Abarikwu et al. 2010, Helfenstein et al. 2010a, Almbro et al. 2011). It has been previously suggested that females could accrue both direct, coming from fertility assurance, and indirect, coming from good genes or sexy sperm, benefits from males that inseminate them with less oxidized sperm (Velando et al. 2008). Therefore, oxidative stress might play an important role in sexual selection, and thus we predicted oxidative stress to be an important constrain to how males allocate resources to either their pre- vs. post-copulatory traits.

Sperm competition models predict males to increase resource investment into post-copulatory traits as their costs to obtain mates increase (Tazzyman et al. 2009, Parker et al. 2013). Indirect evidence for these models comes from a study in Scorpionflies (*Panorpa cognate*), were a negative genetic correlation between attractiveness and mating investment was found (Engqvist 2011). However, direct evidence with the actual resources being traded-off between somatic and germline functioning is still lacking. Based on (1) the predictions of sperm competition models (See box 1 in the General introduction), (2) the adverse effects of oxidative stress on ejaculate traits (see general introduction), and (3) the different access to fertile females by House Sparrow males occupying different social ranks, I predicted that as males would be more subordinate they should increase their antioxidant investment into the production of good quality ejaculates. While I did not observed any differences in sperm design between males (Chapter 2), I found that dominant male house sparrows produced ejaculates with lower proportions of motile sperm (Chapter 1, Fig. 1), lower longevity (Chapter 1, Fig. 2A), lower curvilinear velocity (data not included in this dissertation) compared to subordinate males, and higher variation in sperm design (Chapter 3, Fig. 1A and 3A). Interestingly, sperm morphological design has been argued to affect sperm functioning in various ways (See introduction in Chapter 2), yet differences in sperm morphological design do not explain differences in ejaculate performance across social ranks (Chapter 2). However, differences across social ranks on the above-mentioned traits strongly suggest that males allocated resources differently into the control and production of good quality ejaculates. Further, I also found that ejaculates of dominant males were more oxidized than those of subordinate males (Chapter 1, Fig. 1), supporting my hypothesis that antioxidants resource being traded-off between somatic and germline functioning. While we do not have direct evidence that any of the ejaculate traits that I measured would provided males with a direct advantage in sperm competition, previous

studies have shown in that sperm velocity (Holt et al. 1997, Gage et al. 2004), proportion of motile sperm (Froman et al. 1999, García-González and Simmons 2005), and sperm longevity (Pizzari et al. 2008b) explain variation in paternities under sperm competition across a variety of taxa, from insects to mammals. Thus, I would expect that subordinate House Sparrows producing faster and more viable ejaculates would outcompete dominant males. It remains yet to be tested whether the measure ejaculate traits result in any fertilization advantage, by experimentally inoculating females with the ejaculates of two males and assigning paternities on the nests.

While my results support the theoretical predictions, they do not fully match them. I found that males at the lower end of the hierarchy produced ejaculates with speeds (data not shown in this dissertation), viabilities (Chapter 1, Fig. 1), and variation in sperm design (Chapter 3, Fig. 1 and 3) similar to the ejaculates of dominant males. There are various non-exclusive reasons why males at the bottom of the hierarchy produce ejaculates with low viability and high oxidation levels, similar to dominant males. First, two mathematical models showed that in cases where the risk of sperm competition (e.g. the probability that a given female copulates with another male) turns into intensity of sperm competition (e.g. the female copulates with more than 2 males) the pay-offs from high ejaculate expenditure decrease rapidly, and as a consequences males are expected to reduce ejaculate expenditure under situations where sperm intensity is high (Parker et al. 1996, Parker et al. 2013). Males at the lower end of the hierarchy protect the less their females from sexual harassment by other males (Møller 1987a, Møller 1990, 1992), and it could be thus hypothesized that they would not benefit of having high ejaculate expenditures. Problematically, the predictions of those models are for single mating events (e.g. how much males are expected to expend in a single ejaculate/copulation with a female) (Parker et al. 1996, Parker et al. 2013), and here we manually collected and pooled various ejaculates from each individual in order to understand general investment into ejaculate quality rather than expenditure into a single copulation event. Further, the cloacal massage impedes males from adjusting how much they would expend into their ejaculation. Furthermore, the most recent model by the same authors (Parker et al. 2013) predicts that resource investment into postcopulatory traits continues to increase with the intensity of sperm competition, despite of expected reductions in ejaculate expenditure. Thus, while we cannot fully rule out the possibility that males at the bottom end of the hierarchy reduce their investment into post-copulatory traits, opposed to the theoretical predictions, it is unlikely that the observed patterns are the result of reduced ejaculate expenditure due to higher intensity of sperm competition.

Two non-exclusive alternative explanations are the effects of increased allostatics overloads due to social interactions and the effects of intrinsic individual capacities to access and use resources. In species where social interactions are based on a hierarchical system, both dominant males, due to constant challenges by other males, and subordinate males, due to their inability to win in male-male interactions, are subject to increased allostatic overloads due to the glucocorticoid response (reviewed in (Goymann and Wingfield 2004)). For instance, higher levels of glucocorticoids are associated with increases in oxidative stress in various vertebrae taxa (Haussmann and Marchetto 2010, Costantini et al. 2011). Further, it has been shown that high levels of glucocorticoids can inhibit reproduction (Nelson 2011). Thus, it could be argued that the observed patterns of low sperm viability (Chapter 1, Fig. 1), slower curvilinear velocities (data not shown in this dissertation), higher sperm design variability (Chapter 3, Fig. 1A and 3A), and higher oxidative stress (Chapter 1, Fig. 1) for both dominant and males and the lower end of the hierarchy are the result of higher costs coming from

allostatic overloads. In House Sparrows it is not clear whether dominant or subordinate males suffer of higher allostatic loads from social interactions. For instance, the only three studies that have explored this have found that smaller dominant males have higher allostatic loads (Lindström et al. 2005), that subordinate males have higher allostatic loads (Buchanan et al. 2010), or both have the similar allostatic loads (Gonzalez et al. 2002). Therefore, it is difficult to infer from the literature whether the patterns of ejaculate quality and oxidative stress that we observed are the results of higher allostatic loads by a given social rank, and further experiments will be needed to determine the role of the glucocorticoid response in limiting strategic antioxidant antioxidant allocation to both pre- and post-copulatory traits.

Male ability to obtain and use resources can play a big role on how males allocate resources to different life history traits (van Noordwijk and de Jong 1986). I propose that limited resource access constrains to certain extend how males allocate resources between pre- and post-copulatory traits. That would explain why males at the bottom end of the hierarchy are unable to protect their ejaculates from oxidative stress (Chapter 5, Fig. 1). For instance, if variation in sperm design can be controlled by resource investment as I suggested in Chapter 3, the large amount of intra-ejaculate variation found in males could be explained by their inability to access resources and further invest them in spermatogenesis control. Moreover, if males have a lower control on their spermatogenesis, that would also explain why males at the lower end of the hierarchy produce ejaculates of lower quality. Further evidence that the intrinsic male ability to obtain and use resources can affect the extent to which they can invest in their ejaculates come from the manipulation with a pro-oxidant in Chapter 5, where the only dominant males were able to protect their ejaculates from the oxidative stress challenge (Chapter 5, Fig. 1). Dominant males for instance might have access to more resources than what they need and thus can spare resources in their ejaculates when needed (e.g. big houses and big cars hypothesis, (Reznick et al. 2000)), while subordinate males play in sperm competition games with a limited budged that does not allow them to further protect their ejaculates when they get an oxidative stress challenge (Chapter 5). To confirm whether subordinate males are resource constrained and dominant males have an excess of the resources that they can spare when needed, further studies should manipulate either the amount of resources that they can access or the quality of such resources.

Adaptive plasticity is the result of phenotypic changes that increase individuals' fitness for a given set of biotic and abiotic conditions (Via et al. 1995). Given that the fertilizing ability of an ejaculate largely determines male reproductive success (as reviewed both in the general introduction and above in the general discussion), it would be expected that males are able to change their ejaculate traits to meet the best pre- vs. post-copulatory investment strategy given the risks of sperm competition that they face. To test whether males could adapt their ejaculate traits to changes in their social environment, I mixed males across aviaries to force them into a new hierarchical position (see Materials and Methods appendix). For instance, previous studies have found that males can plastically change their ejaculate traits given the risk of sperm competition that they are facing (Cornwallis and Birkhead 2007), yet the physiological proximate mechanisms regulating such changes were yet to be uncovered. Given that I oxidative stress affects ejaculate quality (Chapters 1 and 5 and references on the General Introduction), I predicted that changes in ejaculate quality should be paralleled by changes in antioxidant allocation into the ejaculate. However, sperm morphology is under tight genetic control (Eddy 2002), and thus changes in sperm morphology are less likely to happen. Notwithstanding, previous studies have shown that sperm morphology

can respond to changes in the pressure of post-copulatory selection. For example, a study on Gouldian Finches (*Erythrura gouldiae*) found that males change their sperm morphological design depending on the social environment (amount of aggressive males in the group)(Immler et al. 2010). Yet, we did not observe any change in sperm morphology (see Chapter 2), which was expected given that social rank could not explain differences across males in sperm morphological design nor morphology explained sperm performance. Interestingly, after manipulating the social environment I found that males matched their proportion of motile sperm to their new social ranks (Chapter 1, Fig. 3), and increases in sperm viability were paralleled by increases in antioxidant allocation into the ejaculate (Chapter 1, Fig. 3) as predicted. These results strongly suggest that changes in ejaculate quality are modulated by strategic antioxidant allocation into post-copulatory traits. Moreover, I also observed that males changed the amount of within-ejaculate variation in sperm design to match their new hierarchical position (Chapter 3, Fig. 3), further supporting the idea that strategic investment into the sperm production control is the likely mechanism regulating post-copulatory traits. Future studies should validate these results in the wild, where male groups are larger and social hierarchies might be less linear. Further, investment in pre-copulatory traits (e.g. badge size) did not predict the social hierarchy established on male-male interactions, and thus I predict that in promiscuous species where large pre-copulatory investment is required to acquire females, a more pronounced evidence for the oxidation based soma vs. germline hypothesis will be found.

Oxidative stress is an unavoidable physiological cost (Finkel and Holbrook 2000, Halliwell and Gutteridge 2007, Hulbert et al. 2007), and it is hypothesized to be a main constraint of life-history evolution (Dowling and Simmons 2009, Monaghan et al. 2009, Metcalfe and Alonso-Alvarez 2010). Further, oxidative stress has been shown to be a proximate cost of reproduction (Wang et al. 2001, Alonso-Alvarez et al. 2004). To my knowledge, I show for the first time the likely physiological mechanism involved in the soma vs. germline trade-off. Given the universality of oxidative stress (Dowling and Simmons 2009) and that it is a major cause of infertility (Aitken 1999, Tremellen 2008), I predict that strategic allocation of antioxidant resources is a more general proximate physiological mechanism to enhance ejaculate quality under sperm competition. Yet, it remains to be further explored how other social-related physiological costs (e.g. allostasis) interplay with oxidative stress to shape males' reproductive strategies. Importantly, my results stress the importance of considering the ability of individuals to monopolize resources, which can largely affect how such resources will be traded-off between different life history traits (van Noordwijk and de Jong 1986). Therefore, it is imperative to measure and manipulate how much resources are being acquired by males at different social ranks, and hence get a better understanding of how resource acquisition can limit investment between soma and germline. As a consequence, further theoretical models should also consider a minimum budged of resources for body maintenance, which is likely to limit expenditure in ejaculate quality on resource-constrained males. Finally but none the less, experimental inseminations are required to better understand which ejaculate traits are important for male fertility in House Sparrows, and thus be able to better understand the selective pressures on post-copulatory traits in this species.

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Materials and Methods Appendix

Ejaculate quality assessment (Chapters 1-4)

I obtained ejaculates (ca. 2.5 µL) by gently massaging male's cloaca (Wolfson 1952). Right after collecting the ejaculates, 0.2 µL of sperm were dissolved into 40 µL of pre-warmed DMEM (Dulbecco modified eagle medium; Sigma Aldrich, USA), and then transferred to a 20 µm depth chamber slide (Leja Products B.V., The Netherlands). I video recorded sperm for 75 seconds at 13 frames per second using an Toshiba CMOS HD camera (TOSHIBA Corporation, Japan) on an Olympus BX43 microscope (Olympus Co., Japan) at 100x magnification under a phase contrast 3, while temperature was maintained at 40°C using a heating plate on the microscope (Minitube HT200 W, MINITÜB GmbH, Germany). The time between the ejaculate collection and the start of the video was recorded. I also collected a subsample of 1 µl of sperm into 9 µL of PBS for MDA assessment, and I stored it at -80°C.

I estimated curvilinear velocity (VCL), average path velocity (VAP), straight-line velocity (VSL), and proportion of swimming sperm from 4 video segments of 2.5 seconds at 0 (hereafter referred to as "initial swimming speed" and "initial proportion of motile sperm"), 15, 30, 45, 60 and 90 seconds after recording started, using a Computer Assisted Sperm Analyser plug-in (Wilson-Leedy and Ingermann 2007) for ImageJ (Schneider et al. 2012). Due to technical problems, the first 2 seconds of video were discarded. A preliminary analysis showed that 2.5 seconds segments maximized the number of sperm tracks detected, while avoiding inflating the percentage of motile sperm due to sperm cells entering the recording field. The minimum and maximum size for a sperm cell were set to 70 and 100 pixels respectively. The minimum tract time was set to 1.31 seconds, and the search radius to 35 pixels. Such settings minimized the risk of counting sperm cells that would enter into the filming area for a short period, while avoiding merging trajectories of crossing sperm into a single track. Sperm cells having a VSL<5 µm/s, a VCL<15 µm/s, or a VAP<10 µm/s were counted as immotile. From the measurements, I obtained the initial sperm swimming speed, sperm swimming endurance (rate at which initial swimming speed decreases through time), and mortality (rate at which initial proportion of swimming sperm decreases through time). Sperm endurance and sperm mortality were derived as individual slopes from a mixed model including curvilinear velocity VCL or the proportion of motile sperm, respectively, as dependent variables, and time (0, 15, 30, 45, 60 and 90 seconds) as a continuous independent variable, with individual identity in interaction with time as a random factor.

Oxidative stress and antioxidant defences

Lipid peroxidation (Chapters 1 and 4)

I assessed the amount of lipid peroxidation by determining the circulating levels of malondialdehyde (MDA) in plasma, erythrocytes, and sperm. Concentrations of MDA, formed by the β-scission of peroxidised fatty acids, were assessed using UHPLC with fluorescence detection, following Moselhy et al. (Moselhy et al. 2013) and Agarwal et al. (Agarwal and Chase 2002) with modifications. All chemicals were of analytical or HPLC grade, and chemical solutions were prepared using ultra pure water (Milli-Q Synthesis; Millipore Corporation, Billerica, MA, USA). To a (plasma or RBC / sperm) 5/8 µl aliquot of sample (0.8 µL of sperm in 7.2 µL of PBS; 0.5 µL of erythrocytes in 4.5 µL of PBS; 5µL of plasma) or standard (1,1,3,3-tetraethoxypropane, TEP; Sigma

Aldrich, USA), 155/152 µl of water, 40 µl of trichloroacetic acid 5% (TCA), and 20 µl thiobarbituric acid (TBA; Sigma Aldrich, USA) solution (42 mM) were added in a 1.5 mL tube. Samples were then vortex mixed for 5 seconds and centrifuged for 14 minutes at 14000 rpm and 4°C. 205 µL of the epiphase was transferred to 2 ml capacity conical-bottom screw-top microcentrifuge tubes, vortex mixed for 5 seconds, then heated at 100°C for exactly 1 hour in a dry bath incubator to allow formation of MDA-$(TBA)_2$ adducts. Samples were then cooled on ice for 5 minutes, and 150 µL of butanol (Sigma Aldrich, USA) were added. Then, the tubes were vortex mixed for 10 seconds and centrifuged for 10 minutes at 14000 rpm and 4°C. Then, 120 µL of the epiphase was recovered in 1.5 mL tubes. A second extraction of the derivatised solution was done by adding 150 µL of butanol, vortex mixed for 10 seconds and centrifuged for 10 minutes at 14000 rpm and 4°C. Then, 140 µL of the epiphase was pooled with the previously recovered epiphase, and then evaporated in a SpeedVac for 60 minutes at 35ºC. Finally, the product was re-suspended in 90 µL of methanol 30% (Sigma Aldrich, USA), and the tubes were sonicated for 5 seconds and then vortex mixed for 10 seconds. A 70 µl aliquot was collected and transferred to an HPLC vial for analysis. Samples (5 µl) were injected into a Dionex Ultimate 3000 Rapid Separation LC system (Dionex Corporation, California, USA) fitted with a Waters (Milford Massachusetts, USA) Acquity UPLC® BEH C18 column (1.7µm, 2.1 x 50 mm) maintained at 30°C. Separation was achieved using gradient elution at a flow rate of 0.4 ml/min with solvent A being 0.05% acetic acid buffered at pH 6 with ammonium hydroxide and solvent B acetonitrile. The gradient was as follows: linear increase from 5% to 100% solvent B over 5 min, followed by 100% solvent B for 1.5 min and re-equilibration at initial conditions (5% B) for 3.2 min. The total analysis time was 9.7 min. The auto-sampler syringe was washed with 700 µl of solvent B after each injection. Data were acquired using a fluorescence detector set at 515 nm (excitation) and 553 nm (emission). For calibration, a standard curve was prepared using a TEP stock solution (5 µM in 40% ethanol) serially diluted using pure water. All the samples were processed blindly of both the identity of the individual and social rank.

Lipid peroxidation (Chapter 5)

I assessed the amount of lipid peroxidation by determining the circulating levels of malondialdehyde (MDA) in plasma, erythrocytes, and sperm. Concentrations of MDA, formed by the β-scission of peroxidized fatty acids, were assessed using UHPLC with fluorescence detection, following (Agarwal and Chase 2002) with modifications. All chemicals were of analytical or HPLC grade, and chemical solutions were prepared using ultra pure water (Milli-Q Synthesis; Millipore Corporation, Billerica, MA, USA). To a 5 µl aliquot of sample (plasma or RBC / sperm) or standard (1,1,3,3-tetraethoxypropane, TEP; Sigma Aldrich, USA), 5 µl butylated hydroxytoluene (BHT) solution (0.05% w/v in 95 % ethanol), 40 µl of phosphoric acid (0.44 M), and 10 µl thiobarbituric acid (TBA; Sigma Aldrich, USA) solution (42 mM) were added in a 1.5 mL screw-top tube. Samples were then heated at 100°C for exactly 1 hour in a dry bath incubator to allow formation of MDA-$(TBA)_2$ adducts. Samples were then cooled on ice for 5 minutes, and 100 µL of n-butanol (Sigma Aldrich, USA) were added. The tubes were vortex mixed for 20 seconds and centrifuged for 4 minutes at 13000 rpm and 4°C. Then, 70 µL of the epiphase were transferred to an HPLC vial for analysis. Samples (5 µl) were injected into a Dionex Ultimate 3000 Rapid Separation LC system (Dionex Corporation, California, USA) fitted with a GL Sciences Inc. (Tokyo, Japan) Inerstil 2µ ODS-4 2.1 x 100 mm column maintained at 37°C. The mobile phase was methanol-buffer (30:70, v/v), the buffer being a 50mM anhydrous solution of potassium

Table S1. Analyte-dependent mass spectrometry parameters. DP, declustering potential, CE, collision energy, CXP, collision cell exit potential.

Analyte	Transitions	DP (V)	CE (eV)	CXP (V)
GSH	308/162	51	25	14
	308/179	51	27	14
	308/84	51	45	10
GSSG	307/130	46	29	10
	307/84	46	35	10
GSEE	336/207	56	19	18
	336/190	56	23	16

monobasic phosphate at pH 6.8 (adjusted using 5M potassium hydroxide solution), running isocratically over 6 min at a flow rate of 0.3 $ml.min^{-1}$. Retention time was 2.15 min. Data were collected using a fluorescence detector set at 515 nm (excitation) and 553 nm (emission). For calibration, a standard curve was prepared using a TEP stock solution (5 µM in 40% ethanol) serially diluted using 40% ethanol. TEP standards were assayed in triplicate and showed high repeatability ($r = 0.996$, $P < 0.0001$, $n = 13$). The repeatability was also high for a subset of plasma samples ($r = 0.90$, $P < 0.0001$, $n = 12$). All the samples were processed blindly of both the identity of the individual and social rank.

Superoxide dismutase activity

I determined superoxide dismutase activity perm ml of tissue in sperm and blood Cayman Chemical, (USA) commercial kit with minor modifications. Specifically, with used dilution of 1:400 for erythrocytes and 1:160 for sperm. Moreover, all the samples were done in duplicate and the %CV was maintained bellow 15% (average %CV: 10.7% for sperm and 9.9% for erythrocytes). All the samples were processed blindly of both the identity of the individual and social rank.

Glutathione concentrations

I determined the levels of glutathione, an intracellular antioxidant in both its reduced (GSH) and oxidized (GSSG) forms, both in sperm and red blood cells following a modified protocol based on Bouligand et al. (Bouligand et al. 2006). I mixed (sperm/RBC) 3/10 µL of aliquot (0.3µL sperm in 2.7 µL of PBS; 1µL of erythrocytes in 9 µL of PBS), 5/25 µL of TCA 5%, 5µL of glutathione ethyl ester (GSHee; Sigma Aldrich, USA) as internal standard (2.25 µg/mL or 20 µg/mL respectively), and 62/0 µL of pure water in a 1.5 mL tube. Then, the solutions were vortex mixed, kept ice-cold for 5 minutes, and centrifuged for 14 minutes at 140000 rpm and 4ºC. After centrifugation, 50/20 µL of the epiphase were diluted in 100/980 µL of water and then 100 µL of the dilution was transferred into glass HPLC vials. Samples (5 µl) were injected on a Dionex Ultimate 3000 Rapid Separation LC system (Dionex Corporation, California, USA) coupled to a 4000 QTRAP mass spectrometer (Sciex, Toronto, Canada) equipped with a Turbo V source. A Waters Acquity UPLC® BEH HSS T3 column (1.8µm, 2.1 x 100 mm) was employed at a flow rate of 0.4 ml/min using the following solvent system: solvent A = milli-Q H_2O with 0.05% formic acid, solvent B = acetonitrile with 0.05% formic acid. The gradient was as follows: 0-20% B in 2 min, 20-100% B in 3 min, 100% B for 3 min, back to 0% B for 5 min. Total analysis time was 13 min. Mass spectrometry detection was achieved using multiple reaction monitoring (MRM) transitions in positive ionization mode (Table S1). A dwell time of 50 ms was applied for all transitions. Ion source parameters were as follows: capillary voltage 5.5 kV, nebulizing gas (GS1) 45 psi, drying gas (GS2) 25 psi at 550°C, curtain gas (CUR)

15 psi. For quantification, a standard curve containing GSH (Sigma Aldrich, USA) and GSSG (Sigma Aldrich, USA) at 0.002, 0.02, 0.1, 0.5, and 2 µg/mL and GSHee at a constant concentration of 0.05 µg/mL was made. All the samples were processed blindly of both the identity of the individual and social rank.

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