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ORIGINAL ARTICLE

Alternative reproductive tactics, sperm mobility and oxidative stress in *Carollia perspicillata* (Seba's short-tailed bat)

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Abstract
In social systems with alternative reproductive tactics, sneakers face a higher level of sperm competition than harem males and hence are predicted to allocate more resources to ejaculates. Antioxidants can protect sperm against oxidative stress, and thus, their allocation to the ejaculate may depend on mating tactic. In this study on the frugivorous bat *Carollia perspicillata*, we assessed, for harem and sneaker males, four sperm mobility traits, blood and ejaculate markers of the redox balance and the ejaculate to blood ratios of the redox markers. Under higher sperm competition, sneaker males should allocate proportionally more antioxidant resources to the protection of sperm than harem males. In contrast, harem males should favour pre-copulatory functions, which comprise the protection of blood. We found significantly higher sperm velocity and sperm survival in sneakers. There was no correlation between sperm mobility and sperm enzymatic antioxidant activity or ejaculate levels of lipid peroxidation (oxidative damage). Ejaculate levels of lipid peroxidation and sperm survival showed a significantly positive correlation, which could be attributed to the role of reactive oxygen species for sperm capacitation. Harem and sneaker males showed similar levels of redox balance markers in ejaculate and blood. However, harem males showed a higher ratio of oxidized over reduced glutathione in blood, which may indicate higher cellular stress due to higher metabolism. Overall, our findings suggest that sneakers of *C. perspicillata* compensate for a higher level of sperm competition by higher sperm mobility.

Significance statement
In social systems with alternative reproductive tactics, sneakers face higher level of sperm competition than harem males and hence are predicted to allocate more resources to ejaculates. Antioxidants can protect sperm against oxidative stress, and thus, their allocation to the ejaculate may depend on mating tactic. In this study on the frugivorous bat *Carollia perspicillata*, we found sperm swimming significantly faster and longer in sneaker males compared to harem males. However, traits other than the investigated antioxidant may favour higher sperm mobility. Measured redox pattern in blood of harem males may indicate higher cellular stress due to higher metabolism. Our results provide support to the current sperm competition models at the intraspecific level, which is still debated for internal fertilizers. This study contributes to better understanding the trade-offs and adaptations resulting from alternative reproductive tactics in mammals.

Keywords Sperm competition · Sperm velocity · Sperm survival · Malondialdehyde · Glutathione · Superoxide dismutase

Nicolas Jean Fasel and Charlotte Wesseling shared first authorship.

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Introduction

Sexual selection drives males to compete for mating opportunities during the pre-copulatory competition and for egg fertilizations during the post-copulatory competition (Birkhead et al. 2008). Alternative reproductive tactics where some males secure privileged access to females while others sneak copulations are frequent in the animal kingdom (Oliveira et al. 2008). For the pre-copulatory competition, sneakers often invest less than harem males in secondary sexual traits (Almada and Robalo 2008), which comprise, for example, horn size, plumage colouration, song rates, courtship rates, guarding behaviours or body condition (Mautz et al. 2013). Sneaker males, however, face a higher level of sperm competition (Flannery et al. 2013) and often suffer reduced fitness (e.g. Heckel and von Helversen 2002; Ortega et al. 2003; Fasel et al. 2016). Theoretical and empirical studies support the hypothesis that under higher level of sperm competition, males should allocate more resources towards testicular and seminal functions to produce more competitive ejaculates (Kilgallon and Simmons 2005; Snook 2005; Ramm and Stockley 2009; Tazzyman et al. 2009; Parker and Pizzari 2010; Fedorka et al. 2011; Parker et al. 2013; Firman et al. 2015). Indeed, studies comparing males facing different sperm competition pressures reported a higher spermatic or seminal production (Stockley et al. 1994; Vladic and Jarvi 2001; Rasotto and Mazzoldi 2002) and a superior sperm mobility such as sperm velocity (Vladic and Jarvi 2001; Burness et al. 2004; Flannery et al. 2013) or motility (i.e. proportion of sperm moving; Vladic and Jarvi 2001; Froman et al. 2002) in sneaker males. However, some other studies found similar or even higher ejaculate quality in males with higher access to females (Kruczek and Styrna 2009; Lemaître et al. 2012; Schradin et al. 2012).

Concurrently, the phenotype-linked fertility hypothesis (Sheldon 1994) predicts that male investment in secondary sexual traits should honestly indicate fertility capacity and should thus positively correlate with sperm mobility (e.g. Birkhead et al. 1994; Malo et al. 2005; Locatello et al. 2006; Helfenstein et al. 2010, see also Mautz et al. 2013). Consequently, in alternative reproductive tactics, the phenotype-linked fertility hypothesis and sperm competition models are compatible only when sneakers invest less resources to the ejaculate in absolute terms but still invest more than harem males relative to the resources invested in functions related to pre-copulatory competition (i.e. secondary sexual traits).

Resources allocated to maintain ejaculate quality are diverse, comprising nutrients, hormones or protective compounds (Perry et al. 2013). Among them, antioxidants are crucial, as they limit the damage caused by reactive oxygen species (ROS), which are generated during the electron chain transport (Finkel and Holbrook 2000). Oxidative stress occurs when ROS overcome the antioxidant capacity and lead to cell apoptosis, lipid peroxidation and DNA fragmentation (Agarwal and Said 2005). Sperm cells, with both a high proportion of poly-unsaturated fatty acids in their membrane and a limited repair machinery, are particularly vulnerable to pro-oxidant insults (Blount et al. 2001). They face greater challenges than any other cell type, as they directly generate a large amount of ROS to sustain swimming capacity (Koppers et al. 2008). The protective function of antioxidants against oxidative stress thus appears pivotal for spermatozoa (Shiva et al. 2011; Tremellen 2012). Indeed, an increase in OS impacts sperm mobility and can lead to male sub-fertility or infertility (reviewed in Blount et al. 2001; Tremellen 2008; Costantini 2014). In an environment with sperm competition, oxidative stress may thus significantly affect male likelihood of paternity (Velando et al. 2008; Almbro et al. 2011).

Within alternative reproductive tactics, sneaker males should thus favour higher ejaculate quality in response to higher level of sperm competition. Therefore, in comparison with harem males, sneakers may allocate more antioxidants to ejaculate protection, relative to the antioxidants allocated to pre-copulatory competition. In contrast, harem males may require a higher metabolism to attract females and defend their social status. They may also endure repetitive injuries during contest, leading to immune responses such as antimicrobial defences and inflammations. These higher reproductive expenditures are expected to induce oxidative costs (Guindre-Parker et al. 2013; Costantini 2014) and favour the allocation of more antioxidants to somatic functions related to pre-copulatory competition and hence less to the protection of sperm cells.

The mating system of the Seba's short-tailed bat (*Carollia perspicillata*) has been described as a resource defence polygyny (Fleming 1988). Between 2 and 7 years of age, a minority of males manage to control the access to high-quality territories where females roost (Fasel et al. 2016) and form harems holding up to 18 females. Males may hold a harem for several years (Williams 1986; Fleming 1988; Knörnschild et al. 2014). The causes for switching of social position are not clear, but males of different social status do not differ in age, basal blood testosterone and cortisol; in testes volume; or in body mass or size (NF unpublished data). Harem males perform courtship displays and aggressive behaviours (Fernandez et al. 2014; Knörnschild et al. 2014) with short rest and foraging periods (Porter 1978; Fleming 1988). Bachelor males do not control a territory and gather at daytime in unstable bachelor groups. Peripheral males are faithful to a territory at variable distance near a harem. They are mostly alone without stable access to females and join occasionally a bachelor group (Fasel et al. 2016). *Post-partum* copulations take place mostly within the harem (Porter 1979), and harem males may mate at a higher frequency compared to other males. Uneven copulation rates among males are indeed expected in polygynous species as reported in the hammer-headed bat (*Hypsignathus monstrosus*), a lekking species (Bradbury 1977).

Selection should favour motile sperm and their protection by seminal fluids in *C. perspicillata*. Indeed, menstruations are periovulatory and uteri of cycling females contain menstrual debris at the time of copulations (Rasweiler et al. 2010a). Fertilization then occurs without prolonged sperm storage (Rasweiler et al. 2010a, b).

Without stable access to females, bachelor and peripheral males differ from harem males in mating tactic, as they have to sneak copulations. They consequently face higher levels of sperm competition compared to harem males. Males of *C. perspicillata* thus show three social statuses (harem, bachelor and peripheral males) but use two mating tactics (harem and sneaker males).

Paternity within the population is shared between males of the three social statuses, which demonstrate the occurrence of sneaked copulations. In our captive population of bats, harem males represent less than a third of all males (in average 40 of 132 males). However, they sire 60% of all the pups born into the population (Fasel et al. 2016). In a study investigating age-related variation in reproductive success across reproductive tactics, harem males were found to benefit from a significantly higher reproductive success than both bachelor and peripheral males, except during a short period from 3.8 to 4.4 years of age and from 2.6 to 4.4 years of age when their reproductive success was similar to that of bachelor and peripheral males, respectively (Fasel et al. 2016).

In the present study, we analysed four sperm mobility traits (velocity, motility, survival and stamina) that are important for sperm competition (Snook 2005; Gomendio and Roldan 2008; Pizzari and Parker 2009; Fitzpatrick and Lüpold 2014). We used a method for sperm collection that is known to provide different values for sperm density and ejaculate volume compared to a natural ejaculation (Mattner and Voglmayr 1962; Moore 1985). Thus, we did not include these two ejaculate traits in the present analysis. Additionally, we measured several markers related to the redox profile in blood and ejaculate, which should indicate investment and costs related to pre- and post-copulatory competitions, respectively. Superoxide dismutase (SOD) is the first line of antioxidant defence against ROS generated during the respiratory chain reaction and the only enzymes catalysing the dismutation of the superoxide anion (O_{2-}) in hydrogen peroxide (H_2O_2) and oxygen (O_2) in eukaryotes (Fridovich 1978). SOD has been shown to play a crucial role in animal survival (Van Raamsdonk and Hekimi 2012). Moreover, SOD is known to protect sperm maturation (Arenas-Ríos et al. 2016) and further functions such as fertilization potential (Yan et al. 2014) and swimming capacity (Kobayashi et al. 1991; Calamera et al. 2003; Shiva et al. 2011) from ROS insults. Malondialdehydes (MDAs) are end products of lipid peroxidation initiated by ROS (Gutteridge 1995). This process leads to functional losses of the cellular membrane (Niki 2009) and can have dramatic effects on sperm mobility (Aitken and Fisher 1994; Suleiman et al. 1996; Alvarez and Aitken 2012). MDA is thus regularly considered as a marker of oxidative damage to the lipids (Monaghan et al. 2009). Finally, reduced glutathione (GSH) is an endogenous tri-peptide, which can be oxidized into glutathione disulphide (GSSG) to reduce ROS via a reaction catalysed by the enzyme glutathione peroxidase. The glutathione ratio GSSG/GSH provides accurate information on the oxidative balance of cells (Cnubben et al. 2001). Ejaculate volumes were small, and for practical reasons, this latter marker was only measured in the blood.

With this study, we predicted superior values of sperm mobility traits in harem males compared to sneaker males (i.e. bachelor and peripheral), as suggested by the phenotype-linked fertility hypothesis. Given the vulnerability of sperm to oxidative stress, we predicted that sperm mobility traits should be positively correlated with ejaculate SOD capacity and negatively with ejaculate MDA. Additionally, based on the phenotype-linked fertility hypothesis, we predicted higher absolute SOD capacity and lower absolute MDA in the blood and ejaculate of harem males than sneaker males. Under the assumption of a trade-off for the allocation of antioxidant resources between pre- and post-copulatory competitions, sperm competition models predict that, compared to harem males, sneaker males should show relatively higher SOD capacity in the ejaculate than in the blood and relatively lower MDA levels in the ejaculate than in the blood. Finally, as harem males are expected to incur higher pre-copulatory costs, their blood cells may face higher levels of oxidative stress and thus may require more intense detoxification (higher oxidized/GSH ratio) compared to sneaker males.

Material and methods

Model species and studied population

The captive population of *C. perspicillata* is housed in a tropical zoo (Papiliorama, Kerzers FR, Switzerland). A colony of approximately 300 adults (sex ratio 0.99 females to 1 male) is accommodated in a 40-m-diameter dome, which mimics a tropical habitat including an artificial cave where bats roost. The cave is open to visitors, and animals are used to human presence. A reversed light cycle enables researchers and visitors to observe nocturnal activities of the bats from 09:30 a.m. to 09:30 p.m. The bats are fed twice a night with a fruit-based mixture.

Capture

Bats used during this present study were caught between February and March 2014 with a harp trap (Faunatech Austbat, Mount Taylor, Australia) mounted at the entrance of the cave, and a small fraction of focal individuals were captured with hand nets. The bats were ringed with a unique

combination of three coloured plastic rings (A.C. Hughes, UK, size XB). Only males with functional i.e. scrotal testes were included in our study. Blood and ejaculate were sampled only of males of known social status. After blood collection, the bats were placed in a cotton bag with food (piece of apple) until ejaculate collection.

Social monitoring

Since May 2013, the social environment of males was monitored twice a month between 9:00 p.m. and 11:00 p.m. at the beginning of the light period. Dawn is the time period where bats are still active but already cluster at their roosts. Videos were taken with camcorders under infrared light and then visualized on the computer to determine group sex ratio and male social status based on spatial distribution of individuals. We defined the social status using spot fidelity and access to females (Fasel et al. 2016). Harem males were frequently observed on harem spots in company of adult females (min 50% of the observations, average observed was 81%). Peripheral males were faithful to a spot at variable distance near a harem but did not have access to females. Bachelor males were faithful members of a bachelor group or occasionally observed close to harems without being faithful to a spot. For the analyses, we merged peripheral and bachelor males and considered them as sneaker males compared to harem males with stable access to mates.

Blood collection

Blood samples were taken within 3 min after capture to avoid potential influence of handling stress on measures of individual redox status, using microtainer tubes for capillary blood collection (Microvette CB 300, Sarstedt) after puncture of the antebrachial vein. The samples were kept on ice until the blood was centrifuged at 21,913*g* during 14 min at 4 °C to allow the separation of red blood cells (RBCs) from plasma, which were then stored in separate tubes (Eppendorf Tubes 3810X) and preserved at −80 °C until analyses. In total, we collected the blood of 27 harem, 19 peripheral and 27 bachelor males.

Ejaculate collection

Ejaculates were sampled by electro-ejaculation (Fasel et al. 2015) on 24 harem 16 peripheral and 19 bachelor males. Males were first anaesthetised using a rodent nosecone non-rebreathing system (Rothacher Medical, CH) of 0.8 l/min oxygen (Carbagas, CH) mixed with 5% of isoflurane (Nicholas Piramal I Ltd, UK) during approximately 5 s, and anaesthesia was maintained with 1 to 2% isoflurane. After manipulation, pure oxygen was provided until bats regained awareness. The monitoring of post-anaesthesia recovery was systematically done by keeping the bats for 1 to 3 h in individual cotton bags provided with food ad libitum (apple pieces). For sperm collection, males were posed dorsally on a warming pad to maintain body temperature. Electric stimulations were generated using two electrodes situated at the end of a probe (ICSB, USA), inserted into the rectum using aqueous lubricant. The electrode was linked to an audio amplifier (JVC A-X2) transmitting three series of regular and increasing electric stimulations (maximally 4 mA). Electrical current was continuously monitored with a milli-ampere meter (Fluke 77 multimeter). In order to avoid desiccation, ejaculates were collected in a 0.5-ml tube (Eppendorf) holding 10 μl of pre-warmed (37 °C) Tris-buffered saline buffer (TBS buffer). Once the complete ejaculate was collected, TBS volume was adjusted to obtain a dilution 1:2 (*v*/*v*). From this mix, 5 μl was transferred in 10 μl of pre-warmed Earle's balanced salt solution (EBSS: SpermWash, Cryos, Denmark) buffer and kept at body temperature (37 °C, Audet and Thomas 1997) for sperm mobility analysis. The remaining volume was stored on dry ice until placed into −80 °C freezer for further analyses.

Sperm mobility analysis

Sperm motion was video-recorded for a first time within 10 min and then every 30 min, until sperm had lost most of their motility (median 90 min, min <10 min, max 210 min). Three microliters of ejaculate mixed with EBSS was placed in a 20-μm-deep chamber slide (SC 20-01-04-B, Leja®, Netherlands) under an Olympus XK41 microscope with dark-field condition and mounted with a Kappa CF 8/5 camera at ×200 magnification. Several 2-s videos (median 9, min 1, max 24) of 30 frames/s with a median number of 126.5 sperm track (min 4, max 1133) were then analysed for each session using a CASA plug-in in ImageJ 1.47v (Rasband, National Institute for Health, USA; Wilson-Leedy and Ingermann 2007) to obtain estimates of sperm swimming parameters such as curvilinear velocity (VCL, μm/s) and motility (proportion of moving sperm). The correlation of VCL with other swimming parameters is highly significant in *C. perspicillata* (Fasel et al. 2015). Sperm cells swimming with a higher velocity than non-sperm particles in the sample, i.e. 6 μm/s, were considered as motile. From those two measures, we further estimated sperm velocity decline (sperm stamina) and motility decline (i.e. sperm survival; see "Statistical analysis" section).

Blood and ejaculate cell homogenization

A volume of 20 μl of RBCs was mixed with 20 μl of phosphate-buffered saline (PBS), sonicated in an ice-cold water bath for 10 min and homogenized with four glass beads for 1 min at 30 Hz using an electric homogenizer. This homogenate was then aliquoted for analysis in 10 μl for MDA, 2 μl for glutathione and 2 μl for SOD assay and stored at −80 °C.

The ejaculate mixed with 1:2 (*v*/*v*) Tris-buffered saline (TBS) was sonicated for 15 min and homogenized with two glass beads for 2 min at 30 Hz using a motorized homogenizer. This homogenate was then aliquoted for analysis in 10 μl for MDA and 1 μl for SOD assay and stored at −80 °C.

Lipid peroxidation

We assessed MDA (nmol/ml) by its reaction with thiobarbituric acid (TBA) to produce a pink derivate measurable using high-performance liquid chromatography (HPLC) with fluorescence detection. MDA concentration estimation was assessed using a method adapted from (Losdat et al. 2014).

All steps were conducted on ice to reduce oxidation. All chemicals were of HPLC grade, and chemical solutions were prepared using ultra pure water H_2OMQ (Milli-Q Synthesis; Millipore, Watford, UK). For calibration of the HPLC, a standard curve was prepared using a 1,1,3,3-tetraethoxypropane (TEP) stock solution (5 μm in 40% ethanol) serially diluted using H_2OMQ, prepared fresh. A volume of 10 μl of homogenized samples or standards was first mixed with 40 μl of trichloroacetic acid (TCA) 5%, allowing the deproteinization of protein-bound MDA, and with 20 μl of 2-thiobarbituric acid (TBA) solution (42 mM) for the acid-catalysed reaction. The TBA solution was prepared fresh by adding 30.89 mg of 98% TBA diluted with 5 ml of H_2OMQ and dissolved on a stirring hot plate at 50 °C. Mixtures of samples or standards, TCA, and TBA were vortexed for 5 s, and 150 μl of H_2OMQ was added and then centrifuged for 14 min at 21,913*g* at 4 °C. The supernatant (205 μl) was transferred in screw-top tubes and incubated for exactly 60 min at 100 °C in a dry bath, allowing the acid-catalysed reaction for the formation of MDA-TBA adducts. The tubes were then cooled on ice for 5 min and vortexed for 10 s. Then, each tube was completed by adding 150 μl of butanol, vortexed and centrifuged for 10 min at 21,913*g* at 4 °C. The epiphase was transferred in Eppendorf tubes. Another 150 μl of butanol was added to each screw-top tube and centrifuged again for 10 min at 21,913*g* at 4 °C. The second supernatant was collected and added to the first one. The Eppendorf tubes containing the supernatant were evaporated in SpeedVac for 60 min at 35 °C and then re-suspended in 90 μl of 30% methanol, sonicated for 5 s and vortexed. Seventy microliters was transferred into HPLC vial inserts (0.250 ml capacity) and stored at −80 °C until HPLC analysis. The samples (5 μl) were injected into an Ultimate 3000 RSLC (Dionex, Thermo) coupled to an Acquity UPLC® BEH C18 column 1.7 μm, 2.1 × 50 mm, with temperature set at 30 °C. Solution A was acetonitrile, and solution B was ammonium acetate pH 6 (acetic acid 0.05% buffered at pH 6 with ammonium). The two solutions ran along a gradient with 5 to 100% solution A in 5 min, followed by 1.7 min with 100% solution A, and then from 100 to 5% solution A in 0.8 min and 5% solution A till the end. Flow rate was 0.4 ml/min and total run time was 10 min. Retention time was 3.35 min, and data were collected using a fluorescence detector (RF2000; Dionex) set at 515 nm (excitation) and 553 nm (emission). For calibration, a standard curve was prepared using a 1,1,3,3-tetraethoxypropane (TEP) stock solution (5 μM in 40% ethanol) serially diluted using 40% ethanol. TEP standards assayed in triplicate showed high repeatability (intraclass correlation coefficient = 0.99, $P < 0.0001$, $n = 12$). Analyses were performed blindly with respect to the males' mating tactic.

Antioxidant capacity

We assessed SOD activity (U/ml) using Cayman's SOD assay kit (Cayman Chemical Company, USA), which is based on the detection of superoxide radicals generated by xanthine oxidase and neutralized by SOD. Homogenates were diluted in PBS 1:1000 (*v*/*v*) for the blood and 1:60 (*v*/*v*) for the ejaculate.

Glutathione

The reduced (GSH, ng/ml) and oxidized (GSSG, ng/ml) forms of glutathione were measured by liquid chromatography tandem mass-spectrometry (LC-MS/MS), according to Bouligand et al. (2006) with some modifications.

All steps were conducted on ice to avoid oxidation. Two microliters of homogenate RBCs was added to 5 μl of 5% TCA and spiked with 5 μl of glutathione ethyl ester (GSH-ee 40 μg/ml) as an internal standard. The samples were vortexed for 5 s and given a short spin before being placed for 5 min on ice to allow protein precipitation. A volume of 38 μl H_2OMQ was then added before centrifugation for 15 min at 21,913*g* at 4 °C. A volume of 10 μl of the epiphase was collected, added to 790 μl TCA 0.05% and 70 μl transferred into HPLC vial inserts and stored at −80 °C until HPLC analysis. The samples were injected into an Ultimate 3000 RSLC (Dionex, Thermo) fitted with API 4000 QTrap (ABSciex) coupled at an Acquity UPLC® HSS T3 column using a HPLC water solvent composed of 0.05% of formic acid and acetonitrile, running isocratically over 8 min at a flow rate of 0.4 ml/min. Data were collected by electrospray ionization (ESI) on a positive ion mode at 308 uma (*m*/*z*). In parallel, standards were prepared by mixing GSH and GSSG solution to GSH-ee solution for final concentrations of 2 to 2 ng/ml for GSH and GSSG and of 50 ng/ml of GSH-ee. We obtained a high repeatability (CV = 0.072, $n = 6$, and intraclass correlation coefficient = 0.966, $n = 8$, $p < 0.001$)

Statistical analysis

The statistical analyses were performed with R (3.1.0) using linear models (function lm). Significance level was set at 5%. Continuous explanatory variables were transformed when

required and centred on the mean of the given mating tactic, providing interpretable intercepts. Comparisons between males of different mating tactics were always performed with the sneaker males as reference.

With regard to our predictions, we first tested if four sperm mobility traits: curvilinear sperm velocity (VCL), sperm motility (proportion of sperm moving; logit transformed), sperm stamina and sperm survival were correlated with male mating tactic, ejaculate redox markers (SOD and MDA) and the interactions between the mating tactic and the ejaculate redox markers. Therefore, we first ran a MANCOVA and then analysed each trait separately. Non-significant interaction terms were removed from the final analysis (Engqvist 2005).

Sperm survival and stamina were both defined by the linear (best data fit) slope of motility (logit transformed) and velocity, respectively, over time. They were estimated with random slope and intercept linear mixed models, with ejaculate identity entered as a random factor (lme, package nlme, R).

Secondly, we tested if the levels of SOD capacity and MDA concentration in the ejaculate may differ proportionally to the red blood cells (RBCs), between males of different mating tactics. We used the ratio of ejaculate SOD capacity over RBC SOD capacity and the ratio of ejaculate MDA concentration over RBC MDA concentration as response variables. The mating tactic was considered as an explanatory variable. Additionally, we compared the absolute ejaculate SOD and MDA levels between males of different mating tactics with a multivariate analysis of variance (MANOVA) and then analysed each trait separately. In order to meet the requirement of normality, ejaculate SOD and both MDA and SOD ratios were log transformed, and ejaculate MDA was square root transformed.

Finally, we investigated the variation of the redox measures in the blood in males of different mating tactics. The levels of MDA, SOD and glutathione ratio GSSG/GSH were therefore analysed as response variables with mating tactic as an explanatory variable. We ran first a MANOVA and then analysed each variable separately. RBC MDA was square root transformed, and RBC SOD and the ratio GSSG/GSH were log transformed.

Models were checked for any deviance in the distribution of residuals and for heteroscedasticity.

Results

Sperm mobility traits

The sperm mobility traits were not significantly influenced by the interactions between the mating tactic and the two redox markers in the ejaculate (status: ejaculate SOD, Pillai's trace = 0.07, $F_{4,36} = 0.64$, $P = 0.634$, status: ejaculate MDA, Pillai's trace = 0.05, $F_{4,36} = 0.52$, $P = 0.720$). The sperm mobility traits were significantly different between males of different mating tactics (Table 1). However, neither ejaculate SOD nor ejaculate MDA did correlate with sperm mobility traits (Table 1).

Sperm velocity (VCL, i.e. the curvilinear distance travelled over time) was significantly lower in harem males (Table 1 and Fig. 1) but did significantly correlate neither with ejaculate SOD nor with ejaculate MDA (Table 1).

Sperm motility (MOT, proportion of sperm moving) did not significantly diverge between males of different mating tactics and did significantly correlate neither with ejaculate SOD nor with ejaculate MDA (Table 1).

Sperm survival (i.e. the slope of decline in motility over time) was significantly lower in harem males (Table 1 and Fig. 2) and significantly positively correlated with ejaculate MDA (Table 1 and Fig. 3) but did not correlate with ejaculate SOD (Table 1).

Sperm stamina (i.e. slope of the decline in sperm velocity over time) did neither significantly diverge between males of

Table 1 Analyses of the sperm mobility (MANOVA and linear models)

Sperm mobility (MANOVA)		Pillai's trace	F_{DF}	P value
Tactic		0.26	$3.27_{4,36}$	0.021
Ejac. SOD (log)		0.09	$0.97_{4,36}$	0.437
Ejac. MDA (sqrt)		0.06	$0.60_{4,36}$	0.662
Velocity	Estimate	SE	F_{DF}	P
Intercept	70.18	4.95	$200.77_{1,41}$	<0.001
Tactic	−21.76	7.09	$9.41_{1,41}$	0.004
Ejac. SOD (log)	3.98	6.32	$0.22_{1,41}$	0.639
Ejac. MDA (sqrt)	−8.27	14.17	$0.34_{1,41}$	0.563
Motility (logit)				
Intercept	−1.18	0.25	$21.49_{1,44}$	<0.001
Tactic	0.16	0.25	$0.17_{1,44}$	0.683
Ejac. SOD (log)	−0.10	0.33	$0.08_{1,44}$	0.773
Ejac. MDA (sqrt)	−1.05	0.76	$1.93_{1,44}$	0.171
Survival				
Intercept				
Tactic	-6.68×10^{-5}	2.93×10^{-5}	$5.25_{1,44}$	0.027
Ejac. SOD (log)	2.99×10^{-5}	2.57×10^{-5}	$1.36_{1,44}$	0.251
Ejac. MDA (sqrt)	1.18×10^{-4}	5.85×10^{-5}	$4.10_{1,44}$	0.049
Stamina				
Intercept	-6.33×10^{-3}	3.35×10^{-3}	$356.11_{1,41}$	<0.001
Tactic	5.05×10^{-4}	4.80×10^{-4}	$1.11_{1,41}$	0.299
Ejac. SOD (log)	-1.15×10^{-4}	4.28×10^{-4}	$0.07_{1,41}$	0.790
Ejac. MDA (sqrt)	8.71×10^{-4}	9.59×10^{-4}	$0.83_{1,41}$	0.369

Comparisons between males of different mating tactics were performed with the sneaker males as reference. Type of data transformation is indicated in brackets. Transformed ejaculate (Ejac.) SOD and MDA values were centred

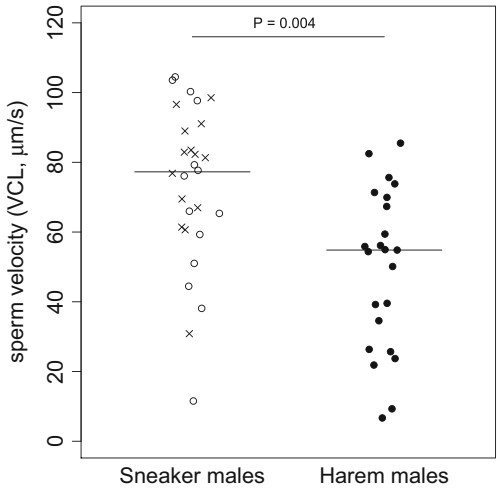

Fig. 1 Sperm velocity (VCL) of harem (*filled circles*) and sneaker males (*empty circles* for bachelors and *crosses* for peripheral males). The *bars* show the medians

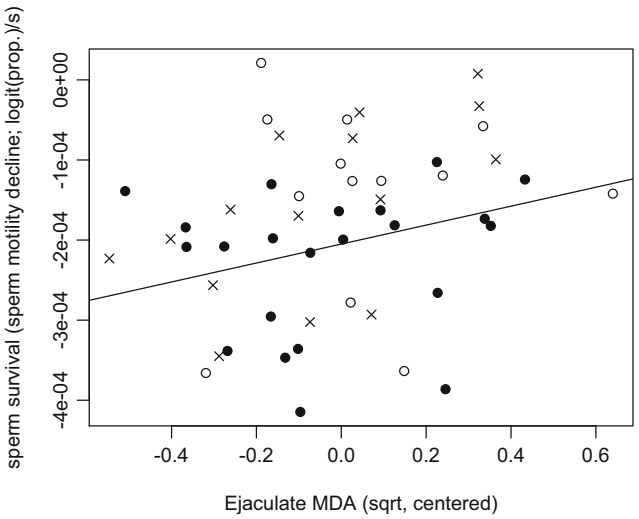

Fig. 3 Significant correlation ($P = 0.049$) between ejaculate MDA and sperm survival for harem (*filled circles*) and sneaker males (*empty circles* for bachelors and *crosses* for peripheral males)

different mating tactics nor correlate with ejaculate SOD nor correlate with ejaculate MDA (Table 1).

Blood redox profile

The blood redox markers showed a significant difference between males of different mating tactics (Table 2).

The ratio GSSG/GSH (oxidized glutathione over GSH) in RBC was significantly larger for harem males (Table 2 and Fig. 4). MDA concentration and SOD capacity in RBC did, however, not significantly diverge between males of different mating tactics (Table 2).

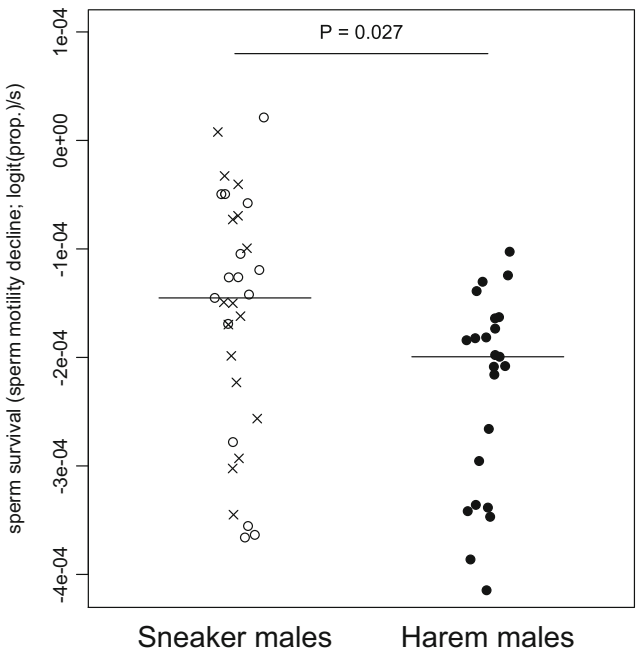

Fig. 2 Sperm survival of harem (*filled circles*) and sneaker males (*empty circles* for bachelors and *crosses* for peripheral males). The *bars* show the medians

Ejaculate redox markers

The ejaculate redox markers did not show a significant difference between males of different mating tactics (Table 3).

When analysed with univariate models, ejaculate SOD capacity and MDA concentration did not significantly diverge between individuals of different mating tactics (Table 3).

Table 2 Analyses of the red blood cell (RBC) redox markers (MANOVA and linear models)

RBC redox markers (MANOVA)		Pillai's trace	F_{DF}	*P* value
Tactic		0.12	$2.92_{1,68}$	0.041
GSSG/GSH (log)	Estimate	SE	F_{DF}	*P*
Intercept	−2.19	0.13	$283.66_{1,70}$	<0.001
Tactic	0.60	0.22	$7.89_{1,70}$	0.007
RBC MDA (sqrt)				
Intercept	0.68	0.04	$15.45_{1,67}$	<0.001
Tactic	−0.02	0.07	$0.05_{1,67}$	0.828
RBC SOD (log)				
Intercept	8.25	0.05	$25{,}208.26_{1,69}$	<0.001
Tactic	0.07	0.08	$0.64_{1,69}$	0.425

Comparisons between males of different mating tactics were performed with the sneaker males as reference. Types of data transformations are indicated in brackets

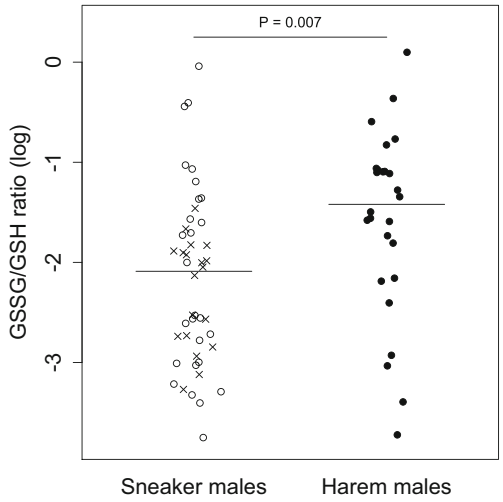

Fig. 4 Blood glutathione ratio of harem (*filled circles*) and sneaker males (*empty circles* for bachelors and *crosses* for peripheral males). The *bars* show the medians

Antioxidant allocation and oxidative stress in sperm vs. soma

Between males of different mating tactics, the ratio of ejaculate SOD capacity over RBC SOD capacity and the ratio of ejaculate MDA concentration over RBC MDA concentration were not significantly different (Table 3).

Discussion

In this study, we analysed putative sperm competitive traits and redox balance in sneaker and harem males of Seba's short-tailed bats. Sneakers comprise bachelor and peripheral males and have reduced access to females. They further face a higher level of sperm competition. When the social statuses (peripheral, bachelor or harem males) were used in the models instead of the mating tactics (sneaker versus harem males), we did not find any significant differences among the sneaker males (results not shown here). This supported us to merge sneaker males in the analyses.

In line with the predictions of sperm competition models and in opposition to the phenotype-linked fertility hypothesis, both sperm velocity and sperm survival were significantly higher in sneaker males. Responses to sperm competition have been observed among external fertilizers (Vladic and Jarvi 2001; Flannery et al. 2013; Locatello et al. 2013) but remain debated for internal fertilizers (Froman et al. 2002; Cornwallis and Birkhead 2006; Pizzari and Parker 2009; Lemaître et al. 2012; Schradin et al. 2012; Burger et al. 2015). Higher sperm velocity led to an enhanced fertilization success in competitive environments (Gage et al. 2004; Malo et al. 2005; Evans et al. 2013). The effect of sperm survival on sperm competition could be important when fertilization is delayed after mating (Snook 2005; Pizzari and Parker 2009; Smith 2012). Without stable access to fertile females, sneaker males may thus rely on high sperm survival to benefit from a longer mating period before the oestrus, compared to harem males. Sperm longevity was repeatedly seen traded off with velocity (Levitan 2000; Gage et al. 2004; Snook 2005; Smith 2012). With sperm both swimming faster and surviving longer, sneaker males of *C. perspicillata* may remain highly competitive even in harsh post-copulatory conditions (high sperm competition and delayed fertilization). However, in this study, the mobility analyses were performed in vitro. We therefore cannot ensure that the measured traits actually correlate with fertilization success in vivo in this species, even though sperm mobility appears to be crucial to reach the uterotubal junction in *C. perspicillata* (Rasweiler et al. 2010a).

Beside sperm mobility, males of different mating tactics may present varying sperm density and ejaculate volume (Stockley et al. 1994; Vladic and Jarvi 2001; Lemaître et al.

Table 3 Analyses of the redox markers in the ejaculate, the antioxidant allocation (Ejac./RBC SOD) and the oxidative damage in sperm versus red blood cells (Ejac./RBC MDA; MANOVA and linear models)

Ejaculate redox markers (MANOVA)		Pillai's trace	F_{DF}	*P* value
Tactic		0.01	$0.07_{2,48}$	0.968
Ejac. SOD (log)	Estimate	SE	F_{DF}	*P*
Intercept	5.47	0.12	$2218.43_{1,52}$	<0.001
Tactic	0.06	0.17	$0.11_{1,52}$	0.741
Ejac. MDA (sqrt)				
Intercept	1.17	0.05	$640.31_{1,53}$	<0.001
Tactic	−0.00	0.07	$0.00_{1,53}$	0.968
Ejac./RBC SOD (log)				
Intercept	−2.77	0.13	$428.12_{1,50}$	<0.001
Tactic	−0.07	0.20	$0.13_{1,50}$	0.725
Ejac./RBC MDA (log)				
Intercept	1.42	0.20	$48.08_{1,49}$	<0.001

Comparisons between males of different mating tactics were performed with the sneaker males as reference. Types of data transformations are indicated in brackets

2012; Schradin et al. 2012; Burger et al. 2015). As mentioned in the "Introduction" section, the method that we used to collect ejaculate provides different values for these traits compared to a natural ejaculation (Mattner and Voglmayr 1962; Moore 1985). Sperm density and ejaculate volume were therefore not considered and did actually not differ between mating tactics (ejaculate volume: Student's t test: $t = -1.58$, $df = 49.0$, $P = 0.121$; ejaculate density: Student's t test; $t = -0.80$, $df = 38.5$, $P = 0.429$).

Sperm competitiveness should be affected by oxidative damage to sperm (e.g. damage to sperm membranes or sperm DNA), caused by oxidative stress, which can be controlled by raising antioxidant defences (e.g. SOD). We thus predicted that sneaker males should allocate a larger portion of antioxidant resources to protect sperm. We did not find any negative correlation between sperm mobility measures and the levels of lipid peroxidation (MDA). Sperm survival even rose with increasing lipid peroxidation at ejaculation time. Reported level of lipid peroxidation may be sufficiently controlled so as to generate no particular detrimental effects on sperm mobility. The positive correlation with sperm survival may highlight the importance of controlled concentrations of ROS for sperm capacitation in mammals (de Lamirande and Cagnon 1993; Aitken and Fisher 1994). This mammalian maturation step renders sperm cells competent for fertilization through a succession of redox regulation events occurring on the cell membrane after the ejaculation (Aitken 2011). Furthermore, lipid peroxidation in the ejaculate was similar among individuals of different mating tactics, which may indicate that tightly controlled levels of lipid peroxidation are required in functional sperm. Beside lipid peroxidation, oxidative damage on sperm DNA may have instead affected the sperm mobility of harem males (Shen et al. 1999).

The ejaculate SOD levels did not differ between mating tactics and did not correlate with sperm mobility traits. Similarly, the ratio between ejaculate SOD capacity over blood SOD capacity did not differ between mating tactics, which shows that sneaker males are not allocating more SOD to the ejaculate than harem males. Sperm protection might have involved other antioxidants than SOD. For example, other endogenous antioxidants such as the enzymes catalase or glutathione peroxidase or dietary antioxidants such as vitamins or carotenoids may contribute to the antioxidant protection of sperm cells. The latter might play an important role in protecting cells against oxidative stress, in particular in frugivorous species such as Seba's short-tailed bat. Indeed, the highest antioxidant concentration and lowest oxidative damage among neotropical bats were found in frugivorous species (Schneeberger et al. 2014), which also showed lower levels of blood SOD compared to other bat species (Wilhelm Filho et al. 2007). Thus, fruit eaters may benefit from more antioxidants provided through their diet and these dietary antioxidants may favour an increase in sperm mobility by potentially affecting the ejaculate redox profile (Suleiman et al. 1996; Eskenazi 2004; Helfenstein et al. 2010; Almbro et al. 2011).

Alternatively, it may be that the difference in sperm mobility between harem and sneaker males does not depend on antioxidant resource allocation but on other types of resources or resources invested in other sperm traits, such as the seminal liquid (Perry et al. 2013), sperm membrane composition (delBarco-Trillo and Roldan 2014; delBarco-Trillo et al. 2015) or sperm morphology (Cornwallis and Birkhead 2006; Firman and Simmons 2010). Moreover, the copulation rate itself, which is expected to be higher for harem than sneaker males (Bradbury 1977; Dixson and Anderson 2004), may affect sperm performance due to a trade-off between sperm mobility and quantity (Cornwallis and Birkhead 2006; Wesseling et al. 2016). Additionally, an increase in the copulation frequency may affect the efficiency of the seminal glands, the prostate or the Cowper's glands (Dixson and Anderson 2004) and thus the quality of the seminal fluid.

Finally, we found assss higher ratio of oxidized glutathione (GSSG) over reduced glutathione (GSH) in the blood of harem males, without significant differences in blood MDA and SOD. This may indicate that securing privileged access to mates, which comprises guarding, courtship and vocalization behaviours, implies higher pre-copulatory investment, such as higher metabolism and elevated cellular detoxification, but does not generate excessive lipid peroxidation in harem males (Alonso-Alvarez et al. 2007; Guindre-Parker et al. 2013).

In conclusion, sneaker males showed higher ejaculate quality in terms of sperm velocity and sperm survival. This was not explained by an increase in SOD allocated to protect the spermatozoa or by a difference in lipid peroxidation in the ejaculate. Exogenous antioxidants, optimal ejaculation rate, adapted seminal products or modified sperm structures are potential alternative explanatory factors for the superior sperm mobility observed in sneaker males. Lastly, harem males seemed to experience higher ROS generation in blood, indicating a higher somatic investment, but appeared to be able to manage it without raising blood SOD capacity.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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