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RESEARCH ARTICLE

Oxidative stress affects sperm performance and ejaculate redox status in subordinate house sparrows

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ABSTRACT

Oxidative stress is the result of random cellular damage caused by reactive oxygen species that leads to cell death, ageing or illness. Most physiological processes can result in oxidative stress, which in turn has been identified as a major cause of infertility. In promiscuous species, the fertilizing ability of the ejaculate partly determines the male reproductive success. When dominance determines access to fertile females, theory predicts that lower ranking males should increase resource investment into enhancing ejaculate quality. We hypothesized that subordinate males should thus prioritize antioxidant protection of their ejaculates to protect them from oxidative stress. We put this hypothesis to the test by chronically dosing wild house sparrows with diquat (~1 mg kg^{-1}), a herbicide that increases pro-oxidant generation. We found that, although they increased their antioxidant levels in the ejaculate, diquat-treated males produced sperm with reduced velocity. Importantly, and contrary to our hypothesis, males at the bottom of the hierarchy suffered the largest reduction in sperm velocity. We suggest that resource access hinders individuals' ability to cope with environmental hazards. Our results point at oxidative stress as a likely physiological mechanism mediating ejaculate quality, while individual ability to access resources may play a role in constraining the extent to which such resources can be allocated into the ejaculate.

KEY WORDS: Social dominance, Soma/germline trade-off, Sperm competition, Sperm velocity, Pollutants

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. gene expression, immune response, etc.), in many instances antioxidants cannot quench all the ROS, resulting in ROS-induced damage known as oxidative stress (OS) (Jones, 2006). 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) are common factors responsible for an increase in OS. Given that all organisms are subject to attacks by ROS, OS is hypothesized to be a main constraint to life history evolution (Dowling and Simmons, 2009; Metcalfe and Alonso-Alvarez, 2010; Monaghan et al., 2009).

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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), making sperm cells particularly vulnerable to OS. Oxidative damage to the sperm membranes leads to impaired cell–egg interactions, reduced ejaculate quality, and subfertility 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 (Aitken and Krausz, 2001; Kemal Duru et al., 2000; Lloyd et al., 1997; Wellejus et al., 2000; Zitzmann et al., 2003), which renders sperm be unable to fertilize the ovum. For the above-mentioned reasons, OS is considered as one of the main causes of male infertility (Aitken and Baker, 2004; Tremellen, 2008).

Competition for the fertilization of 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 male reproductive success (Birkhead and Pizzari, 2002; Fitzpatrick and Lüpold, 2014; Simmons and Fitzpatrick, 2012; Snook, 2005). Ejaculate traits that confer a fertilizing advantage on males are referred to as ejaculate quality (reviewed in Snook, 2005), and among them sperm velocity (e.g. Gage et al., 2004; Holt et al., 1997), viability (e.g. Froman et al., 1999; García-González and Simmons, 2005) and longevity (e.g. Pizzari et al., 2008) have been found to be important determinants of paternity. Theoretical models have explored how males may optimize the trade-off between investing in pre-copulatory traits to enhance mating success and investing in post-copulatory traits that enhance the fertilizing ability of their ejaculate. Such models have predicted a negative correlation between mating success (e.g. favoured versus disfavoured males) and ejaculate quality (Parker, 1998; Parker and Pizzari, 2010). The predictions of those models have been tested in several taxa (e.g. Cornwallis and Birkhead, 2007; Evans, 2010; Lemaître et al., 2012; Preston et al., 2001; Rudolfsen et al., 2006; Thomas and Simmons, 2009), although these studies analysed only discontinuous roles (e.g. sneaker versus territorial, dominant versus subordinate). More recent models predict a continuous increase in resource investment into ejaculate quality as mating opportunities decrease – or mating costs increase (Parker et al., 2013; Tazzyman et al., 2009) – resulting in a continuous soma versus germline resource allocation trade-off (see fig. 2B in Parker et al., 2013). Some support for these models comes from a study by Engqvist (2011), who found negative genetic co-variation between attractiveness and mating investment in scorpionflies *Panorpa cognata*.

OS and/or antioxidant availability have also been observed to affect ejaculate traits in various organisms (e.g. Almbro et al., 2011; Bréque et al., 2003; Chitra et al., 2003; Ciereszko and Dabrowski, 1995; Helfenstein et al., 2010; Mitre et al., 2004). Therefore, it

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List of abbreviations

GSH	reduced glutathione
GSSG	oxidized glutathione
MDA	malondialdehyde
OS	oxidative stress
PUFA	polyunsaturated fatty acid
ROS	reactive oxygen species
SOD	superoxide dismutase
VCL	curvilinear velocity

could be hypothesized that the more males are disfavoured by females and thus the higher the level of sperm competition they face, the more antioxidant resources they should invest in the production of high quality ejaculates, i.e. the oxidation-based soma versus germline allocation trade-off hypothesis. House sparrows, *Passer domesticus*, are socially monogamous passerines exhibiting from 12% to 15% extra-pair paternity (Møller, 1987; Møller and Birkhead, 1994; Wetton et al., 1995; Wetton and Parkin, 1991), and where dominance hierarchy is linked to mate guarding intensity, copulation rate and body condition (Anderson, 2006). In a previous study, we found that subordinate male house sparrows produced less-oxidized and better quality ejaculates than dominant males, and that variation in antioxidant allocation paralleled adjustments in ejaculate quality after an experimental manipulation of the males' social ranks (Rojas Mora, 2016). Thus, strategic allocation of antioxidant resources between somatic functions (e.g. ability to monopolize resources, aggressiveness, etc.) and germline functions (e.g. ejaculate quality, DNA integrity) might underlie such rank-related differences in ejaculate oxidative status and quality.

In their natural environment, individuals may be subjected to increased levels of ROS, e.g. through reproductive costs (Alonso-Alvarez et al., 2004; Wang et al., 2001), and/or exposure to pollutants (Banerjee et al., 2001; Li et al., 2003). Here, we tested whether exposure to a pro-oxidant contaminant would change rank-related differences in antioxidant allocation and 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. The use and appropriate dosage of diquat were determined in a pilot where males were dosed with either diquat or paraquat, as the two herbicides exploit the same pathway to increase pro-oxidant molecules (Bus and Gibson, 1984; Saeed et al., 2001). Dominant males can face different physiological costs from subordinate males (Goymann and Wingfield, 2004; Sapolsky, 2005), which can result in higher OS for dominant males (e.g. higher OS susceptibility in *Acrocephalus sechellensis* dominant males during the reproductive season; van de Crommenacker et al., 2011). Thus, under an oxidative challenge, we predicted that subordinate males would prioritize the antioxidant protection of their ejaculate at the expense of their soma, thus enhancing their paternities through sperm competition.

MATERIALS AND METHODS

Pilot study

In order to manipulate the levels of oxidative damage in male house sparrows *Passer domesticus* (Linnaeus 1758), we exposed the study individuals to two potent pro-oxidants: diquat and paraquat (Koch and Hill, 2017). These compounds are non-selective herbicides that enter into a cycling reaction with, among others, nicotinamide adenine dinucleotide (NAD^+/NADH) and oxygen molecules that are broken into singlet oxygen (Koch and Hill, 2017). However, while we were interested in the potential effects of OS on sperm traits, it is worth noting that both paraquat- and diquat-induced ROS can also pleiotropically affect several physiological traits (Adachi et al., 2003; Kimura et al., 2007; Shibata et al., 2010). Paraquat and diquat are broadly used under the brand names Gramoxone® (Paraquat, Syngenta AG, Basel, Switzerland) and Reglone® (Diquat 20%, Syngenta AG), 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: 1.5 m, width: 1.5 m, height: 2.5 m). The birds were then left to acclimatize to the aviary conditions for 1 week. After acclimatization, we orally dosed individuals using a 1 ml syringe with 0.1 ml of paraquat (5, 10, 15 or 20 mg kg^{-1} in 0.8% NaCl), diquat (0.5, 1.0, 1.5 or 2.0 mg kg^{-1} in 0.1 ml of 0.8% NaCl) or physiological saline (0.8% NaCl) every second day for 2 weeks. Treatment doses were administered with the bird's beak in an upward position by introducing the tip of the syringe in the throat, avoiding any harm on the soft tissues. After administering the treatment, the birds were held with their beak upwards for a couple of seconds until we observed swallowing. The doses were based on previous studies, which used these substances to generate OS insults (Alonso-Alvarez and Galván, 2011; Galván and Alonso-Alvarez, 2009; Isaksson and Andersson, 2008). We assigned two aviaries to each of the nine 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 min. We determined the levels of malondialdehyde (MDA) from the plasma and erythrocyte samples following standard procedures using derivatization with thiobarbituric acid followed by ultra-high-performance 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 (Fig. S1A; dose×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 (Fig. S1B; dose: $F_{4,4.8}$=5.22, P=0.052; dose×time: $F_{12,42.7}$=1.50, P=0.16). Additionally, the birds experienced very adverse effects when treated with 2.0 mg kg^{-1} of diquat (e.g. sudden loss of roughly 10% of their body mass accompanied by crop fibrosis that caused the death of two out of four individuals in that treatment after 2–3 doses), and the treatment was stopped after the death of the first individual in this treatment. Individuals on the other treatments did not show any evident health problems at release, and some of them were found to be in good condition during the bird captures for the main experiment. Based on the fact that the doses of diquat necessary to cause lipid peroxidation were tenfold less than those of paraquat – and hence were more likely to be ingested in agricultural environments – and that a dose of 1.0 mg kg^{-1} of diquat caused a large increase in plasma MDA (Fig. S1), we decided to use a 1.0 mg kg^{-1} 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 during early May 2013 in Ariège, France. We measured body mass and tarsus length of each individual, and then transferred the birds into 18 mixed outdoor aviaries (1.5 m×4 m×3.5 m) at the CRNS Experimental Ecology Station (Moulis, France). Individuals

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were given coloured rings with unique within-aviary combinations. Three males and three 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 g 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 the good health of all individuals before starting the experiment.

Experimental design

After acclimation, we split males and females into 27 unisex aviaries (18 male and 11 female aviaries) each containing three males or three females. Aviary disposition was arranged so that males had visual and acoustic contact with females (i.e. each male aviary was adjacent to a female aviary), and males remained in the aviaries where they had become acclimated. We maintained only 33 females during the experiment, and the other 21 females were released. Birds were given a controlled amount of food on a single feeding plate (~60 g per aviary per day of the mix used during the acclimation period) and water *ad libitum*. On the first day of the experiment, we used a 1 ml syringe to orally dose males in nine of the aviaries with 0.1 ml of physiological saline (0.8% NaCl) containing 0.024 mg of diquat (~1 mg kg^{-1}), while the males of the other nine aviaries were given 0.1 ml of physiological saline (0.8% NaCl) alone. Oral doses were administered as described in the pilot study. We repeated the 0.1 ml oral dose of either diquat or physiological saline every second day for a total of 18 days (Fig. 1). For logistical reasons, the sampling was divided into two batches that were processed 1 day apart. After the 18 day experiment, we released all the individuals at their original locality.

Behavioural observations

To assess the male hierarchy in each aviary, we recorded antagonistic interactions between individuals. Males were given a single feeding station consisting of a small plastic plate placed over a large plastic plate with a plastic mesh, and thus any spilt seeds were inaccessible. During the acclimation period, we observed each aviary for 1 h, three times a week. Additionally, every second morning we filmed each aviary for 1 h right after feeding the birds (at ca. 06:00 h GMT+1) using a GoPro® camera mounted on the door of the aviary. From both the direct observations and the videos, we counted the number of encounters between individuals, and assigned the bird that would retreat after being aggressed or challenged as the loser. Using this information, we estimated a David's score (Gammell et al., 2003) to assign the higher ranked males as dominant, middle ranked males as subordinate-1 and lower ranked males as subordinate-2. These ranks were used as the hierarchical position of each individual in its aviary.

Blood samples

We obtained blood samples every 6 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, Sarstedt, Nümbrecht, Germany). An initial sample was taken on the first day of the experiment before males received their first treatment dose (Fig. 1). 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

We collected sperm samples every second day for each individual, and the first sample was obtained on the day the birds received the first treatment dose (Fig. 1). Ejaculates (ca. 2 µl for 2–3 pooled ejaculates) were obtained by gently massaging the male's cloaca (Wolfson, 1952), and immediately collected into 5 µl capillary tubes (intraMark 5 µl, Blaubrand®). We noted the time elapsed between the collection of the ejaculate and the start of the video. However, if after 5–7 min of massage an ejaculate was not obtained, we assumed the bird to be sperm depleted. For such cases, we confirmed whether males were still producing sperm by visual inspection under a microscope of a faecal sample collected at the end of the cloacal massage. Following the collection of ejaculates, 0.2 µl of sperm was dissolved into 40 µl of pre-warmed DMEM (Dulbecco's modified Eagle's medium, 4500 mg l^{-1} glucose, 110 mg l^{-1} sodium pyruvate and L-glutamine), and then transferred to a 20 µm-deep chamber slide (Leja Products B.V., Nieuw-Vennep, The Netherlands). We video recorded sperm for 75 s at 16 frames s^{-1} using an Olympus SC100 camera (Olympus Co., Tokyo, Japan) fitted on an Olympus BX43 microscope (Olympus Co.) at 100× magnification under dark-field conditions generated by a phase-3 annular ring. Temperature was maintained at 40°C using a heating plate on the microscope (Minitube HT 200, Tiefenbach, Germany). A subsample of 1 µl of sperm was collected into 9 µl of PBS and stored at −80°C for later assessment of MDA concentration, activity of superoxide dismutase (SOD) and concentrations of reduced and oxidized glutathione (GSH and GSSG).

From each sample, we estimated curvilinear velocity (total point-to-point distance travelled by the sperm over the time period analysed averaged to a per second value, µm s^{-1}) and the proportion of swimming sperm from four video segments of 2.5 s (at 0, 20, 40 and 60 s after recording started) using a Computer Assisted Sperm Analyser plug-in (Wilson-Leedy and Ingermann, 2007) for ImageJ (Schneider et al., 2012). This allowed us to determine sperm stamina (the rate at which sperm swimming speed decreases over time) and sperm longevity (the rate at which the proportion of swimming sperm decreases through time) in each ejaculate by modelling both the proportion of motile sperm and sperm velocity using a linear mixed model with random slopes and

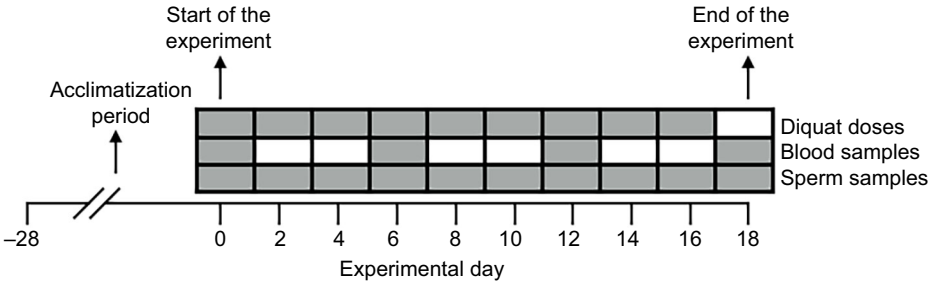

Fig. 1. Diagram of the experimental plan used for the current study. Shaded cells correspond to a manipulation on the given day. All birds were released the day after the end of the experiment at their original locations.

intercepts for each sample, while controlling for the time elapsed before the start of the video.

Biomarkers of OS and antioxidant defences

Lipid peroxidation

MDAs are end products of lipid peroxidation that reflect the amount of lipid damage due to ROS attacks (Halliwell and Gutteridge, 2007; Monaghan et al., 2009). We assessed the amount of lipid peroxidation by determining the circulating levels of 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 red blood cells/sperm) or standard [1,1,3,3-tetraethoxypropane (TEP), Sigma-Aldrich, St Louis, MO, USA], 5 µl butylated hydroxytoluene (BHT) solution (0.05% w/v in 95% ethanol), 40 µl of phosphoric acid (0.44 mol l^{-1}) and 10 µl thiobarbituric acid (TBA; Sigma-Aldrich) solution (42 mmol l^{-1}) were added in a 1.5 ml screw-top tube. Samples were then heated at 100°C for exactly 1 h in a dry bath incubator to allow formation of MDA-$(TBA)_2$ adducts. Samples were then cooled on ice for 5 min, and 100 µl of *n*-butanol (Sigma-Aldrich) was added. The tubes were vortexed for 20 s and centrifuged for 4 min at 13,000 rpm and 4°C. Then, 70 µl of the epiphase was transferred to an HPLC vial for analysis. Samples (5 µl) were injected into a Dionex Ultimate 3000 Rapid Separation LC system (Sunnyvale, CA, USA) fitted with a GL Sciences Inc. (Tokyo, Japan) Inerstil 2µ ODS-4 2.1×100 mm column maintained at 37°C. The mobile phase was methanol-buffer (30:70, v/v), the buffer being a 50 mmol l^{-1} anhydrous solution of potassium monobasic phosphate at pH 6.8 (adjusted using 5 mol l^{-1} 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 µmol l^{-1} 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 blind to both the identity of the individual and social rank.

SOD activity

SOD is an endogenous enzymatic antioxidant, which catalyses the dismutation of superoxide anions into hydrogen peroxide (Halliwell and Gutteridge, 2007). We determined superoxide dismutase activity per ml of tissue in both sperm and red blood cells using a commercial kit with minor modifications (Cayman Chemical, Ann Arbor, MI, USA). We used a dilution of 1:400 for erythrocytes and 1:160 for sperm, and then followed the standard procedures of the kit. All the samples were run in duplicate while ensuring that the coefficient of variation (CV) was maintained bellow 15% (average %CV: 8.67% for sperm and 5.09% for erythrocytes). All the samples were processed blind to both the identity of the individual and social rank.

Glutathione

GSH is an endogenous intracellular tripeptide, 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 ratio of reduced glutathione to oxidized glutathione (GSH:GSSG) provides accurate information about 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 (for details, see Rojas Mora et al., 2016).

Statistical analyses

We performed all analyses using R v.3.1.1 (http://www.R-project.org/). We ran linear mixed models with restricted maximum likelihood for parameter estimation using the R package lme4 (Bates et al., 2014 preprint), and analyses of variance were assessed using a Kenward–Roger approximation for the degrees of freedom using the R package lmerTest (https://CRAN.R-project.org/package=lmerTest) with α (type I error) set at 0.05. The identity of each individual, the sampling date and the aviary in which each individual was housed were included as random intercepts, while a random slope was used 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-sine 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. Apart from removing these two covariates, we did not perform further model selection on the main effects to reduce the risk of type I errors associated with multiple testing during model selection (Forstmeier et al., 2016).

We cannot rule out type I errors with *P*-values either below or just above 0.05; therefore, we cautiously present and discuss *P*-values above such a cut-off.

RESULTS

Ejaculate quality

In total, we recovered 387 ejaculates (dominant=123, subordinate-1=138, subordinate-2=126) from 45 males in 18 days, and we were unable to get any sperm samples from nine males (three dominants, two subordinate-1 and four subordinate-2) with very small cloacal protuberances (44.0±17.5 mm^3, mean±s.d.) compared with males from which we obtained sperm samples (107.2±57.5 mm^3, mean±s.d.). First, we tested whether any differences between groups were present on the day that males received their first treatment dose, and confirmed that none of the traits differed between diquat or control males (sperm velocity: $F_{1,14.4}$=1.62, P=0.22; sperm longevity: $F_{1,11.6}$=0.003, P=0.96; proportion of motile sperm: $F_{1,11.6}$=0.85, P=0.38; sperm stamina: $F_{1,12.3}$=0.16, P=0.69) and social ranks (sperm velocity: $F_{2,16.4}$=1.01, P=0.39; sperm longevity: $F_{2,20.6}$=0.54, P=0.59; proportion of motile sperm: $F_{2,20.6}$=1.21, P=0.32; sperm stamina: $F_{2,16.2}$=1.47, P=0.26) before the treatment was applied.

We found that ejaculate speed was reduced in birds chronically dosed with diquat ($F_{1,19.5}$=5.31, P=0.032; means±s.d.: control 51.17±12.57 µm s^{-1}, diquat 45.76±12.77 µm s^{-1}). We did not find any effect of diquat treatment on the proportion of motile sperm, sperm longevity or sperm stamina (Table 1); similarly, males of different hierarchical ranks did not differ in the proportion of motile sperm, longevity or stamina (Table 1). Nevertheless, we observed that males of different ranks differed in their ejaculate velocity (Fig. 2A–C, Table 1; means±s.d.: dominant 51.4±13.2 µm s^{-1}, subordinate-1 45.3±12.2 µm s^{-1}, subordinate-2 49.4±12.7 µm s^{-1}).

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Table 1. Linear mixed models for measurements of ejaculate quality fitted with a restricted maximum likelihood and a Kenward–Roger approximation for the degrees of freedom

	VCL				Proportion of motile sperm				Sperm longevity				Sperm endurance			
Fixed effects	Estimate±s.d.	*F*	d.f.	*P*	Estimate±s.d.	*F*	d.f.	*P*	Estimate±s.d.	*F*	d.f.	*P*	Estimate±s.d.	*F*	d.f.	*P*
Intercept	50.88±3.57				0.253±0.267				0.26±0.03				11.15±4.21			
Treatment	−0.37±4.41	5.3	1,19.5	**0.032**	−0.227±0.256	2.1	1,21.6	0.17	0.002±0.041	0.06	1,32.9	0.8	4.74±5.08	0.03	1,23	0.86
Rank		3.6	2,35.8	**0.037**		2.8	2,31.6	*0.077*		2	2,36.4	0.15		0.2	2,28.2	0.81
S1	−0.92±3.44				−0.195±0.226				−0.0586±0.0404				3.35±4.14			
S2	8.1±3.73				−0.002±0.243				−0.0332±0.0424				2.94±4.5			
Day	0.09±0.28	1	1,16.5	0.33	0.041±0.029	3.6	1,16.9	*0.077*	0±0.0024	2.8	1,16.4	0.11	0.55±0.44	0.96	1,16.6	0.34
Day^2	−0.04±0.06	2.3	1,16.6	0.14	−0.003±0.006	2.3	1,17.1	0.15	−0.0004±0.0006	1.8	1,19.8	0.20	−0.1±0.09	1.8	1,17.4	0.20
Treatment×rank		3.3	2,35.8	**0.047**		0.6	2,31.6	0.56		0.03	2,36.4	0.97		1.1	2,28.2	0.33
Diquat×S1	−8.17±5.01				−0.189±0.331				0.0002±0.0582				−6.67±6.11			
Diquat×S2	−13.42±5.19				0.169±0.341				0.0127±0.0595				−9.37±6.33			
Treatment×day	0.28±0.3	0.1	1,302.2	0.78	−0.006±0.026	0.3	1,305.9	0.6	−0.0026±0.0031	0.1	1,308	0.72	−0.1±0.51	1.6	1,309	0.20
Treatment×day^2	−0.03±0.06	2.2	1,36.8	0.15	−0.003±0.005	0.2	1,37.3	0.66	−0.0008±0.0007	0.001	1,43.7	0.97	0.03±0.11	0.4	1,36	0.55
Rank×day		0.2	2,301.4	0.78		0.4	2,304.7	0.68		0.5	2,308	0.63		0.8	2,307.2	0.47
S1	0.14±0.28				−0.004±0.024				−0.004±0.003				−0.16±0.48			
S2	0.14±0.29				0.005±0.025				−0.0039±0.0031				−0.06±0.5			
Rank×day^2		0.2	2,36.3	0.80		1.4	2,36.8	0.27		3.5	2,43.5	**0.041**		0.4	2,35.5	0.64
S1	−0.07±0.06				−0.007±0.005				0.0007±0.0007				−0.03±0.1			
S2	−0.08±0.06				−0.007±0.005				−0.0007±0.0007				0.05±0.11			
Treatment×rank×day		0.7	2,303.3	0.51		0.2	2,306.4	0.82		1	2,309	0.38		0.7	2,308.1	0.50
Diquat×S1	−0.21±0.41				0.018±0.035				0.0061±0.0043				−0.06±0.7			
Diquat×S2	−0.49±0.42				0.021±0.036				0.0035±0.0044				−0.75±0.71			
Treatment×rank×day^2		1.2	2,36.5	0.30		1.4	2,37	0.26		0.9	2,43.6	0.41		0.02	2,35.4	0.98
Diquat×S1	0.13±0.08				0.011±0.007				0.0009±0.001				0.02±0.15			
Diquat×S2	0.08±0.08				0.002±0.007				0.0014±0.001				−0.001±0.153			

Treatment and rank contrasts were done with control and dominant individuals, respectively. In all models, both aviaries and sampling dates were allowed to have random intercepts, while each individual had a random slope and intercept. Significant differences are in bold, whereas trends are in italics.
S1/S2, subordinate-1/2; VCL, curvilinear velocity.

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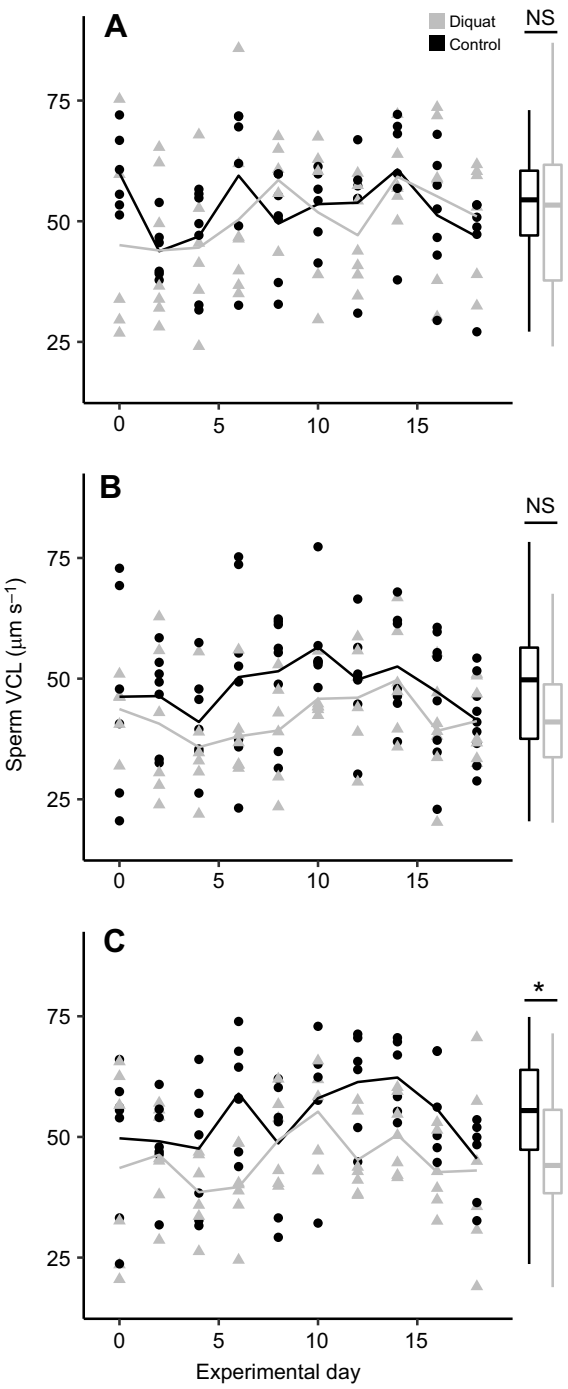

Fig. 2. Effects of chronic diquat treatment on sperm velocity in ejaculates of males of different social rank. Curvilinear velocity (VCL) of sperm of (A) dominant, (B) subordinate-1 and (C) subordinate-2 males. Lines join the means of each treatment per day. Marginal boxplots summarize the sperm velocity between control (black) and diquat-treated (grey) males; outliers are not plotted as they can be seen in the distribution of data in the scatterplot. *Post hoc* pairwise comparisons: NS, not significant, $P>0.05$; *$P<0.05$.

More interestingly, the effect of diquat treatment on sperm velocity depended on male rank (Fig. 2, Table 1). In order to 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 sampling date and 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 ANOVA: dominant treatment versus control, $F_{1,14.7}=0.19$, $P>0.1$, control 52.4±11.7 μm s^{-1}, diquat 50.4±14.6 μm s^{-1}; subordinate-1 treatment versus control, $F_{1,13.6}=1.16$, $P>0.1$, control 45.3±12.2 μm s^{-1}, diquat 41.8±10.4 μm s^{-1}; subordinate-2 treatment versus control, $F_{1,13.4}=8.10$, $P=0.013$, control 53.5±12.4 μm s^{-1}, diquat 45.2±11.7 μm s^{-1}).

Oxidative stress and antioxidant allocation

As a result of 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. At the onset of the experiment, we found rank-related differences in ejaculate levels of total glutathione (Fig. 3; tGSH; $F_{2,17.9}=5.57$, $P=0.013$), while tGSH in red blood cells was positively related to body mass (slope=0.27±0.10, $F_{1,42.3}=6.85$, $P=0.012$). None of the remaining redox markers differed between treatment groups at the onset of the experiment for neither sperm (tGSH: $F_{1,12.5}=0.29$, $P=0.60$; GSH:GSSG: $F_{1,12.1}=0.55$, $P=0.47$; MDA: $F_{1,12.1}=1.26$, $P=0.28$; SOD: $F_{1,12.4}=0.04$, $P=0.84$) or blood markers (tGSH: $F_{1,15.3}=0.16$, $P=0.69$; GSH:GSSG: $F_{1,15.4}=0.02$, $P=0.89$; MDA: $F_{1,15.4}=1.28$, $P=0.27$; SOD: $F_{1,15.8}=0.01$, $P=0.91$). Nor did we find further differences between social ranks in redox markers in sperm (GSH:GSSG: $F_{2,16.7}=0.26$, $P=0.77$; MDA: $F_{2,18.3}=0.12$, $P=0.88$; SOD: $F_{2,18.8}=1.69$, $P=0.21$) or blood markers (tGSH: $F_{2,30.2}=0.66$, $P=0.52$; GSH:GSSG: $F_{2,28.0}=0.49$, $P=0.62$; MDA: $F_{2,30.7}=1.04$, $P=0.37$; SOD: $F_{2,30.2}=0.03$, $P=0.97$). Finally, we found no rank-related differences in body mass before the experiment ($F_{1,15.1}=0.02$, $P=0.90$), and body mass was positively correlated to tarsus length (slope=1.07±0.24, $F_{1,40.5}=18.28$, $P=0.0001$).

The males that received diquat increased tGSH in their ejaculates (Fig. 4A, Table 2), yet the GSH:GSSG ratio remained unchanged (Fig. 4B, Table 2). SOD activity and levels of MDA in the ejaculates were unaffected by the diquat treatment (Table 2). However,

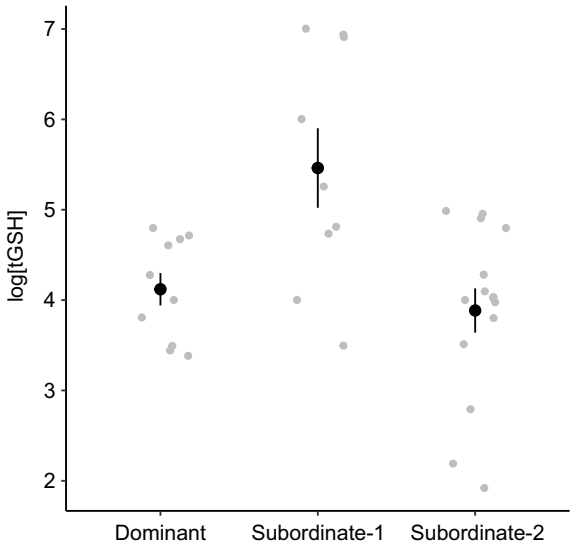

Fig. 3. Levels of total glutathione (tGSH) in sperm of males of different social rank prior to experimental treatment. tGSH was measured in mg ml^{-1}. Means±s.e.m. are shown in black.

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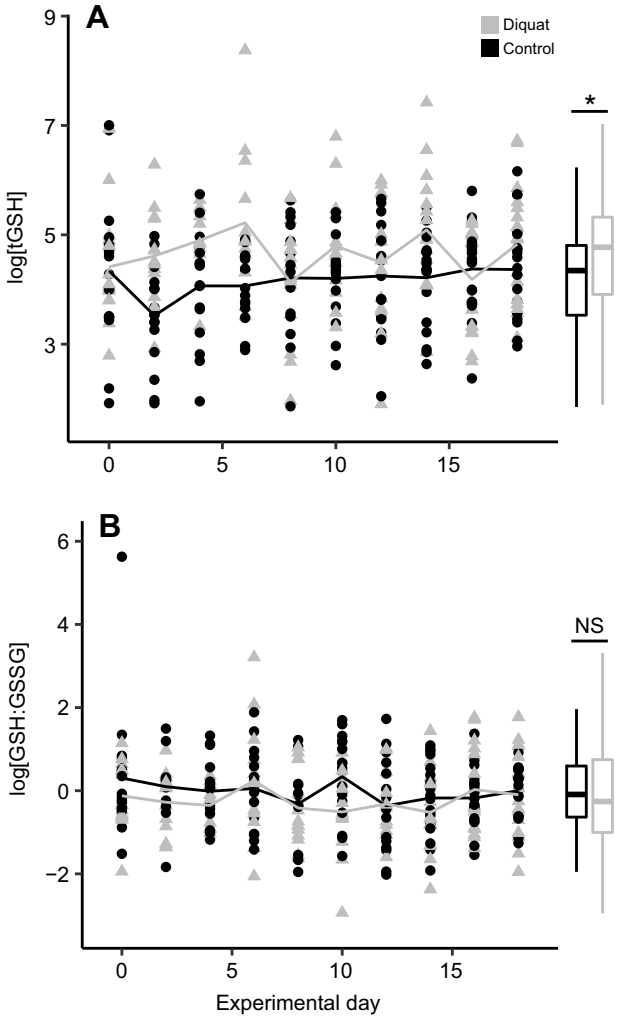

Fig. 4. Effects of chronic diquat treatment on glutathione levels in sperm. (A) tGSH levels in sperm (mg ml^{-1}). (B) Oxidized to reduced glutathione ratio (GSSG:GSH) in sperm. Lines join the means of each treatment per day. Marginal boxplots summarize the *y*-axis between control (black) and diquat-treated (grey) males, and outliers are not plotted as they can be seen in the distribution of data in the scatterplot. NS, not significant, $P>0.05$; *$P<0.05$.

diquat-treated males tended to reduce the levels of MDA in their ejaculate as the experiment progressed (Fig. S2; Table 2).

The redox equilibrium in the soma (red blood cells and plasma) appeared to be unaffected by the diquat treatment, with none of the redox biomarkers (MDA, tGSH, GSH:GSSG or SOD activity) differing between the groups (Table 3). However, the GSH:GSSH ratio in red blood cells was positively related to body mass (Table 3), and diquat-treated males had a slightly higher body mass as the experiment progressed (Fig. S3; treatment×quadratic time interaction: $F_{1,47.5}=5.25$, $P=0.026$). Male hierarchical rank did not covary with any of the redox biomarkers, either in interaction with the treatment or alone, either in the sperm (Table 2) or the blood (Table 3).

DISCUSSION

In the present study, we chronically dosed wild-caught house sparrows with diquat (~1 mg kg^{-1}) for 18 days. At the onset of the experiment, we found that males in the middle of the hierarchy invested more antioxidant into their ejaculates (Fig. 3), yet this was not reflected in a better ejaculate motility or velocity. This partly supports other studies in house sparrows, where it was found that males in the middle of the hierarchy produce ejaculates better protected against ROS, of higher quality and with less morphologically variable sperm (Rojas Mora, 2016). After chronically dosing males with diquat, we observed rank-related reductions in sperm velocity (Fig. 2). Further, we found that diquat-treated males increased their allocation of tGSH to their ejaculates (Fig. 4A), thus managing to maintain a stable GSH:GSSG ratio in their ejaculates (Fig. 4B). However, this augmentation of tGSH in the ejaculate was achieved at no apparent oxidative cost to the soma as none of the redox markers in the blood were affected by diquat treatment (see Results; Table 3). Previous studies have shown that birds can change their blood antioxidant capacity in short periods of time in response to OS (e.g. Bertrand et al., 2006; Costantini and Dell'Omo, 2006; Lin et al., 2004). It has been shown that rank-related OS costs are both sex and season dependent (e.g. Beaulieu et al., 2014; Cram et al., 2015). We found increases in MDA levels in plasma during the pilot study, which was conducted before the reproductive season (during winter). It is likely that birds changed their physiology to cope with their reproductive costs (e.g. higher metabolism, higher basal corticosterone, etc.). However, we only measured a single marker of oxidative damage (MDA), and differences in somatic OS might lie in other types of ROS-induced damage (e.g. damage to DNA and proteins), as found in another study using paraquat to generate OS (e.g. increased DNA damage in paraquat-treated green finches, *Carduelis chloris*; Meitern et al., 2013).

We predicted that under a higher risk of oxidation, subordinate males would prioritize the antioxidant protection of their ejaculate at the expense of their somatic balance. Opposite to our predictions, we observed that the sperm velocity of males at the bottom of the hierarchy (subordinate-2) was significantly impacted by diquat treatment, while the sperm velocity of dominant and subordinate-1 males was not affected by the treatment (Fig. 2). Sperm velocity has been shown to be an important ejaculate parameter determining the outcome of sperm competition (e.g. Birkhead et al., 1999; Gage et al., 2004; Holt et al., 1997). However, whether the differences in velocity found in the present study are sufficient to cause rank-related reductions in fertilization success remains to be investigated. Rank-related differences in the glucocorticoid stress response could result in higher allostatic costs for subordinate males (Goymann et al., 2010), which in turn could translate into OS insults (Costantini et al., 2011). Such physiological costs of subordination could prevent males at the lower end of the hierarchy from further investing in the protection of their ejaculates. In house sparrows, however, it is not clear whether dominant or subordinate males incur higher allostatic costs (e.g. contrasting results between the following studies: Buchanan et al., 2010; Gonzalez et al., 2002; Lindström et al., 2005), and thus we cannot rule out the possibility that subordinate-2 males were constrained by their social status-related physiological costs.

Alternatively, dominant males might be better at monopolizing resources, leaving males at the bottom of the hierarchy with a lower resource budget to be traded-off between somatic and germline functions. For instance, rank-related differences in access to resources could result in dominant males having better antioxidant defences (Catoni et al., 2008; Cohen et al., 2009). Hence, dominant males may be able to defend both their soma and their ejaculates from OS (e.g. big houses, big cars; Reznick et al., 2000). In another study, we found that both dominant males and males at the lower end of the hierarchy have the highest

Table 2. Linear mixed models for measurements of OS and antioxidant defences in sperm fitted with a restricted maximum likelihood and a Kenward–Roger approximation for the degrees of freedom

	tGSH				GSSG:tGSH				SOD				MDA			
Fixed effects	Estimate±s.d.	*F*	d.f.	*P*	Estimate±s.d.	*F*	d.f.	*P*	Estimate±s.d.	*F*	d.f.	*P*	Estimate±s.d.	*F*	d.f.	*P*
Intercept	4.07±0.25				0.75±0.13				5.95±0.22				−0.4±0.24			
Treatment	0.733±0.36	8.52	1,24.5	**0.007**	−0.298±0.193	1.71	1,21.6	0.20	0.196±0.305	0.25	1,38.4	0.62	0.513±0.34	0.03	1,22.4	0.86
Rank		0.1	2,30.9	0.90		1.28	2,30.9	0.29		1.24	2,54.2	0.30		0.77	2,26.8	0.47
S1	−0.031±0.343				0.029±0.181				−0.076±0.3				0.68±0.312			
S2	0.076±0.341				0.092±0.182				0.057±0.305				0.352±0.322			
Day	0.034±0.022	0.24	1,15.5	0.63	−0.009±0.012	0.72	1,15.9	0.41	0.029±0.014	19.88	1,18.4	**0.0003**	0.049±0.023	0.03	1,15	0.85
Day^2	0.002±0.004	0.16	1,11.5	0.69	−0.001±0.002	0.82	1,10.9	0.38	−0.007±0.004	0.84	1,31.8	0.37	0.006±0.005	0.13	1,12.1	0.72
Treatment×rank		0.37	2,31	0.70		1.06	2,30.9	0.36		2	2,54.3	0.15		2.07	2,26.6	0.15
Diquat×S1	0.012±0.504				0.372±0.266				−0.068±0.43				−0.891±0.458			
Diquat×S2	−0.375±0.509				0.08±0.271				−0.786±0.433				−0.758±0.462			
Treatment×day	−0.043±0.033	2.2	1,253.5	0.14	0.023±0.019	2.09	1,239.9	0.15	0.012±0.021	2.12	1,240.5	0.15	−0.074±0.034	3.06	1,228.4	*0.08*
Treatment×day^2	−0.008±0.007	0.38	1,32.4	0.54	0.005±0.004	0.03	1,31.6	0.86	0.003±0.005	0.43	1,62.4	0.52	−0.0054±0.007	0.14	1,32.1	0.71
Rank×day		2.66	2,254.3	*0.072*		1.05	2,236.1	0.35		4.54	2,236.2	**0.012**		0.75	2,227.5	0.47
S1	−0.053±0.03				−0.015±0.017				0.005±0.018				−0.048±0.031			
S2	0.007±0.031				0.005±0.017				0.024±0.018				−0.054±0.031			
Rank×day^2		3.23	2,32.3	*0.053*		0.24	2,31.5	0.79		5.14	2,62.3	**0.009**		0.36	2,32.2	0.70
S1	0.005±0.006				0.004±0.003				0.009±0.005				−0.008±0.007			
S2	−0.005±0.006				0.001±0.003				0.004±0.005				−0.008±0.006			
Treatment×rank×day		0.2	2,254	0.82		0.23	2,237.8	0.79		2.76	2,236.4	*0.065*		1.06	2,226.7	0.35
Diquat×S1	0.03±0.047				−0.006±0.026				−0.066±0.029				0.069±0.048			
Diquat×S2	0.015±0.046				−0.017±0.025				−0.021±0.028				0.05±0.047			
Treatment×rank×day^2		0.59	2,32.3	0.56		2.29	2,31.5	0.12		1.06	2,62.4	0.35		0.85	2,32	0.44
Diquat×S1	0.009±0.009				−0.011±0.005				0.004±0.008				0.008±0.01			
Diquat×S2	0.008±0.009				−0.003±0.005				−0.007±0.007				0.012±0.009			

Treatment and rank contrasts are done with control and dominant individuals, respectively. In all models, both aviaries and sampling dates were allowed to have random intercepts, while each individual had a random slope and intercept. Significant differences are bold, whereas trends are in italics.

OS, oxidative stress; tGSH, total glutathione; GSSG:tGSH, oxidized to reduced total glutathione ratio; SOD, superoxide dismutase; MDA, malondialdehyde; S1/S2, subordinate-1/2.

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Table 3. Linear mixed models for measurements of OS and antioxidant defences in blood fitted with a restricted maximum likelihood and a Kenward–Roger approximation for the degrees of freedom

	tGSH				GSSG:tGSH*				SOD				MDA			
Fixed effects	Estimate±s.d.	*F*	d.f.	*P*	Estimate±s.d.	*F*	d.f.	*P*	Estimate±s.d.	*F*	d.f.	*P*	Estimate±s.d.	*F*	d.f.	*P*
Intercept	7.5±0.19				0.55±1.48				7.2±0.17				1.56±0.16			
Treatment	−0.107±0.173	1.05	1,30	0.31	0.095±0.26	0.08	1,28.6	0.79	0.16±0.232	0.2	1,40.4	0.66	0.088±0.223	0.4	1,36.8	0.53
Rank		0.8	2,39.9	0.46		1.77	2,38.6	0.18		0.33	2,44.3	0.72		0.66	2,44.4	0.52
S1	0.028±0.167				0.356±0.247				0.064±0.223				0.133±0.215			
S2	−0.184±0.17				−0.004±0.252				0.147±0.225				0.146±0.218			
Day	0.09±0.018	46.84	1,4.7	**0.001**	0.005±0.021	0.79	1,4.6	0.42	0.045±0.012	33.67	1,4.1	**0.004**	0.004±0.012	0.58	1,4	0.49
Day^2	−0.013±0.004	20.65	1,5.4	**0.005**	−0.001±0.004	0.92	1,5.1	0.38	0.003±0.003	0.69	1,5.5	0.44	0.003±0.002	0.27	1,4.6	0.63
Treatment×rank		0.28	2,39.9	0.75		0.25	2,37.7	0.78		0.17	2,44.3	0.84		0.15	2,44.4	0.86
Diquat×S1	−0.091±0.24				−0.24±0.353				−0.185±0.315				0.082±0.304			
Diquat×S2	0.09±0.24				−0.18±0.352				−0.104±0.317				−0.085±0.306			
Treatment×day	0.009±0.016	1.24	1,95.7	0.27	−0.021±0.023	0.03	1,95.3	0.86	−0.005±0.016	0.03	1,99.3	0.86	0.014±0.017	2.29	1,100.6	0.13
Treatment×day^2	0.0003±0.004	0.49	1,54.5	0.49	−0.0007±0.005	0.37	1,49	0.55	−0.003±0.003	0.35	1,51	0.56	−0.003±0.003	0.8	1,49.3	0.37
Rank×day		0.74	2,97.5	0.48		0.53	2,98.3	0.59		0.12	2,103.3	0.88		2.26	2,103.9	0.11
S1	0.011±0.015				−0.032±0.023				0.002±0.016				−0.032±0.017			
S2	−0.002±0.016				−0.019±0.023				−0.017±0.016				−0.012±0.017			
Rank×day^2		0.27	2,60	0.77		3.23	2,52.3	**0.048**		0.24	2,52.3	0.79		0.17	2,50.8	0.84
S1	−0.002±0.004				−0.007±0.005				−0.001±0.003				−0.001±0.003			
S2	0.001±0.004				0.005±0.005				−0.001±0.003				−0.003±0.003			
Treatment×rank×day		0.01	2,97.4	0.99		0.48	2,98.5	0.62		0.98	2,103.2	0.38		1.07	2,103.8	0.35
Diquat×S1	−0.0002±0.022				0.03±0.033				−0.005±0.022				0.018±0.024			
Diquat×S2	0.003±0.023				0.026±0.033				0.025±0.022				−0.017±0.024			
Treatment×rank×day^2		0.23	2,59.9	0.79		0.74	2,52.2	0.48		0.46	2,52.3	0.63		0.48	2,50.8	0.62
Diquat×S1	0.003±0.005				0.003±0.007				0.004±0.005				−0.00003±0.005			
Diquat×S2	0.0004±0.005				−0.0056±0.007				0.001±0.005				0.004±0.005			
Body mass	–	–	–	–	0.098±0.042	4.96	1,57.8	**0.03**	–	–	–	–	–	–	–	–
Tarsus length	–	–	–	–	−0.02±0.083	0.06	1,43.2	0.81	–	–	–	–	–	–	–	–

Treatment and rank contrasts were done with control and dominant individuals, respectively. In all models, both aviaries and sampling dates were allowed to have random intercepts, while each individual had a random slope and intercept. Significant differences are bold, whereas trends are in italics. *In this model the inclusion of body mass and tarsus length increased the fit (χ^2=6.70, d.f.=2, *P*=0.035).
OS, oxidative stress; tGSH, total glutathione; GSSG:tGSH, oxidized to reduced total glutathione ratio; SOD, superoxide dismutase; MDA, malondialdehyde; S1/S2, subordinate-1/2.

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within-ejaculate morphological variation, suggesting that those males exerted a reduced control over spermatogenesis (Rojas Mora, 2016). This suggests that males at the lower end of the hierarchy cannot invest as much as predicted by sperm competition models in the control and quality of ejaculate production. Thus, while dominant males may be able to protect both their ejaculates and their soma, males at the bottom of the hierarchy may not be able to afford the extra costs of maintaining their soma above a minimum threshold of body condition that would still allow them to get some mates. Our results thus suggest that sperm production is condition dependent, and the intrinsic ability to monopolize resources in general, and to use antioxidant resources in particular, is a likely constraint in the evolution of status-dependent reproductive tactics. Importantly, using our markers of OS in the blood, we found no apparent cost to the greater allocation of antioxidants into the ejaculate, and potential costs to the soma remain to be explored and identified.

There are at least three, non-exclusive mechanistic explanations for the observed reduction in sperm velocity, particularly in the lowest ranked males. It has previously been observed in great tits, *Parus major*, that higher levels of MDA in the ejaculate are negatively correlated with ejaculate swimming ability measured as a principal component analysis comprising various sperm velocity parameters (Helfenstein et al., 2010; Losdat et al., 2011). Thus, reduced sperm velocity in diquat-treated males could arise from oxidative damage to the membranes. However, MDA levels in ejaculates of diquat-treated males tended to decrease as the experiment progressed (Fig. S2). This result could arise from a reduction in the proportion of PUFAs in the sperm membrane, which in turn would lower the risk of oxidative damage to the spermatozoa (delBarco-Trillo and Roldan, 2014; Wathes et al., 2007). Noticeably, a lower proportion of PUFAs in the sperm membrane could lead to lower sperm performance (Asturiano et al., 2001; Mitre et al., 2004). Alternatively, the disruption of membrane proteins in the mitochondria as a result of increased ROS production induced by diquat could reduce ATP production (Hulbert et al., 2007), hence reducing the energy budget available to sperm for swimming. Despite the observation of an upregulation of tGSH in ejaculates of diquat-treated males – suggesting an immediate need to protect the ejaculate from ROS (Fig. 4) – further research is necessary to understand which of these potential mechanisms are responsible for the observed reduction in sperm velocity following treatment with diquat.

Here, we showed that exposure to a pro-oxidant could greatly affect sperm swimming ability, with males occupying a disfavoured social role suffering the largest reductions in ejaculate speed. Although the extent to which such reductions will affect sperm competitiveness is still unclear, it seems reasonable to assume that it exerts a significant pressure on such males that already have a low mating rate, because males at the bottom of the hierarchy could lose out on their few mating attempts through sperm competition. Previous studies on other animals, including humans, have found that pollutants can affect ejaculate quality (e.g. Abarikwu et al., 2010; Dauwe et al., 2004; Lacoume et al., 2009). 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; Ottinger et al., 2002). Thus, we encourage further research to test the extent to which rank-related effects of OS caused by pollutants can limit male reproductive success under sperm competition, especially for those males that invest in postcopulatory traits as a reproductive tactic.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: A.R.M., F.H.; Methodology: A.R.M., F.H.; Formal analysis: A.R.M., A.V.; Investigation: A.R.M., A.F., S.B.; Resources: A.V.; Data curation: A.R.M.; Writing - original draft: A.R.M.; Writing - review & editing: A.R.M., F.H.; Supervision: F.H.; Project administration: F.H.; Funding acquisition: F.H.

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Data availability
Data are available from figshare: 10.6084/m9.figshare.5117575

Supplementary information
Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.154799.supplemental

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Supplementary figures

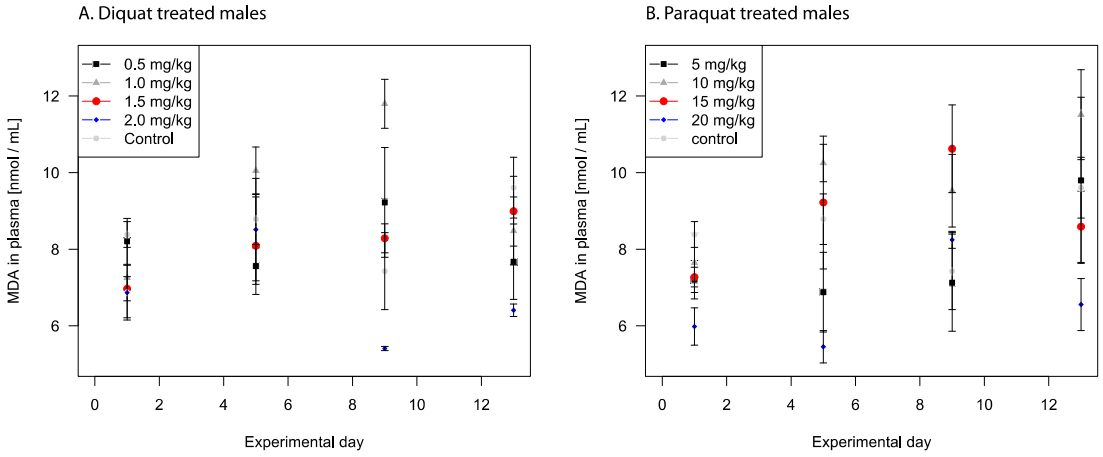

Figure S1. Levels of MDA in plasma found during the pilot study for (A) diquat treated and (B) paraquat treated males. Males receiving the 2 mg/kg dose of diquat did not receive any further treatment after day 6. Points represent the mean ± SEM.

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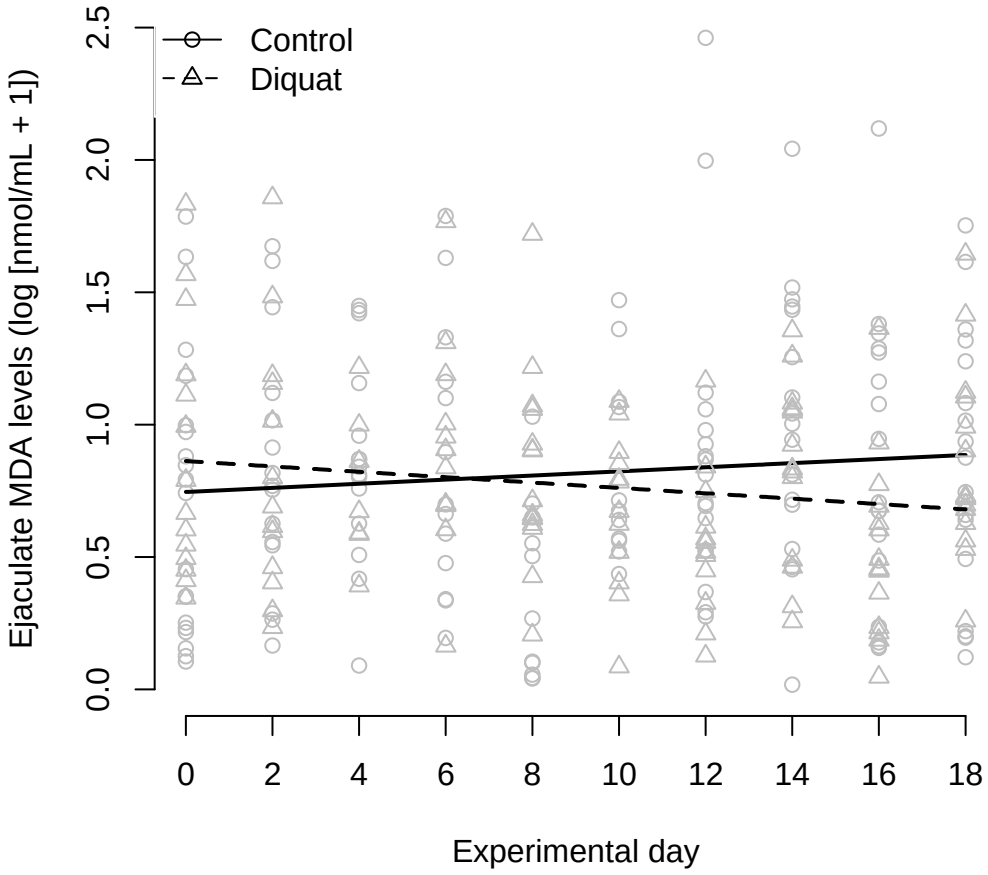

Figure S2. Levels of MDA in the ejaculate for diquat treated (triangles and dashed line) and control males (circles and solid line) as the experiment progressed. The lines represent a linear regression.

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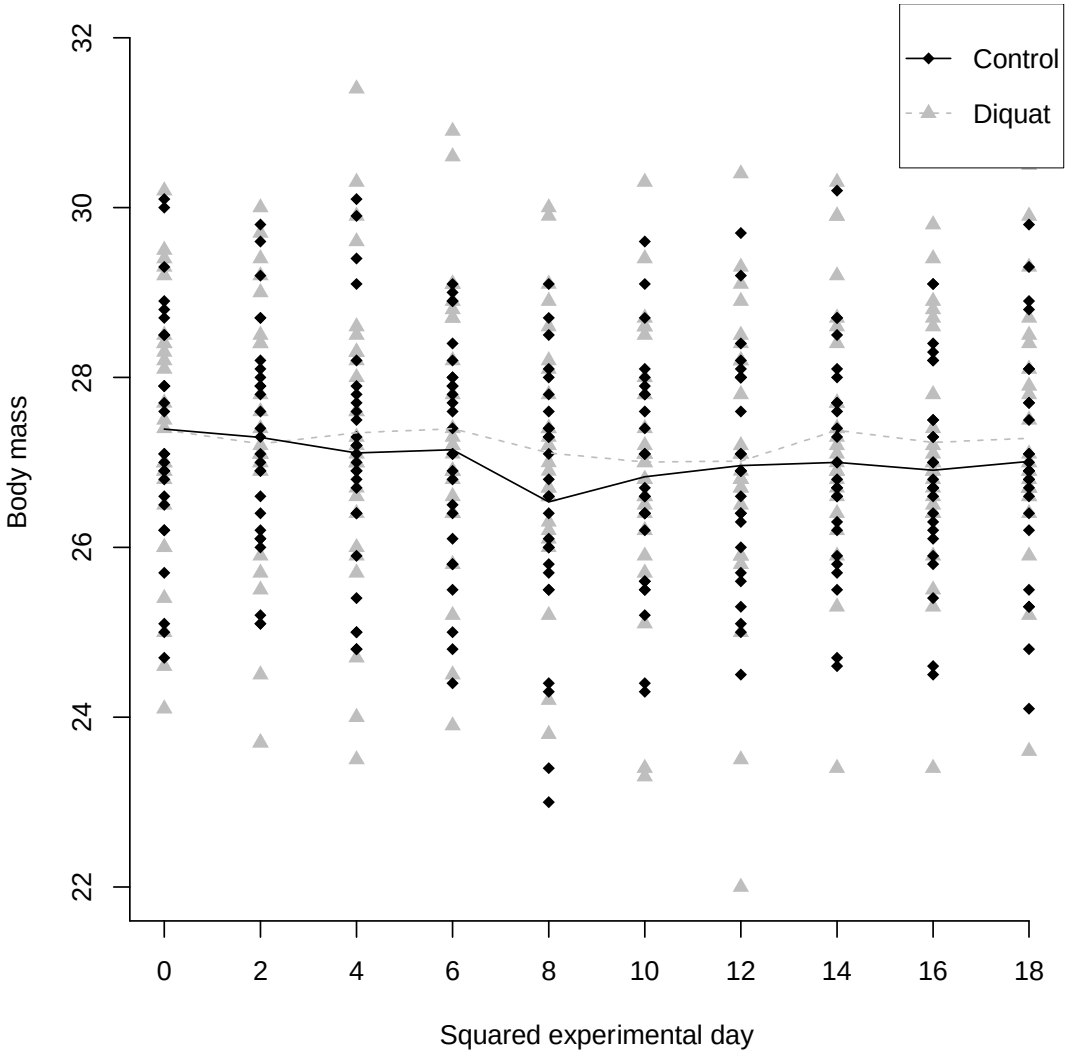

Figure S3. Body mass (g) of diquat treated (triangles and dashed line) and control males (diamonds and solid line) as the experiment progressed. The lines represent the mean values observed for each group.

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