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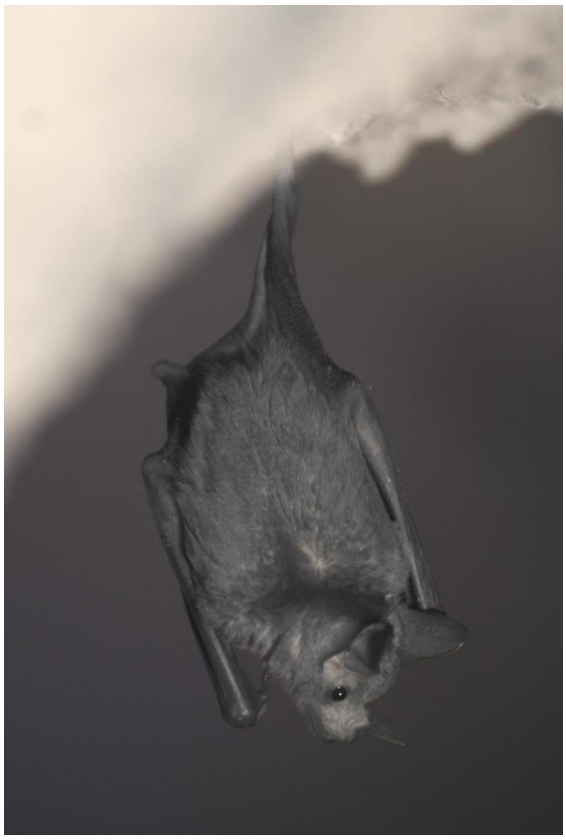
Oxidative stress as a life history constraint?

- Perspectives from a bat with Alternative Reproductive Tactics -

Magali Meniri



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UNIVERSITÉ DE NEUCHÂTEL

Faculté des Sciences
Secrétariat-décanat de Faculté
Rue Emile-Argand 11
2000 Neuchâtel – Suisse
Tél : + 41 (0)32 718 21 00
E-mail : secretariat.sciences@unine.ch

IMPRIMATUR POUR THESE DE DOCTORAT

La Faculté des sciences de l'Université de Neuchâtel autorise l'impression de la présente thèse soutenue par

Madame Magali MENIRI

Titre:

"Oxidative stress as a life history constraint : perspectives from a bat species with alternative reproductive tactics"

sur le rapport des membres du jury composé comme suit:

- Prof. Fabrice Helfenstein, directeur de thèse, Université de Neuchâtel, Suisse
- Dr Nicolas Fasel, co-directeur de thèse, IZW, Berlin, Allemagne
- Prof. Jacob Koella, Université de Neuchâtel, Suisse
- Prof. Andrea Pilastro, University of Padova, Italie
- Prof. Jonathan Blount, University of Exeter, UK

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Le Doyen, Prof. P. Felber

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General summary

In this thesis, I investigated the role of oxidative stress (OS) as a constraint for life history traits, using Seba's short-tailed bats (*Carollia perspicillata*) as a model species. In this species, males exhibit three alternative reproductive tactics having unequal pay-offs, and whose acquisition could be impacted by early life conditions. First, I studied the effect of OS as a constraint during early life. I induced early adverse conditions using food restriction followed by *ad libitum* feeding. Although we found that growth generated oxidative damage, we did not find physiological short-term costs (OS and glucocorticoids) to compensatory growth. Moreover, survival during the first year was not significantly impacted by our treatment. These results suggest that pups were able to efficiently mitigate the short-term consequences of early-life adverse conditions. Then, I tested the role of OS as a constraint for reproduction and investigated whether antioxidant protection could mediate the trade-off between pre- and post-copulatory traits, and thus explain the higher sperm quality found previously in sneaker males. In an experiment where we manipulated males' reproductive tactic, we found that all males, regardless of their tactic, exhibited similar sperm quality (sperm swimming performance and sperm morphology). Although oxidative damage negatively impacted sperm swimming performance, the redox profile of blood and ejaculates were similar for all males. Overall, our results suggest that a trade-off between investing in the soma or in the ejaculate might not occur. As other studies before, we did not find a correlation between sperm swimming performance and sperm morphology, which questions the existence of a functional link between those two traits. Finally, we propose that males may apply a "gametic bet-hedging" strategy, whereby they would produce highly morphologically variable sperm to optimize their sperm fertilizing abilities across varying sperm competition risks.

In conclusion, we found that early life was associated with elevated levels of OS, although compensatory growth did not entail physiological costs on the short-term. Moreover, oxidative stress did not seem to constraint the expression of alternative reproductive tactics. Overall, I suggest that OS might not represent a strong constraint in Seba's short-tailed bats. I suggest that harem males might invest in both pre- and post-copulatory traits to both attract females and secure fertilizations. Finally, I advocate for experimental studies to be conducted in the natural environment rather than in cages, and I designed a selective trap for that purpose.

General introduction

1. Oxidative stress

1.1 Oxygen, a great molecule for energy production, but at what costs?

About 2.45 billion years ago, the atmospheric concentration of oxygen increased suddenly, mostly due to the evolution of photosynthesis by cyanobacteria. This phenomenon known as the Great Oxidation Event (Sessions et al., 2009), allowed the evolution of aerobic metabolism, more energy efficient than the anaerobic pathways used until then. Oxidative phosphorylation, which takes place in the mitochondria, is a major source of ATP (cf Fig.1). Through multiple redox reactions, electrons get transported along the electron transport chain until the final electron acceptor, oxygen. Coupled with the electron transport chain, a proton gradient is created, used for the formation of ATP via the ATP-synthase. However, along the electron transport chain, the leakage of electrons leads to an incomplete reduction of O_2, and thus to the formation of the superoxide anion O_2^-. Reactive oxygen species (ROS) include oxygen radicals such as superoxide O_2^- and non-radicals derivatives of O_2 such as hydrogen peroxide H_2O_2, formed by dismutation of the superoxide anion (Halliwell and Gutteridge, 2007). ROS are very reactive, and can cause damage to DNA, proteins and lipids (Monaghan et al., 2009). Although the majority of ROS are generated as by-products of normal metabolic processes (Balaban et al., 2005), about 10% are formed by specific enzymes and have necessary functions in the organism. For example, ROS are involved in cell communication via redox signaling (Dröge, 2002). ROS also play an essential role in the innate immunity against pathogens (Bedard and Krause, 2007; Sadd and Siva-Jothy, 2006).

1.2 Antioxidant defense mechanisms

Antioxidants have evolved as defense mechanisms to cope with ROS. Antioxidants can be broadly defined as any mechanism, structure and/or substance that prevents, delays, removes or protects against oxidative non-enzymatic chemical modification (damage) to a target molecule (Pamplona and Costantini, 2011). The major molecular antioxidants are the enzymes superoxide dismustase

(SOD), which catalyze the dismutation of the superoxide anion into oxygen and hydrogen peroxide (Halliwell and Gutteridge, 2007), and the enzymes catalase (CAT) and glutathione peroxidase (GPx), which decompose hydrogen peroxide into water. GPx catalyze the oxidation of glutathione (GSH) to oxidized glutathione (GSSG), followed by the regeneration of GSH from GSSG using glutathione reductase (GR) and NADPH. Dietary antioxidants such as vitamins and carotenoids also play an important role against the damaging effect of ROS. Notably, tocopherols, also known as vitamin E, are lipophile molecules embedded in cell membranes. They can stop the chain reaction of lipid peroxidation occurring in cell membrane.

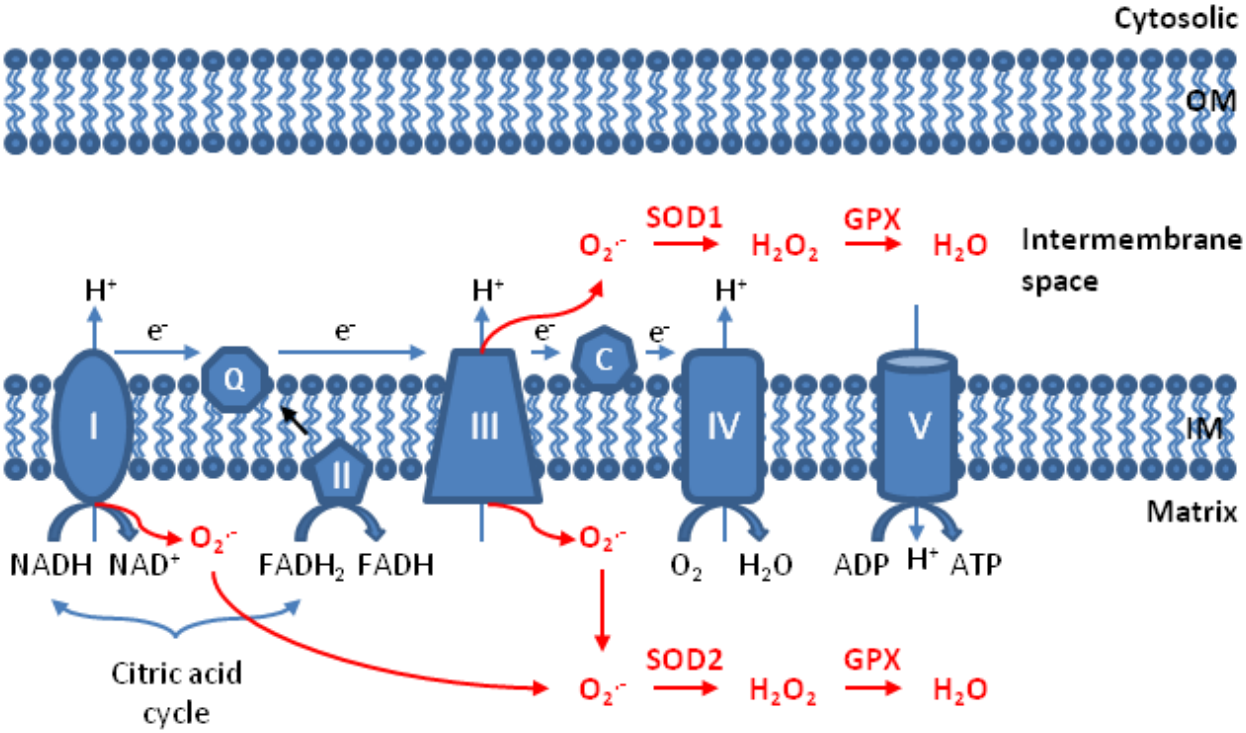


Figure 1: Schematic representation of oxidative phosphorylation. From Li et al. (2013)

1.3 Oxidative stress as a life history constraint?

Oxidative stress arises from an imbalance between the production of ROS and the antioxidant defense mechanisms (Halliwell and Gutteridge, 2007). Oxidative stress has been proposed to represent a potential mediator of major life history trade-offs (Metcalfe and Alonso-Alvarez, 2010; Monaghan et al., 2009). Indeed, the deleterious effects of ROS have an influence on many

aspects of an individual life history: early life (reviewed in Metcalfe and Alonso-Alvarez, 2010), reproduction (reviewed in Metcalfe and Monaghan, 2013), immunity (Schneeberger et al., 2013), ageing (reviewed in Finkel and Holbrook, 2000).

2. Early life conditions and oxidative stress

Adverse conditions during early life, either during gestation or after birth, can arise from a variety of factors, such as sibling rivalry (Nilsson and Gårdmark, 2001), inadequate temperature (Bizuayehu et al., 2015), suboptimal time of birth (Monclús et al., 2014) or strong predator pressure (Bell et al., 2011). When encountering sub-optimal conditions during early life, elevated levels of oxidative stress can be expected for a number of reasons.

2.1 Growth and oxidative stress

Often, adverse early life conditions result in a momentary decrease in growth rate, which is likely to occur regularly in the wild. Therefore, most species exhibit compensatory mechanisms to avoid detrimental consequences of a smaller adult size (Hector and Nakagawa, 2012). Mate acquisition, ability to defend a territory and susceptibility to predation are a few of the traits that could be negatively impacted by a smaller adult size (reviewed in Metcalfe and Monaghan, 2001). A common mechanism used to adjust phenotypic development in response to adverse conditions is compensatory growth (Metcalfe and Monaghan, 2001). Compensatory growth is defined as an acceleration of the growth rate, without necessarily reaching control size. Catch-up growth however, implies to reach the control size, which could be achieved solely by growing for longer (Hector and Nakagawa, 2012). Both mechanisms can act together to attain adult size. Normal growth rate is considered to be optimal, by opposition to maximal growth rate. The latter is more demanding and may lead to short and long-term effects on individuals (Mangel and Munch, 2005; Metcalfe and Monaghan, 2001).

On the short-term, compensatory growth is expected to lead to higher level of oxidative stress, as high metabolic activity is required to sustain increased growth rate (Monaghan et al., 2009). An experiment in the zebra finches *Taeniopygia guttata* found that growth rate was negatively correlated to the susceptibility to oxidative damage during the compensatory growth phase (Alonso-Alvarez et al., 2007). In damselflies *Lestes viridis,* individuals exhibited higher

levels of antioxidant at the end of the compensation phase, suggesting an oxidative challenge linked to higher growth rate (De Block and Stoks, 2008). Compensatory growth was also found to accelerate telomere attrition rate and oxidative stress in 3 months old rats *Rattus norvegicus* (Tarry-Adkins et al., 2008, 2009).

2.2 Glucocorticoids and oxidative stress

Hormones play a major role in mediating the effect of environmental factors on the organism. The Hypothalamus-Pituitary-Adrenal (HPA) axis coordinates the baseline level production of glucocorticoids and helps sustain normal energetic requirement. Moreover, the HPA axis, together with the Sympathetic Adreno-Medullary (SAM) system, mediates the acute stress response (cf box 1). Despite the numerous studies investigating the impact of glucocorticoids levels on fitness (Henderson et al., 2017; Ouyang et al., 2013; Romero and Wikelski, 2010; Vitousek et al., 2014), it remains complex to draw conclusions. Indeed, glucocorticoids levels vary dynamically with the environment and are highly variable between individuals (Bonier et al., 2009; Crespi et al., 2013; Dantzer et al., 2016). Individual variation may partly rise from early life conditions experienced by the individual. Indeed, early life conditions have been shown to program the HPA axis durably (reviewed in van Bodegom et al., 2017). Programming of the HPA axis involves modifications in the basal level of circulating glucocorticoids (Rice et al., 2008), but also in the magnitude of the response in case of an acute stress (Hayward et al., 2006). This early life programming may lead to either a down-regulation or an up-regulation of the stress response physiology, depending on factors such as sex, timing of the stressor during an individual's development, and its duration (Levine, 2005). Therefore, more longitudinal studies are required to understand how glucocorticoids levels are impacted by early life adversity, and the long-term consequences of such programing.

Importantly, glucocorticoids are predicted to induce oxidative stress (Costantini et al., 2011). When facing early life adversity, individuals are likely to experience elevated levels of glucocorticoids, and thus to be submitted to the detrimental consequences of elevated level of oxidative stress. Glucocorticoids may generate oxidative stress via an increase in ROS production (You et al., 2009), a decrease in antioxidant protection mechanism (Stojiljković et al., 2009) and a decrease in repair mechanism (reviewed in Haussman and Marchetto, 2010). The pre-natal manipulation of glucocorticoids in chickens *Gallus domesticus* led to increased levels of ROS, and shorter telomeres (Haussmann et al., 2012)

Moreover, in both control and treated individuals, Haussemann et al. (2012) showed an increase in oxidative stress during an acute stress response. In humans, early life stress such as exposure to violence and maltreatment has also been linked to accelerated telomere shortening (Asok et al., 2013; Shalev, 2012). Thus, oxidative stress could represent a physiological mechanism to the deleterious consequences of chronic stress (Zafir and Banu, 2009).

2.3 Long term consequences of early life adversity

Depending on the timing and the intensity of the compensatory growth, negative consequences can also arise on the long-term. One-year old rats that had previously undergone compensatory growth showed higher oxidative stress in muscles, associated with increased telomere shortening compared to control individuals (Tarry-Adkins et al., 2016). Interestingly, after a compensatory growth, accelerated telomere attrition (Geiger et al., 2012; Tarry-Adkins et al., 2016) and reduced lifespan (Lee et al., 2013b; Ozanne and Hales, 2005) are often reported, and have been linked to elevated oxidative stress (von Zglinicki, 2002). Several studies have shown that singing performances in adult birds are negatively impacted by developmental stress (Buchanan et al., 2003; Nowicki et al., 2002), suggesting impaired cognition. In zebra finches, compensatory growth was associated with a negative impact on cognitive abilities (Fisher et al., 2006). It has been hypothesized that oxidative stress might be associated with cognitive impairment, as highlighted by cognitive decline during aging in humans (Baierle et al., 2015; Revel et al., 2015). Furthermore, a study conducted on rats has showed that vitamin E supplementation could prevent the negative impact caused by oxidative stress on cognitive abilities (Fukui et al., 2006). Finally, a lot of attention has recently been devoted to the impact of early life conditions on the ontogeny of behavior. For example, coping behaviors, *i.e.* behaviors exhibited to respond to environmental challenges, are expected to be affected by early life conditions (reviewed in Langenhof and Komdeur, 2018). In a cooperatively breeding fish *Neolamprologus pulcher*, early social environment has been shown to impact the response to a social challenge as adults (Nyman et al., 2017). In males' rock pigeons *Columba livia*, food restriction during early life has been shown to impair courtship behaviors and pair-bonding behavior (Hsu et al., 2017). In humans, studies have shown that early life stress increases the likelihood of mental disorders later in life (Carr et al., 2013; Schiavone et al., 2015). Importantly, effects of early conditions can also be indirect and influence future generations, as reviewed by Burton and Metcalfe, (2014), sometimes without affecting the individuals experiencing those conditions. In zebra finches, body size of the offspring

was impacted by the early environment experienced by their mother, in a sex-specific way (Naguib and Gil, 2005).

Overall, early life conditions have been found to affect a wide range of morphological, physiological and behavioral traits, and the negative consequences can often be linked to an increase in oxidative stress.

Box 1: Acute stress response

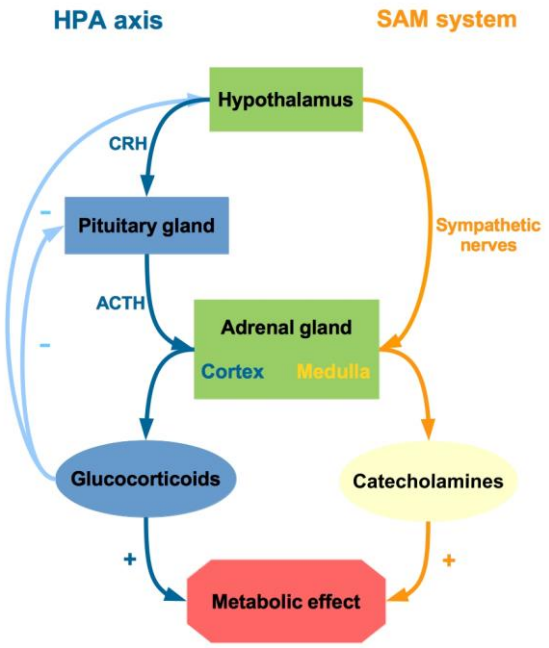


Figure 2: Acute stress response

Activation of the HPA axis

The hypothalamus secretes Corticotropin Releasing Hormone (CRH), which will act on the pituitary gland to promote the secretion of AdrenoCorticoTropic Hormones, which will stimulate the production of glucocorticoids in the cortex part of the adrenal gland. A negative feedback loop, triggered by glucocorticoids, acts on the pituitary gland and the hypothalamus to regulate the production of glucocorticoids.

Activation of the SAM system

The sympathetic nervous system gets activated, leading to the production of catecholamines in the medulla part of the adrenal gland.

A stressor can broadly be defined as "conditions where an environmental demand exceeds the natural regulatory capacity of an organism, in particular situations that include unpredictability and uncontrollability" (Koolhaas et al., 2011).

When a stressor is perceived, two systems act in coordination in a process called stress response. The Sympathetic Adreno Medullary (SAM) system allows for a quick response, referred to as the *"Fight or flight response",* term coined by Cannon (1927). Once a threat is detected, hypothalamus leads via the sympathetic nerves to the production in the adrenal medulla of catecholamines epinephrine (adrenaline) and norepinephrine (noradrenaline), to quickly provide metabolic support in order to face the threat: increase of blood sugar, increase heart rat, etc. The HPA axis mediated response takes place after 1 minute, and the peak response is seen after about 20 min. It modulates the release of glucocorticoids, which in concert with catecholamines will enhance and prolong for several hours the metabolic support, and lead to physiological and behavioral changes, such as increased cognition, and awareness. Physiological responses include a decrease in non-essential functions of the organism strong suppressive effect on the immune system, decrease reproductive physiology, digestion, in order to cope with the stressor (Sapolsky et al., 2000). The HPA axis, in opposition to SAM system, is tightly controlled by a negative feedback loop, which acts to get levels of circulating glucocorticoids back to baseline level. Glucocorticoids and mineralocorticoids receptors are distributed ubiquitously throughout the body. The timing, duration and intensity of the stress response vary widely between individuals and are expected to be programmed by early life conditions.

3. Oxidative stress and reproduction in males

Reproduction is physiologically costly, and high investment in current reproduction is expected to reduce future fecundity or survival (Stearns, 1992; Williams, 1996). Oxidative stress has been hypothesized to represent a cost of reproduction (Alonso-Alvarez et al., 2004; Metcalfe and Monaghan, 2013). In a wild population of Florida scrub jays *Aphelocoma coerulescens*, Heiss and Schoech, (2012) showed that males with lower pre-breeding levels of oxidative damage presented higher subsequent reproductive effort, and that the post-breeding levels of oxidative damage were significantly higher compared to pre-breeding ones. Losdat et al. (2011) showed in great tits *Parus major* that experimentally increased brood size resulted in reduced antioxidant capacity in males. Another study found an increase of oxidative damage with litter size in female chipmunks *Tamias stratus* (Bergeron et al., 2011). Here, we focus on the traits associated with obtaining a mate and fertilizing the eggs (cf box 2), and how oxidative stress may impact the relative investment to both types of traits.

3.1 Oxidative stress and ejaculate quality

Sperm competition (cf box 2) represents a major force shaping the evolution of ejaculate fertilization abilities in a competitive environment, for example by selecting for improved sperm quality (Fitzpatrick and Lüpold, 2014; Simmons and Fitzpatrick, 2012). Sperm quality refers to a wide range of traits related to male fertility (Snook, 2005). However, oxidative stress might represent a constraint for ejaculate quality, as sperm cells are very prone to oxidation.

3.2 What makes a good ejaculate?

If sperm competition is considered to be a fair raffle, the more tickets a male buys, *i.e.* the more spermatozoa he inseminates, the more likely he is to win the lottery, *i.e.* to fertilize the eggs (Parker, 1990). Although sperm competition does not always correspond to a fair raffle, sperm number is then expected to be a key component of fertilization success as shown in the guppy *Poecilia reticulata* (Boschetto et al., 2011), and in the bluegill sunfish *Lepomis macrochirus* (Neff et al., 2003). Besides sperm number, sperm swimming abilities are expected to also impact fertilization success (Simmons and Fitzpatrick, 2012; Snook, 2005). For example, sperm velocity has been shown to positively impact fertilization (Gasparini et al., 2010; Malo et al., 2005a). The

proportion of motile sperm has also been linked to higher fertilization efficiency (Asa et al., 2007; Pusch, 1987), and has been shown to increase with the level of sperm competition (Montoto et al., 2011). In species with sperm storage, *i.e.* where copulation and fertilization are decoupled, sperm longevity is expected be an important determinant of fertilization efficiency. Notably, velocity and longevity are predicted to be traded off against one another (Levitan, 2000).

Sperm morphology is extremely diverse across species and is also expected to evolve under sperm competition pressure. Normal sperm morphology is important for fertilization (Sakkas et al., 2015). Moreover, the percentage of abnormal sperm has been shown to be negatively correlated with the level of sperm competition (Rowe and Pruett-Jones, 2011). Sperm length has been shown to increase with the level of sperm competition inter-specifically (*frogs:* Byrne et al., 2003; *fishes:* Fitzpatrick et al., 2009; *butterflies:* Gage, 1994; *mammals:* Gomendio and Roldan, 1991; *snakes:* Tourmente et al., 2009). To explain the increase in sperm length, one hypothesis proposed that longer sperm swims faster (Gomendio and Roldan, 1991), as a longer flagellum could provide more propelling force, and a longer mid-piece generate more energy. However so far, results remain very inconclusive, despite active research. Indeed, comparative studies have found a correlation between sperm length and sperm swimming abilities in several taxa (*fishes:* Fitzpatrick et al., 2009; *mammals:* Gomendio and Roldan, 2008; Tourmente et al., 2011; but see in *birds* Lüpold et al., 2009). However, intra-specifically, results are very mixed. Some studies have found positive associations (Firman and Simmons, 2010; Lifjeld et al., 2012; Losdat and Helfenstein, 2018; Malo et al., 2006; Mossman et al., 2009), while others show negative correlations (Cramer et al., 2015), or even a lack of correlation (Denk et al., 2005). Overall, more research in more species is required to get a better understanding of the potential link between sperm morphology and sperm swimming abilities.

Recently, seminal fluid composition has attracted attention, as it became clear that its composition could impact sperm fertilization abilities in many ways (Perry et al., 2013; Poiani, 2006). For example, Bartlett et al. (2017) have shown that ejaculate quality, and specifically rapid changes in sperm velocity, are mediated by seminal fluid in the chinook salmon *Oncorhynchus tshawytscha.* Moreover, in fruit flies, seminal proteins transferred in the ejaculates can influence female's remating behavior (Chapman and Davies, 2004).

3.3 Impact of oxidative stress on ejaculate quality

Sperm cells are extremely susceptible to ROS, partly because of the high content in polyunsaturated fatty acids (PUFAs) of their membrane, less resistant to peroxidation than monounsaturated or saturated fatty acids. PUFAs contribute to membrane fluidity, required for sperm-oocyte fusion and therefore fertilization (Halliwell and Gutteridge, 2007). The peroxidation of PUFAs has also been linked to a loss in viability and motility of the sperm (De Lamirande and Gagnon, 1992; Gomez et al., 1998). Moreover, the limited DNA repair occurring in sperm cells also exacerbates the vulnerability to oxidative stress (Lewis and Aitken, 2005). The deleterious effect of ROS on sperm can impair fertilization ability, and if fertilization does occur, it may decrease the rate of implantation, and increase the rate of post-implantation loss (O et al., 1988), leading to failure of embryo development (Morris et al., 2002). In humans, oxidative damage could be a major cause of infertility (Tremellen, 2008).

However, at low concentrations, ROS may play a key role in controlling normal sperm function, leading to successful fertilization (Baker and Aitken, 2004; Halliwell and Gutteridge, 2007). Indeed, ROS may have positive effects on the sperm cell during maturation, as well as the post-ejaculatory capacitation, the acrosome reaction, and the sperm-oocyte fusion (Wagner et al., 2018). In humans, it has been shown that a decrease in hydrogen peroxide level leads to a decrease in tyrosine phosphorylation, associated with a loss of fertilizing capacity (Aitken et al., 1995). Overall, oxidative stress may be a key factor shaping the fertilization success of the sperm (Silva et al., 2010; Wagner et al., 2018).

4. Oxidative stress and the pre- vs. post-copulatory traits trade-off

Researchers have tried to understand the relative investment between pre-copulatory sexually selected traits, *i.e.* traits impacting the ability to acquire a mate, and post-copulatory sexually selected traits, *i.e.* traits impacting the probability to fertilize the egg. We propose that oxidative stress could represent a constraint for both types of traits, and therefore that strategic antioxidant resource allocation between them may be modulated according to individual reproductive tactics.

4.1 Be attractive, have good ejaculates, or do both?

Are individuals able to maximize both type of traits, or do they trade them off? What are the consequences of this relative expenditure on reproductive success? The phenotype-linked fertility hypothesis proposes that more attractive males should also exhibit sperm with higher fertilizing abilities, as phenotype is expected to be an honest predictor of the male's fertility (Sheldon, 1994). For example, in the guppies *Poecilia reticulata,* more colorful males have sperm of higher quality (Locatello et al., 2006). In the red deer *Cervus elaphus*, antlers size is positively correlated with sperm quality (Malo et al., 2005b). In opposition, sperm competition models assume that relative investment to pre and post copulatory selected traits trade-off against each other. Empirical evidence of this trade-off has been found in several taxa (*birds:* Froman et al., 2002; *reptiles:* Kahrl et al., 2016; *beetles:* Simmons and Emlen, 2006; *fishes:* Young et al., 2013). Recently, Lüpold et al. (2014) proposed that the phenotype-linked fertility hypothesis and the sperm competition models might be two sides of the same coin. They showed that whether a positive or negative relationship occurs between pre and post-copulatory traits is mediated by the ability of males to monopolize females. In species were dominant males are able to monopolize females, they are not expected to invest heavily in both types of traits, but to favor pre-copulatory traits. However, in species were males are unable to do so, and therefore might face sperm competition, they are expected to invest also in sperm fertilizing abilities, therefore exhibiting a positive correlation between the two types of traits. Therefore, the phenotype-linked fertility hypothesis might only describe a particular situation where males are unable to monopolize females, and therefore have to invest in both types of traits in order to maximize their fitness.

4.2 Sperm competition models

First, sperm competition models predict a different investment in pre vs post-copulatory selected traits depending on male's relative access to females. To maximize their fitness, males with a lower access to females (*eg:* subordinate males) are predicted to invest relatively more in their ejaculate quality. Conversely, males with a favored access to females are predicted to maximize their reproductive success by investing relatively more towards pre-copulatory selected traits. In chinook salmons, sneaker males have been shown to have higher fertilization efficiency than dominant males in a competitive setting (Young et al., 2013). Second, post-copulatory expenditure is expected to increase with increasing level of sperm competition. Comparative studies showed

that testes mass relative to body mass increases with the level of sperm competition (Kenagy and Trombulak, 1986; Short, 1979). However, sperm competition models distinguish between the *risk* of sperm competition, when the ejaculates of one to two males compete for fertilization, and the *intensity* of sperm competition, where more than two males' ejaculate compete for fertilization. Investment per ejaculate is predicted to be highest at intermediate level of risk, and to decrease as the intensity of sperm competition increases. Indeed, in two species of goby *Zosterisessor ophiocephalus* and *Gobius niger,* sneaker males have been found to reduce the number of sperm released as the intensity of sperm competition increased (Pilastro et al., 2002). More drastically, in the promiscuous thirteen-line ground squirrels *Ictidomys tridecemlineatus* in which fertilization is characterized by first male precedence, males have been shown to avoid copulation with mated females when the fertilization pay-off is lower that what could be achieved with other females (Schwagmeyer and Parker, 1990).

Box 2: Reproduction, a complex story

Finding a mate …

Listening to birds relentlessly singing their complex trills. Being mesmerized by the courtship of the peacock spider. Admiring tropical fishes, with their colorful and intricate patterns of coloration. Watching two powerful elephants seals fiercely fighting. These are a few examples of the many spectacular events that can be observed in nature. The existence of such extravagant traits, which rather than increasing survival are likely to carry great costs, deeply startled Darwin for years. He even wrote to a colleague "the sight of a feather in a peacock's tail, whenever I gaze at it, makes me sick!". Nevertheless, a few years later, he proposed the theory of sexual selection to explain the evolution of such elaborated ornaments, as a force that would promote traits increasing mating success (Darwin, 1871). One century later, it is well accepted that the evolution of extravagant features, so-called secondary sexual characters, are driven by sexual selection. Darwin proposed that sexual selection could operate at two levels: (1) intrasexual selection: competition between individuals of the same sex to acquire a mate, for example leading to the evolution of exaggerated weapons; and (2) intersexual selection: mate choice from individuals of the opposite sex, which promotes the evolution of elaborated courtship, with behavioral and morphological ornaments. The idea that one sex, often females, could discriminate and choose between males was not readily accepted at the time. Nowadays, it is well recognized that mate choice occurs, from the female, but also from the male. For example, in the Red-necked phalaropes *Phalaropus lobatus*, males provide paternal care alone, and are the choosy sex, while females are more colorful, and more aggressive than males (Reynolds, 1987).

… is not always enough to reproduce!

Contrary to recent beliefs, once individuals acquire a mate, they don't always live happily ever after. Actually, more often that not, females copulate with more than one male during a single reproductive bout. Thus, selection continues after mating in a process known as post-copulatory sexual selection. Post-copulatory selection can act through sperm competition, which occurs when ejaculates from different males compete to fertilize a given set of ova, as first conceptualized by Parker (1970). Cryptic female choice is another mechanism of post-copulatory selection. It describes the selection operated by females regarding the sperm cells that will fertilize her eggs, to bias the outcome of sperm competition. This process is cryptic, as it occurs inside the female's reproductive tract and remains poorly understood (Eberhard 1996).

Figure 3: a. Males Arabian oryx *Oryx leucoryx* fighting. b. Display of peacock spider *Maratus caeruleus*, picture by Jurgen Otto. c. Sperm competition in action! *Drosophila melanogaster* female's reproductive tract filled with ejaculates of two competing genetically modified males to produce either red or green fluorescent sperm cells. Picture from http://www.lupoldlab.net/research.html

4.3 Alternative reproductive tactics

In many species, in order to maximize their lifetime reproductive success, males adopt alternative reproductive tactics. ARTs refer to discontinuous behavioral and other traits selected to maximize fitness in two or more alternative ways – mutually exclusive – in the context of intraspecific and intrasexual reproductive competition (Taborsky et al., 2008). Generally, two types of tactics can be found within a species: a *bourgeois* tactic, where individuals will invest resources in order to attract mates such as ornaments or the defense of a breeding territory, and an alternative *sneaker* tactic, which may exploit this investment.

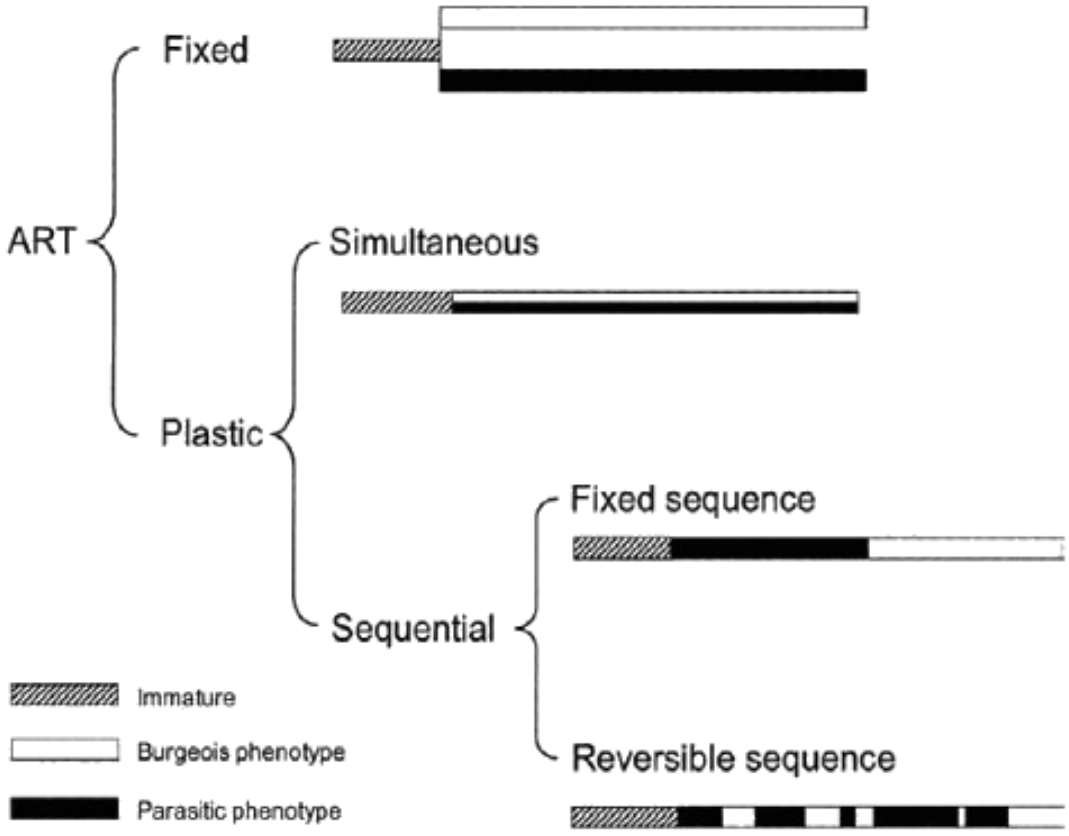


Figure 4: Alternative reproductive tactics. From Taborsky et al. 2008

ARTs can be fixed throughout an individual lifespan, either determined genetically, as for the dwarf male tactic in *Lamprologus callipterus* (von Kuerthy et al., 2016), or based on the conditions during ontogeny as for the dung beetles *Onthophagus taurus* (Moczek et al., 2002). Alternatively, ARTs can be plastic, where individuals can switch between tactics, either once or

reversely during their life (cf. Fig. 4). The North American green tree frog *Hyla cinerea* is an example of flexible tactics, where males can switch between the bourgeois tactic consisting of singing to attract females, or sneaking copulations with females attracted by a singing male (Humfeld, 2013). Males adopt the optimal tactic considering the current social and ecological constraints. When ARTs are based on environmental or phenotypic factors, they can result in equal pay-offs. In the damselfly *Mnais pruinosa costalis*, males exhibit two morph types: orange winged males, territorials, and clear-winged males, mimicking females. Even though clear-winged males have lower daily reproductive success, they live longer than orange winged males and therefore, there is no difference on the lifetime reproductive success between the two morphs (Tsubaki et al., 1997). On the other hand, for ARTs with unequal pay-offs, one tactic results in the most mating opportunities, and the males adopting the alternative tactic have to "make the best of a bad job" (Eberhard, 1982). Such ARTs are conditionally dependent of the frequency of dominant males, and the subordinate will adopt an alternative tactic that provides some immediate reproductive success or makes them more competitive for future reproductive success (Oliveira et al., 2008a). For example, in sassaby antelopes *Damaliscus lunatus* some males adopt a resource defense polygyny tactic, defined as a mating tactic where one male copulates with several females, that are attracted by the ressources defended by the male (Emlen and Oring, 1977), in this case territories containing food resources. The other males join leks, *i.e.* aggregations of males displaying for females. In the leks, males sassaby antelopes spend more time in contest behavior and obtain fewer matings compared to males defending a territory. These smaller lek males might be unable to acquire resource territories (Gosling and Petrie, 1990).

The factors leading to the evolution of ARTs are poorly understood (Taborsky and Brockmann, 2010). Generally, the occurrence of ART seems to be related to the intensity of sexual selection, and to be favored by the possibility of exploiting the investment of same sex conspecific competitors to acquire mates or fertilizations (Taborsky and Brockmann, 2010). Interestingly, the factors leading to the occurrence of genetic and conditional tactics are different, the former being facilitated by disruptive selection (Engqvist and Taborsky, 2016).

The physiological mechanisms underlying the expression of ARTs are likely to differ whether the tactics are fixed or can be switched sequentially. For flexible tactics, a multiple switch mechanism is required, whereas a permanent organization is expected for fixed tactics (Oliveira et al., 2008b). Hormones could mediate the switch to another tactic, as they can have both an organizational effect, for example when expressed during early life, or a transient, activational

effect. It is interesting to note that since hormones are influenced by the environment, they can interact with the tactic exhibited, leading to multiple possible hormonal profile.

4.4 What about oxidative stress?

According to the sperm competition models, males are predicted to invest their resources towards pre and post-copulatory traits differently based on their access to females. We propose that oxidative stress could mediate such a trade-off, as it is expected to represent a major constraint for both types of traits. Indeed, pre-copulatory traits such as courtship behaviors (Friesen et al., 2017; Mowles and Jepson, 2015) or territory defense (Marler et al., 1995; Ros et al., 2006) have been shown to generate high metabolic expenses, which might lead to enhanced ROS production, requiring increased somatic maintenance. Moreover, ejaculate quality is highly susceptible to oxidative stress (Lewis and Aitken, 2005). Therefore, we postulate that antioxidant resource allocation could differ between reproductive tactics. Sneaker males, which always experience sperm competition, are expected to favor the antioxidant protection of their ejaculates to optimize the fertilizing ability of their sperm. In contrast, dominant, bourgeois males are expected to favor the antioxidant protection of their soma. The trade-off between soma vs ejaculate quality has recently been shown to be mediated by oxidative stress in house sparrows *Passer domesticus,* a species in which social status determines the number of copulations (Mora et al., 2017). Mora et al. (2017) found that antioxidant resource allocation varied according to the social status, and modulated sperm quality, both before and after experimental manipulation of the social status. Overall, subordinate males exhibited higher sperm quality and better antioxidant protection of their ejaculates compared to dominant males. A similar pattern has been shown in zebra finches, where individuals that exhibited initially a more intense carotenoid-based coloration suffered a decrease in sperm quality when facing an oxidative challenge (Tomášek et al., 2017).

Box 3: Sperm competition and reproductive success in Seba's short-tailed bats

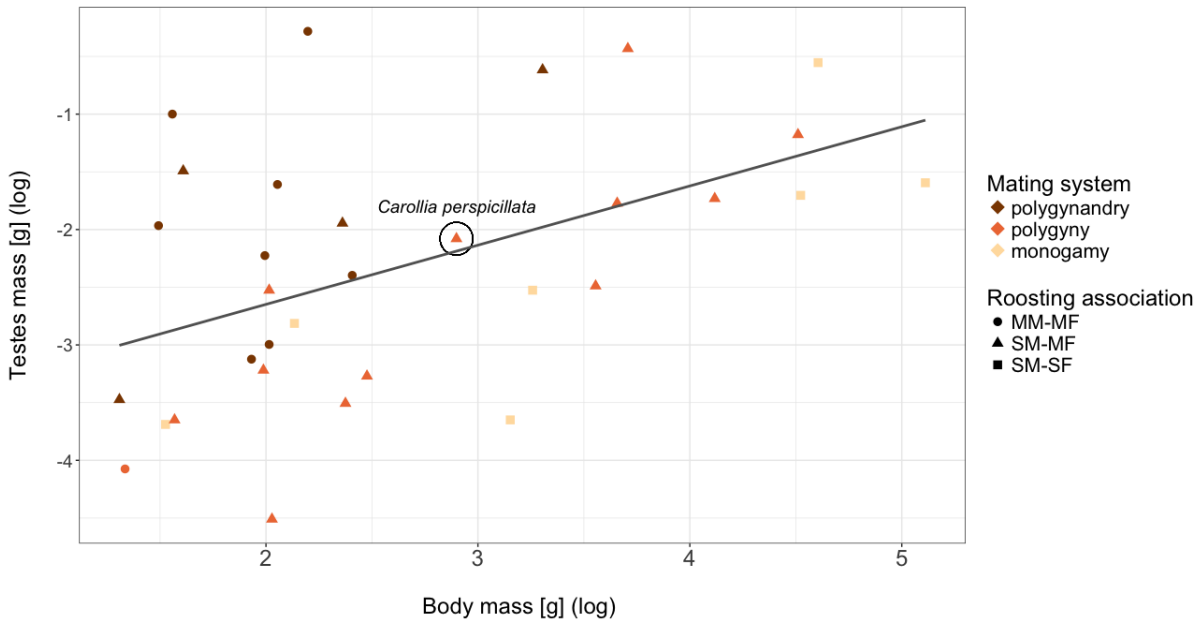
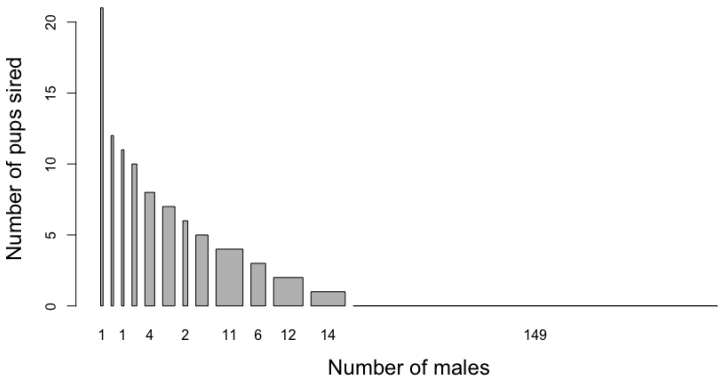


Figure 5: Relationship between body mass and testes mass in bats, described by mating system and roosting associations (MM-MF: Multiple Males-Multiple Females; SM-MF: Single Male-Multiple Females; SM-SF: Single Male-Single Female). Data adapted from Pitnick et al., 2006.

The testes size relative to body size is expected to increase with the level of sperm competition (Kenagy and Trombulak, 1986 ; Short, 1979), to allow for a higher production of sperm, leading to higher volume ejaculates with an increased sperm concentration (Møller, 1988). Therefore, relative testes size can be used as a proxy for the level of sperm competition. Compared to other species of bats, the relative testes size of *C. perspicillata* is slightly above the regression line, indicating possible risks, but low intensity of sperm competition.



Reproductive success is highly skewed towards a few males in the population.

Figure 6: Reproductive success over a period of 2.5 years for males of the population we monitor.

Box 3: Sperm competition and reproductive success in Seba's short-tailed bats

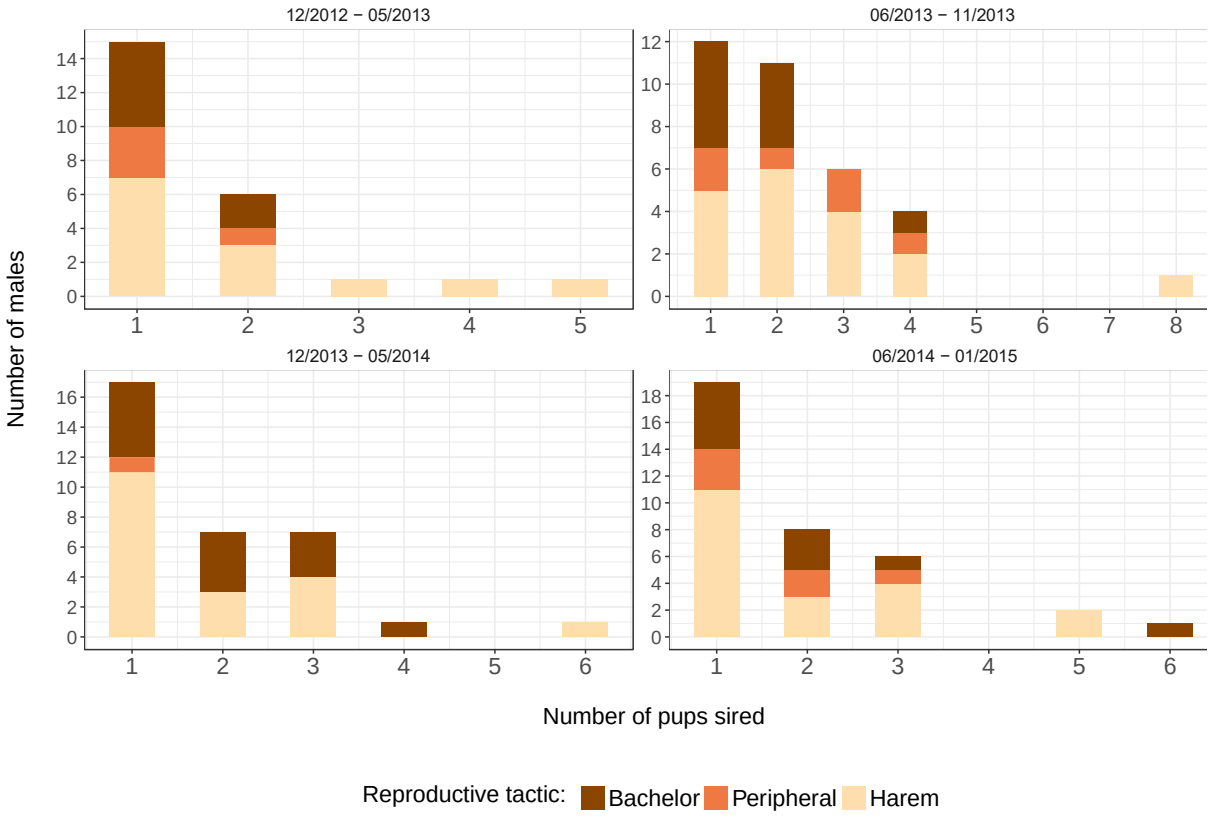


Figure 7: Reproductive success shown by reproductive tactic in our population

Most males that reproduce are only able to sire 1 to 2 offspring per 6 months period. Peripheral and bachelors males are able to sire a total of 40% of the offspring over the 2.5 years period. This shows that sperm competition is present in our population.

Overall, these results indicate that sperm competition does occur in our population, but is likely limited to the "risk" range, whereby the sperm of sneaker male are expected to compete against the sperm of one rival, the harem male, whereas harem males are rarely expected to face sperm competition at all.

5. Study species

The Seba's short-tailed bats *Carollia perspicillata* is a common species found in the Neotropics, belonging to the Phyllostomidae family. Frugivorous, they mainly feed on piper fruits, but their diet includes about 50 species of fruits, and therefore, they play an important ecological role as seed dispersers. Females present a seasonal bimodal polyoestry with post-partum oestrus, giving birth to a single pup per oestrus. The first birth is closely followed by a post-partum oestrus and thereafter females are simultaneously pregnant and lactating.

Seba's short tailed bats are gregarious, roosting in colonies of 10-100 in caves and hollow trees. Their mating system is described as resource defense polygyny (Williams, 1986), and males exhibit three ART's. First, harem males defend a territory used by females to roost, and therefore have stable access to females. In contrast, bachelor males, which gather in large groups during the day, and peripheral males which show high spot fidelity at the periphery of a harem territory, but are rarely observed with females, are both expected to sneak copulations. In the Seba's short-tailed bats, ARTs are flexible, but the probability of transition within a 6-months period from a bachelor tactic to a harem one is only of about 10 %, where peripheral males have about 50 % probability to transit to both tactic. When given the opportunity, males can rapidly switch tactics to become territorial males, within a few days (pers. observation). However, it is unknown whether physiological changes occur in relation to the tactic switch, and how long such changes might be detectable. Importantly, neither age nor size are explaining the acquisition of a particular ART, and testosterone level does not differ between individuals of different reproductive tactic. Moreover, ARTs have unequal pay-offs, with harem males siring 60% of the pups in our population, thus with 40% of the pups being sired by sneakers (Fasel et al., 2016). All males, regardless of their tactics, exhibit similar testes size (unpublished data). These results were previously interpreted as a sign that sperm competition is very high in Seba's short-tailed bats. However, the relative testes mass is only slightly higher than predicted by the regression of testes mass to body mass (cf box 3, Fig. 5). Further, the reproductive success is highly skewed in the population (cf box 3, Fig. 6), although some sneaker males are able to achieve fertilization and paternity (cf box 3, Fig. 7). Therefore, I suggest that in the Seba's short-tailed bats, levels of sperm competition are limited to a variation in the risk, rather than the intensity of sperm competition. In such a system, sneaker males are expected to face a high risk that their sperm have to compete with the sperm of one rival male, the harem male, whereas harem males are expected to be rarely exposed to sperm competition. As predicted by the sperm competition models (Parker,

1990; Parker et al., 2013), a previous study conducted on the Seba's short-tailed bats has found that sneakers exhibit higher sperm quality than harem males (Fasel et al., 2017).

6. Study site

Two captive populations of *Carollia perspicillata* are housed at the Papillorama (Kerzers, Switzerland), a tropical zoo. Both colonies can fly freely in a 40m diameter dome, and roost in an artificial cave. The main colony comprises about 400 bats, which are monitored according to the long-term monitoring described below. In this dome, the light cycle is reversed, the night time being from 9:30am to 9:30pm. The food, a fruit-based mixture, is provided twice a day. For the smaller colony of about 200 bats, the light cycle is not reversed. Moreover, the individuals in this population are not monitored.

7. Long term monitoring

The main population has been followed since 2011 using a mark recapture protocol. All adult individuals are marked in the population, with only few exceptions. Bats are ringed with a unique combination of three plastic rings on their forearm, allowing individual recognition. Moreover, they are equipped with PIT-tags, inserted between the scapulas. An opportunistic trapping is performed monthly, where measurements were conducted on marked individuals, whereas unknown juveniles were ringed and PIT-tagged. Using a wing biopsy, a pedigree of the population was characterized from 2012 until 2015. Moreover, the social environment was monitored monthly, by filming groups during the resting period of the bats and reporting individual identity and geographical localization.

8. Measuring oxidative stress

When measuring oxidative stress, the importance of considering the two sides of the oxidative balance, *i.e*. both pro- and antioxidants has been highlighted (Costantini and Verhulst, 2009; Hõrak and Cohen, 2010; Monaghan et al., 2009). However, ROS are extremely reactive and are thus difficult to measure. Instead, measuring damage caused by the ROS can be an efficient way to monitor the pro-oxidant side of the oxidative balance. An increase in pro-oxidants does not necessary indicate a state of oxidative stress, for example if antioxidant defenses were also up-regulated (Monaghan et al., 2009).

In this thesis, we chose to measure an array of biomarkers, each considered to be key components of the oxidative stress balance. For the antioxidant defense, we measured an endogenous one, the activity of the enzyme (SOD) and an exogenous antioxidant, the level of tocopherol. We also quantified the ratio of reduced to oxidized glutathione as a marker of cellular oxidation. Finally, we measured a marker of the level of lipid peroxidation, *i.e.* damage to the lipids caused by ROS, using malondialdehyde (MDA) both in the red blood cells and in the soma. Interestingly, many studies have found that following an oxidative challenge, only some of the measured biomarkers show a response (Christensen et al., 2015). To get a more comprehensive picture, in some studies, more general assays have been used, such as the total antioxidant capacity assay (TAC) (Christensen et al., 2016) or the non-enzymatic antioxidant capacity OXY assay (Costantini et al., 2012), or the diacron reactive oxygen metabolites (d-ROMs) test, which quantifies metabolites generated by oxidative stress (Costantini, 2016). However, the lack of the specificity of such assays has sometimes been criticized (Kilk et al., 2014). Moreover, even such global assays often measure different molecules, and therefore do not necessarily correlate with one another (Jansen and Ruskovska, 2013).

In addition to the variation found in the biomarkers response to an oxidative challenge, studies have also reported variation in the amount of oxidative stress exhibited by different tissues. Indeed, depending on the tissues used to monitor the oxidative balance, whether it is kidney, brain, liver, muscles or blood, the results might not correlate to one-another (Costantini, 2008). Many ecological studies use blood to study somatic oxidative stress (including ours), as it is a tissue easy to collect non-invasively. However, it remains poorly understood how blood actually reflects the level of oxidative stress in other tissues. Several studies have found that depending on the biomarkers, the level of oxidative stress was not always comparable between blood and other tissues (Argüelles et al., 2004; Blount et al., 2015; Veskoukis et al., 2009). However, Veskoukis

et al. (2009) found that blood seemed to provide a more comprehensive picture compared to liver or muscle tissue for example, which only provided biomarker specific responses. Similarly, another meta-analysis suggests that most biomarkers of oxidative stress (with the notable exception of the reduced to oxidized glutathione ratio) measured in the blood reflect the level of oxidative stress found in other tissues (Margaritelis et al., 2015).

To conclude, these results highlights the need for an array of biomarkers to be used, while keeping in mind that tissue-specific responses might not be unraveled when using solely blood as the tissue of investigation.

9. Objectives of the thesis

The goal of my thesis was to evaluate the role of oxidative stress as a constraint for life history traits. We studied this question at two key moments of an individual's life history: during early life, and during reproduction. The Seba's short-tailed bats are particularly suited as a model species, as males exhibit alternative reproductive tactics, and hence experience different risks of sperm competition, allowing us to test the predictions of the sperm competition models. Moreover, the factors determining the acquisition of reproductive tactics are unknown so far and could potentially be impacted by early life conditions.

First, we tested the prediction that early life adverse conditions can lead to both short and long-term negative consequences, which can be caused by elevated oxidative stress. In chapter 1, I present an experiment where we created adverse early conditions by food restricting the mothers of unweaned pups for 10 days, followed by *ad libitum* feeding. We evaluated the short-term consequences of adverse early life conditions on morphological traits *i.e.* growth rate and wing morphometry (unpublished results). We also monitored physiological traits, by looking at the redox balance in the blood (MDA, SOD activity, tocopherol and the ratio of oxidized to reduced glutathione), the level of glucocorticoids in the hairs, and telomere length. Moreover, we looked at the exploratory behavior of the pups, and their ability to find their mothers in a maze, both during and at the end of growth (unpublished results). We further explored the relationship between physiological parameters at the end of growth and the likelihood to survive over one year.

Second, we tested the hypothesis that oxidative stress could mediate the pre vs post copulatory trade-off. We studied whether antioxidant resource allocation between the soma and the ejaculates differed between males of different reproductive tactics. Males exhibiting a bourgeois tactic, *i.e.* harem males are expected to favor soma maintenance, whereas sneaker males are expected to invest resources into antioxidant protection of their ejaculates. In chapter 2, I present the results of an experiment where we manipulated males' reproductive tactics and monitored sperm quality and the redox balance in the blood and in the ejaculates (MDA, SOD activity, tocopherol and the ratio of oxidized to reduced glutathione), before and after the manipulation of reproductive tactic. In chapter 3, we further tested whether sperm morphology varied according to male reproductive tactics, and whether sperm swimming abilities is linked to sperm morphology in the Seba's short-tailed bats.

Finally, in chapter 4, I present a selective trap based on radio frequency identification that we designed and built. Indeed, we decided to develop a way to efficiently recapture individuals within the dome. It will also allow experiments to take place within the dome, without restraining individuals in cages.

Chapter 1: Compensating for a bad start does not affect glucocorticoids levels and does not entail oxidative costs in a fruit-eating bat

Magali Meniri[1], Doriane Hebinger[1], Mahaut Sorlin[1], Marine Ramirez[1], Emilie Kauffmann[1], Nicolas Fasel*[2] and Fabrice Helfenstein*[1]

1: Laboratory of Evolutionary Ecophysiology, Institute of Biology, University of Neuchâtel, Switzerland

2: Leibniz Institute for Zoo and Wildlife Research, Berlin, Germany

*: Shared last authorship

Abstract

Early life adverse conditions can have major consequences on individual's life history traits both on the short-term and on the long-term. We postulate that oxidative stress could represent a mechanism underlying the negative consequences of early life stress.

To test that hypothesis, we restricted the quantity of food available for Seba's short-tailed bat (*Carollia perspicillata*) mothers of unweaned pups for 10 days, followed by *ad libitum* provisioning. We also had a control, unrestricted group. We explored the morphological and physiological short-term consequences of early adverse conditions by measuring growth rate (size and mass) from birth until the end of growth 48 days later. We also measured four markers of blood oxidative balance immediately after the food restriction and at the end of growth. We assessed the level of cortisol, and its inactive form cortisone, in the hair of the pups at the end of growth. Finally, we monitored survival during the first year.

Food restriction triggered a slowdown in growth followed by compensatory growth when *ad libitum* was restored, leading to full compensation in size compared to control individuals, but not body mass. We found that growth was associated with an increase in oxidative damage. However, we found no evidence for physiological costs specific to the compensatory growth, both for glucocorticoids and oxidative stress. Yet, the oxidative balance was correlated to the glucocorticoids level. Survival after a year was not impacted by the treatment, the oxidative balance or the level of glucocorticoids at the end of growth.

In conclusion, our results show that individuals were able to efficiently mitigate the short-term consequences of adverse early life conditions, both morphologically and physiologically. However, consequences might arise on the long-term, and could impact reproductive success or lifespan.

Keywords: *Compensatory growth, oxidative stress, Carollia perspicillata, early life, glucocorticoids*

1. Introduction

Individual phenotype and behavior can permanently be affected during pre- or post-natal development by adverse conditions, such as low resource availability (Ohlsson and Smith, 2001), inadequate temperature (Bizuayehu et al., 2015), or strong predator pressure (Bell et al., 2011). Early life experiences can thus have long lasting consequences on a wide range of traits, for example on adult size (Hopwood et al., 2014), ornamentation (Walker et al., 2013), singing performances (Nowicki et al., 2002), reproductive success (Pigeon et al., 2017), adult metabolic rate (Criscuolo et al., 2008), coping behavior (reviewed in Langenhof and Komdeur, 2018), aging rate (Nussey et al., 2007) and also survival (Lee et al., 2013a). Moreover, early life conditions might not only impact the individual experiencing them-selves, but also their offspring (reviewed in Burton and Metcalfe, 2014).

It remains largely debated whether or not such changes in the phenotype of an individual are adaptive (reviewed in Monaghan, 2008). First, the environmental matching models, which includes the predictive adaptive response hypothesis, postulate that early life experiences are used as predictors of the quality of the future environment, to program an individual to perform best as an adult in an environment of similar quality (Gluckman et al., 2005). Therefore, an individual born in a low quality environment will perform better as an adult in a low quality environment compared to an individual born in a high quality environment (e.g.: *butterfly:* van den Heuvel et al., 2013). However, support for this hypothesis is scarce (Douhard et al., 2014). The hypothesis itself is mostly used in medicine, to explain the emergence of metabolic diseases later in life (Wells, 2012). Alternatively, the silver spoon effect (Grafen, 1988) proposes that individuals born in a high quality environment will always perform better than individuals born in a low quality environment, regardless of the quality of the environment encountered as adults (e.g.: *lady bird beetle*: Dmitriew and Rowe, 2011; *earwig:* Wong and Kölliker, 2014).

Environmental effects on organisms are partly mediated by the hormones. The hypothalamus-pituitary-adrenal axis (HPA) is the principal neuro-endocrine system mediating the stress response. In response to perceived challenges, the HPA axis modulates the release of glucocorticoids, which promote physiological changes such as the release of glucose in the blood flow, and inhibition of non-essential functions, such as growth (Dantzer et al., 2016; Harris and Seckl, 2011), immunity (Bellavance and Rivest, 2014) and reproduction (Joseph and Whirledge, 2017). Numerous studies support the prediction that early life experiences can program the HPA axis, and hence modify the long-term physiology of the stress response (reviewed in van Bodegom

et al., 2017). Programming of the HPA axis involves modifications in the basal level of circulating glucocorticoids (Rice et al., 2008), but also in the magnitude of the response in case of an acute stress (Hayward et al., 2006), both aspects being impacted by early life. The impact on the HPA axis can vary depending on the sex of the individual, on the timing of the stressor during an individual's development, and on its duration. Ultimately, impacts on the HPA axis may lead to either high resilience or to extreme sensitivity to stress (Levine, 2005).

Developing in a suboptimal early-environment often leads to a temporary decrease in growth rate, which can sometimes be compensated (Hector and Nakagawa, 2012). If the growth delay is not compensated, individuals will exhibit smaller adult size, and may bear strong fitness costs. Indeed, smaller body size may lead to higher predation pressure, lower foraging abilities or lower reproductive success (Blanckenhorn, 2005). Once conditions improve, individuals may attempt to grow faster and/or for longer (Metcalfe and Monaghan, 2001), and hence enjoy the benefits associated with a "normal" size. Compensatory growth has been reported in wild populations (Bize et al., 2006; Bjorndal et al., 2003), and following experimental manipulation of environmental factors, such as food availability (Xu et al., 2014) or temperature (Hostins et al., 2015). Growing at a faster rate than the optimal growth rate of the species, although providing immediate benefits, might also carry costs that are paid later in life (Metcalfe and Monaghan, 2001). Indeed, compensatory growth has been associated with reduced cognitive performances (Fisher et al., 2006), higher rate of telomere attrition (Geiger et al., 2012) and reduced lifespan (Lee et al., 2013b). Thus, one possible mechanism by which compensatory growth entails costs is oxidative stress (Alonso-Alvarez et al., 2007; De Block and Stoks, 2008). Oxidative stress is defined as an imbalance between the production of reactive oxygen species (ROS), a by-product of normal metabolism generated during the electron transport chain, and the antioxidant machinery (Halliwell and Gutteridge, 2007). Oxidative stress has been proposed to represent a mediator of life history trade-offs (Metcalfe and Alonso-Alvarez, 2010; Monaghan et al., 2009), and might represent a cost of compensatory growth. Indeed, an increased growth rate is associated with higher metabolism, which could generate more ROS. Without an efficient mitigation by the antioxidant machinery, this increase in oxidative potential might lead to oxidative stress. Moreover, elevated levels of glucocorticoids, likely to occur in individuals experiencing early adverse conditions, is suggested to induce oxidative stress (reviewed in Costantini et al., 2011). Indeed, glucocorticoids may increase ROS production (You et al., 2009), decrease antioxidant protection mechanism (Stojiljković et al., 2009) and decrease repair mechanism (reviewed in Haussman and Marchetto, 2010).

Seba's short tailed bats *(Carollia perspicillata)*, as a tropical species, may face substantial spatial and temporal variation in food availability, and these variations might amplify in the future, since the quality of their natural habitat decreases at a fast rate due to climate change and habitat fragmentation (Opdam and Wascher, 2004; Vázquez et al., 2015). Therefore, the number of individuals experiencing adverse early conditions such as a transient food shortage is likely to increase, potentially having important consequences both for individuals and for population dynamics (Beckerman et al., 2002). As long-lived mammals, bats might provide important insights on the short and long-term effect of early life conditions. Newborns weight about 25% of their mother's body mass, with fur and eyes open. It suggests that they likely experience strong constraints to grow fast, potentially in order quickly reach flight capacities. Also, in *C. perspicillata*, reproductive success is skewed towards a few males (Fasel et al., 2016), and they exhibit resource defense polygyny, a common mating system in tropical bats. Resource defense polygyny leads to high level of male-male competition to obtain and keep a territory (Fernandez et al., 2014). As a result, early life conditions may constrain the expression of reproductive tactics (English et al., 2013), and hence may play a major role on male reproductive success later in life.

To understand the morphological and physiological consequences of adverse early conditions, we restricted the food availability of mothers of unweaned pups during 10 days after parturition, followed by *ad libitum* provisioning. We also had a control, unrestricted group. We aimed at exploring whether adverse early conditions (1) lead to compensatory growth, (2) increased the level of glucocorticoids, (3) generated oxidative stress and (4) whether a link exists between oxidative stress and level of glucocorticoids. For this purpose, we measured growth rate (size and mass) from birth until the end of growth 48 days later, assessed the level of glucocorticoids in the hairs of the pups as markers of the hypothalamus-pituitary-adrenal stress response, and measured four markers of blood oxidative balance (level of antioxidants and oxidative damage) immediately after the food restriction and at the end of growth. Lastly, (5) we evaluated the first-year survival in relation to the sex and the early life conditions.

2. Material and methods

2.1 Model species and studied population

The study was conducted with individuals captured from two captive breeding colonies of *Carollia perspicillata*, hosted in a tropical zoo (Papiliorama) located in Switzerland. One population of about 350 individuals fly freely in a 40m diameter dome, open to the public, and are fed with a fruit-based mixture. All adult bats are marked with a unique combination of three colored rings on their forearm (A.C Hugues, UK, size XB), and equipped with PIT-tags, as part of a long-term monitoring. A smaller colony of about 200 bats live in another dome under similar condition, but unmonitored.

In the wild, *C. perspicillata* present a seasonal bimodal polyoestry with post-partum oestrus. The gestation period lasts 4 months, which can be extended up to 6 months. They give birth to a single pup, fed with heavy lactation for the first month, and weaned at about 2.5 months (Fleming, 1988). Pups fly around the age of 24 days.

2.2 Timeline of experiment

The experiment was conducted between February and October 2016. Gravid females were trapped from both colonies using either a harp-trap (Faunatech Ausbat, Mount Taylor, Australia) for the bigger colony, or a hand net for the smaller one. Females in late gestation can easily be detected both visually and with gentle abdominal pressure, allowing us to select females ready to give birth within the next 3 weeks. Upon capture, gravid females were transferred to a common experimental cage (1m*2m*2m and provided with food and water *ad libitum*). Females were kept an average of 2 weeks before giving birth (Total time spend in cages: 67.86 days ± 12.12 days), and after a month without giving birth, females where released. All bats were visually checked daily for welfare and to detect newborn pups. All newborn were detected within 24 h of their birth, as attested by the presence of the umbilical cord. The day a newborn pup was detected was considered as day 0, and the pup was weighed to the nearest 0.1g and its forearm was measured to the nearest 0.1 mm, as a proxy of size. From day 0, pups were measured every 2 days until day 48, which corresponds to the end of growth. At day 2, the mother-pup pair, irrespective of their treatment, was transferred in an individual cage, to allow precise monitoring of the food consumption. The treatment, i.e. food restriction or control, started on day 4 for 10 days, which

corresponds to the period of linear growth of the pups, lasting about 20 days (Fleming, 1988). Since pups are feeding solely on their mothers' milk during that time, food restriction was applied on the mothers, in order to decrease milk quality/quantity to generate adverse early-life conditions) for the pups. Mother-pup pairs were attributed randomly to the treatment, by assigning alternatively one pair to the control group and the next pair to the restricted group. Since sex ratio is biased toward males at birth (Fleming, 1988), and in order to maintain a balanced distribution within sex, alternating attribution to a group was sex-specific. Food restricted mothers were given 80% of their normal food intake, which corresponds to 1.5 times their body mass per day, as determined during a preliminary study. During restriction, if pups lost weight or did not gain weight for 4 consecutive days, the amount of food given was increased. The control pairs were fed *ad libitum*. On day 14, the mother-pup pair were transferred back to a common cage, and provided with food *ad-libitum*. Pups were identified by marking them using nail polish on their toe nails. Access to water was always provided *ad libitum*.

Two blood samplings were performed on the pup, the first one on day 14 (end of treatment) and the second one on day 48 (end of growth). On day 49, pups were ringed and PIT-tagged to allow future identification. The same day, a sample of hair was clipped from the lower back of the pups, to allow glucocorticoids dosage. All individuals were then released back to the main colony. A total of 49 pups were manipulated during the experiment, with 9 control females and 10 restricted, and 15 control males and 15 restricted.

2.3 Blood sampling

Blood samples were drawn from the antebrachial wing vein by puncturing with a sterile needle, and collecting the droplet of blood using a Microvette (CB 300 Hep-Li, Sarstedt). Samples were stored on ice until transportation to the lab. Back to the lab, blood samples were centrifuged at 2’000 G for 5 minutes at 4°C to separate cells from plasma. The plasma was collected and aliquoted for later analyses. Samples were stored at –80°C.

2.4 Hair sampling

Glucocorticoids were measured from a sample of hair clipped from the lower back of the pups. Pups are born with a thin fur, which gets thicker as they grow older. Therefore, the level of glucocorticoids contained in the hair represents the entire early-life of the pup, up until clipping, with an emphasis on the linear growth period, as it is when the most intense fur growth takes place. Samples were stored in the dark at ambient temperature until analysis.

2.5 Survival monitoring

The main colony was monitored during one year after the experiment. First, approximately once a month, we filmed the colony, and thanks to a unique combination of color rings on their forearm, we could identify individuals. Second, we opportunistically trapped individuals using a harp-trap at once every two weeks during the experiment, and once a month one year after, during 2h at the beginning of their activity period. Third, every cadaver that was found in the dome was kept and registered. Combining all three approaches, the probability of detection of an individual per year was of 80% (mean= 0.8, sd= 0.02). Finally, during the summer 2017, we recorded visits at the feeder using a modified feeder equipped with RFID identification technology, based on their PIT-tag (chapter 4).

2.6 Lab protocol

Redox markers

Lab analyses were performed blindly with respect to sample identity and the experimental design. All steps were conducted on ice to reduce oxidation. All chemicals were HPLC grade, and chemical solutions were prepared using ultra pure water H_2O MQ (Milli-Q Synthesis; Millipore, Watford, UK).

Blood sample preparation

A homogenate of red blood cells (RBC) was prepared by diluting 10 µl of cell pellet into 10 µl of phosphate buffer saline (PBS). The mixture was homogenized with four glass beads for 1 minute at 30 Hz using a mixer mill, immersed in an ice-cold bath and sonicated for 5 minutes, and centrifuged for 5 minutes at 11’200 G at 4°C. Of the supernatant 5 µl of RBC homogenate were

transferred in an Eppendorf tube for MDA quantification. Another 2 µl were diluted with 748 µl of PBS, vortex-mixed and centrifuged for 15 minutes at 10'000 G and 4°C (final dilution RBC 1:750 v:v) for SOD colorimetric assays. Finally, 2 µl were used immediately to quantify the reduced (GSH, ng/ml) and oxidized (glutathione disulfide GSSG, ng/ml) forms of glutathione (see glutathione quantification). We also quantified α-tocopherol using 10 µl of plasma, and MDA in 5 µl of plasma.

Quantification of redox markers

MDA quantification

We assessed MDA (nmol/ml) in the RBC and in the plasma by ultra-high performance liquid chromatography tandem mass spectrophotometry (UHPLC-MS/MS), according to (Mendonça et al., 2017). Details about the method can be found in the Annex.

SOD activity

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.

Glutathione quantification

The reduced (GSH, ng/ml) and oxidized (glutathione disulfide 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. Further details about the method can be found in the Annex.

Tocopherol quantification

We quantified α-tocopherol in the plasma by ultra-high performance liquid chromatography tandem mass spectrophotometry (UHPLC-MS/MS). More details about the method can be found in the Annex.

Glucocorticoids analysis

We quantified cortisol and cortisone in the hair by liquid chromatography tandem mass-spectrometry (LC-MS/MS). More details about the method can be found in the Annex.

2.7 Statistical analysis

Morphological consequences of a bad start

Body mass and size

First, we checked whether individuals were similar between groups at day 4, before starting the treatment. We ran a linear model with either body mass or forearm length at day 4 as the response variable, and treatment, sex, and their interaction as explanatory variables.

Then, we verified that food restriction had impacted growth by the end of the treatment, at day 14. We ran a linear model with either body mass or forearm length at day 14 as the response variable, and treatment, sex, and their interaction as explanatory variables.

Finally, we looked at the growth parameters after the treatment, to detect possible compensation for the adverse early conditions. Using the package "grofit" (Kahm and Kschischo, 2015), we fitted growth curves after the period of treatment i.e. from day 16 to day 48, in order to detect potential compensation for the bad start. We chose to apply model-free spline provided by the package "grofit", as it fitted the data better than the conventional parametric growth curve model such as Logistic growth, Gompertz growth or Richards. We then retrieved two of the characteristic parameters of the growth curves, the maximum growth rate μ and the asymptotic value A. We computed two sets of models. The first one used data of the growth parameters for the pup body mass. We ran linear models with either maximum growth rate μ or the maximum value A as the response variables, and treatment, sex and their interaction as explanatory variables. The second set of models used the growth parameters for forearm length, for which we ran linear models with either maximum growth rate μ or the maximum value A as response variables, with treatment, sex and their interaction as explanatory variables.

Age at first flight

We looked at whether the treatment had an effect on the age at first flight using linear regression. We included age at first flight as the response variable, and treatment, sex and their interaction as explanatory variables.

Physiological consequences of a bad start

Redox markers

To test the prediction that compensatory growth may represent an oxidative challenge, we analysed data of both sampling days together. First, we ran a PCA on the five redox markers (MDA $_{RBC}$, MDA $_{plasma}$, SOD $_{RBC}$, ratio GSSG/GSH $_{RBC}$, α-tocopherol $_{plasma}$). The first principal component explained 31.84% of the variance, and was positively loaded with both MDA $_{RBC}$ ($r=0.87$, $t=13.88$, p-value<0.001) and MDA $_{plasma}$ ($r=0.72$, $t=8.31$, p-value<0.001). Hence, the first principal component described the amount of oxidative damage to the lipids, an individual with a higher score having more oxidative damage. The second principal component explained 24.99% of the variance, and was positively loaded with both the level of SOD $_{RBC}$ activity ($r=0.69$, $t=7.63$, p-value<0.001) and α-tocopherol $_{plasma}$ ($r=0.69$, $t=7.66$, p-value<0.001), while being negatively loaded with the ratio GSSG/GSH $_{RBC}$ ($r=-0.51$, $t=-4.71$, p-value<0.001). Therefore, the second principal component was interpreted as the "redox profile" of the cell, an individual with a higher score having less oxidatively stressed red blood cells and more antioxidant defences (more plasmatic tocopherol and more SOD activity in red blood cells).

We ran linear mixed-effects models with redox profile or oxidative damage as the response variable, and treatment, sampling day, sex and their interactions as explanatory variables. To account for repeated measures, individual identity was included as a random factor.

Glucocorticoids

To test the impact of the treatment on the glucocorticoids levels of the pups, we ran linear models with either level of cortisol or cortisone plasma levels as the response variable, and treatment, sex and their interaction as explanatory variables.

Link between oxidative stress and glucocorticoids

To investigate a potential link between oxidative stress and glucocorticoids levels, we ran linear regression, using redox markers assessed on day 48, because glucocorticoids values are expected to mostly represent the time period between day 16 and day 48, as most of the hair growth occurred then. We used the redox profile or the oxidative damage as the response variable, and cortisol (log transformed) and cortisone (log transformed) concentrations as explanatory variables.

Survival after a year

To explain survival after a year, we fitted a general linear model with as response variable the survival after a year coded as a binary variable, and treatment, sex and their interaction as explanatory variables. We also tested whether the physiological status at the end of growth could explain survival. For the oxidative balance, we fitted a general linear model with as response variable the survival after a year, and the redox profile and oxidative damage at day 48 as explanatory variables. For the glucocorticoids, we fitted a similar model with cortisol (log-transformed) and cortisone (log-transformed) as explanatory variables. For all models, we used the binomial family, with the logit as a link function. We found that the colony of origin did not impact the survival probability, and therefore did not include it in the analysis (LR Chisq=0.77, P-value=0.38).

Data were analysed using R, version 3.3.3. The "nlme" package was used for the linear mixed-effects models, and we used the "lsmeans" package to run post-hoc tests based on least-square means with Tukey correction for test multiplicity. The significance level was set at 0.05.

2.8 Ethical statement

This study was performed under the authorization 2015_43_ FR, delivered by the veterinary office of the Canton Fribourg, Switzerland after examination by its ethical committee.

3. Results

Morphological consequences of a bad start

Before the start of the treatment, at day 4, there were no differences in body mass or forearm length between individuals of each treatment, sex, or their interaction. After the treatment, at day 14, individuals differed significantly in their body mass, and their forearm length according to the treatment, with control individual being bigger and heavier than restricted individuals (cf Fig. 1, Table I).

Then, we looked at the possible compensation in body mass and forearm length from day 16 until the end of growth, at day 48 (cf Fig. 2, Table II). The maximum growth rate calculated

during that period was significantly different between the treatments, for both body mass, and forearm length (cf Fig. 2, Table II). There were no differences between the sexes (cf, Table II).

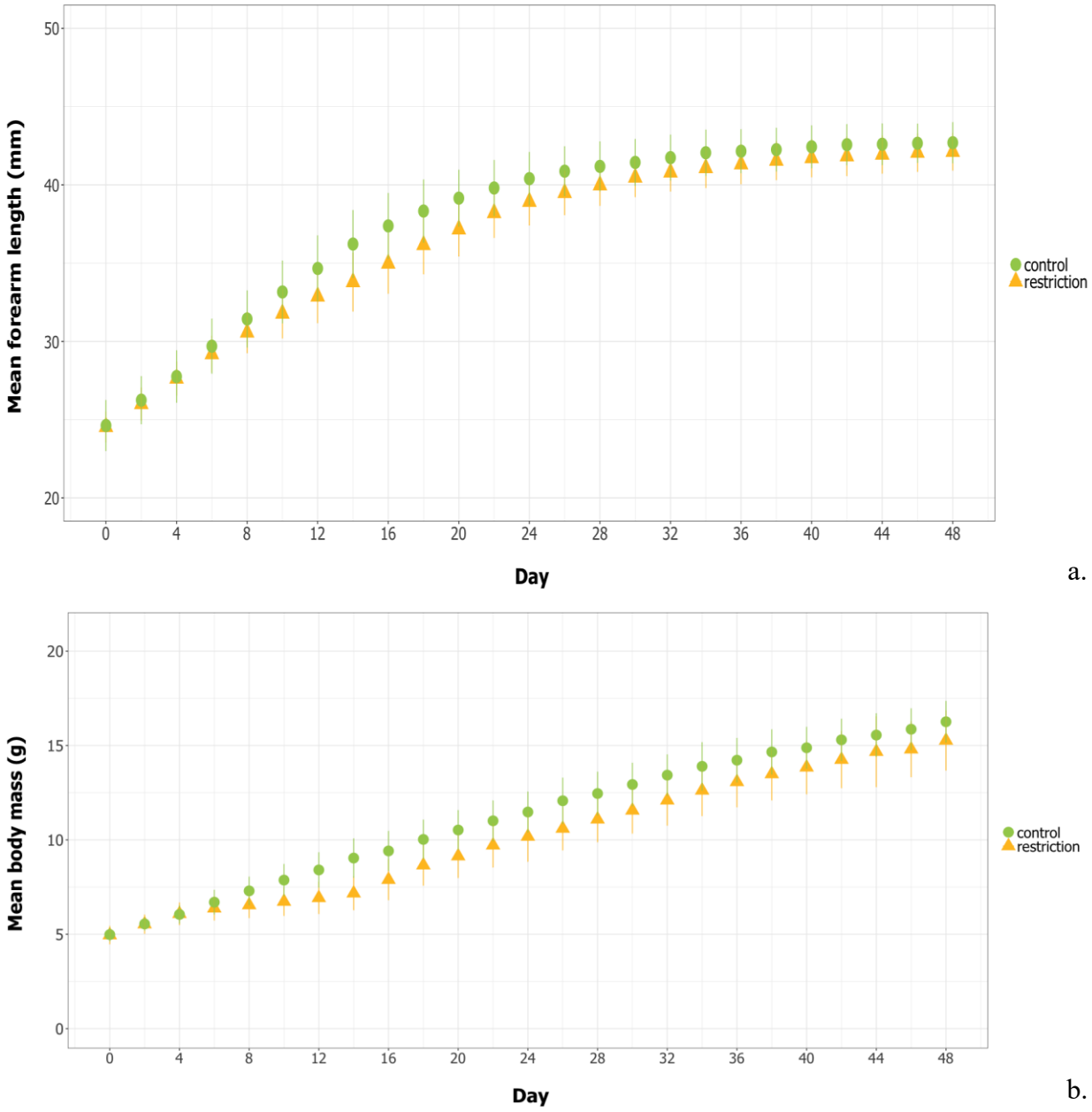


Figure 1: Mean growth curve for forearm length (a), and body mass (b). Vertical bars represent standard deviation around the mean for the group. Treatment took place from day 4 until day 14.

The maximum body mass (on day 48) was significantly different between the treatments, with control individuals being heavier than the restricted but not according to the sex, nor the interaction between sex and treatment (cf Fig. 2, Table II). The forearm length was not

significantly different between the treatments, but females had a significantly longer forearm than males did (cf Fig. 3, Table II). There was no interaction with the treatment. The age at first flight was significantly delayed by the restriction (F-value = 15; P-value <0.00.1; cf Fig. 4), but not affected by sex (F-value = 0.71; P-value = 0.4).

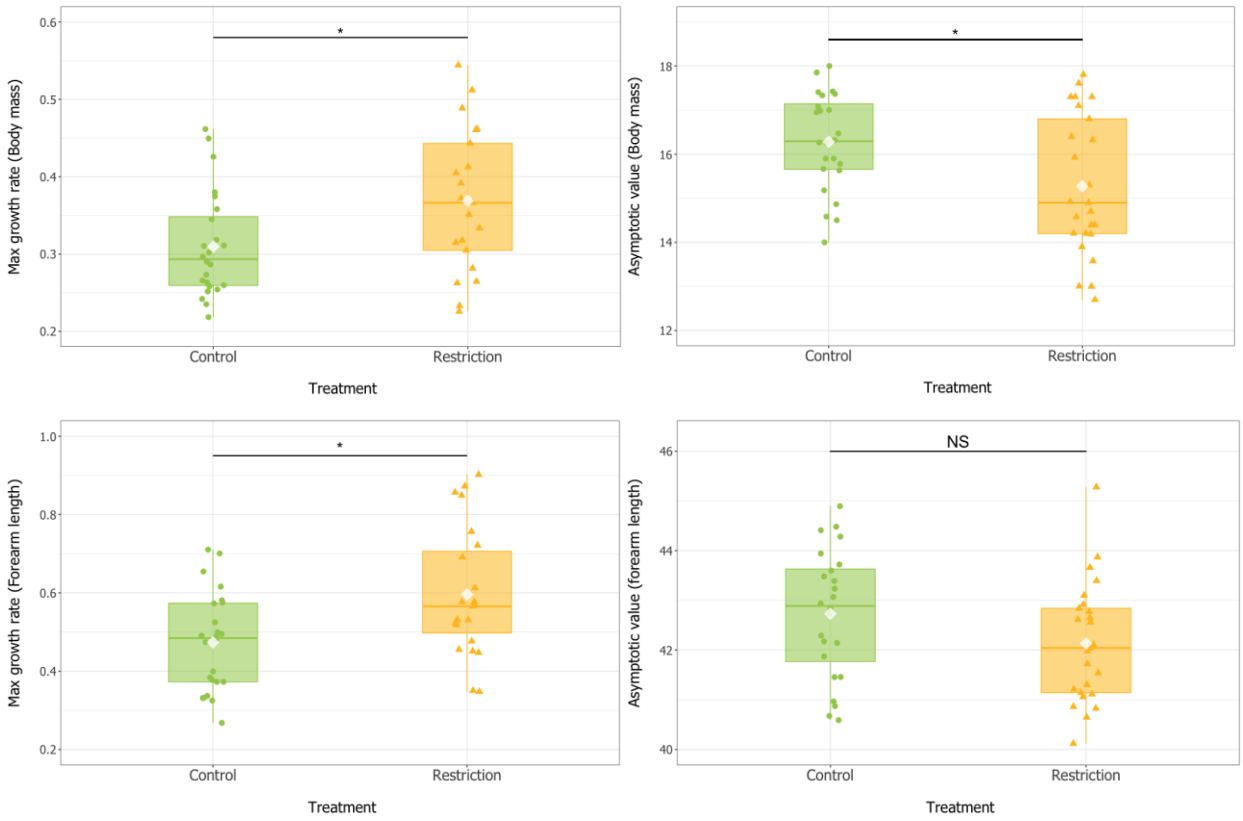


Figure 2: Growth parameters according to the treatment, computed between day 16 and day 48. Points represent the distribution of the data, and the white diamonds the group means. Horizontal bars and asterisks indicate statistical differences between groups (*p-value≤0.05).

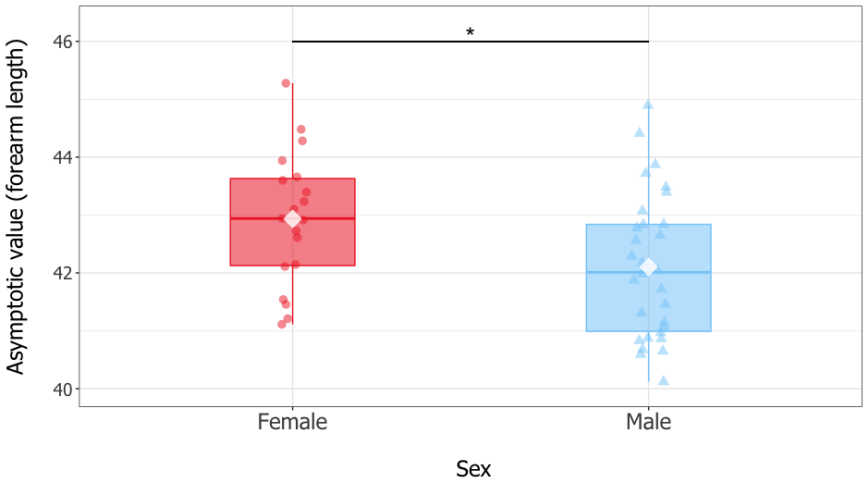


Figure 3: Asymptotic value for forearm length according to the sex (day 48). Points represent the distribution of the data, and the white diamonds the group means. Horizontal bars and asterisks indicate statistical differences between groups (*p-value≤0.05).

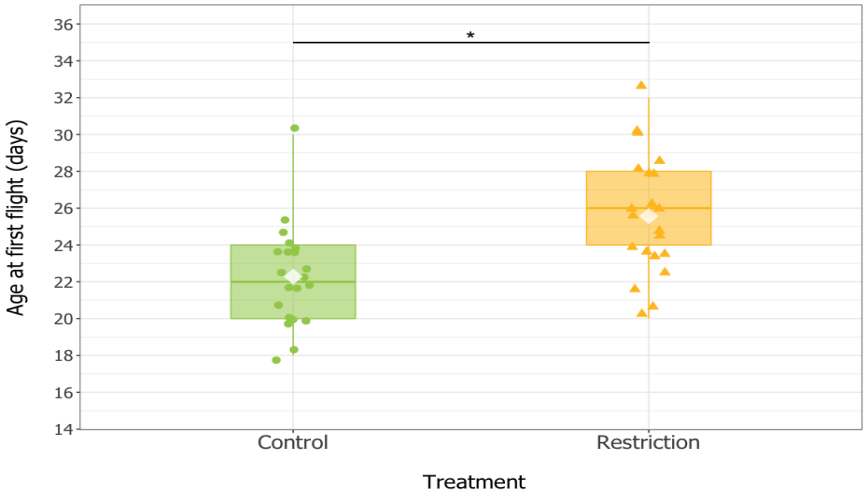


Figure 4: Age at first flight according to the treatment. Points represent the distribution of the data, and the white diamonds the group means. Horizontal bars and asterisks indicate statistical differences between groups (*p-value≤0.05).

Table I: Linear regression exploring the relationship between body mass, size and treatment, sex and their interaction, before (day 4) and after the treatment (day 14).

	Body mass		**Forearm length**	
Explanatory variables	**F-value**	**P-value**	**F-value**	**P-value**
Day 4				
Treatment	0.05	0.82	0.12	0.72
Sex	0.04	0.83	0.99	0.32
Treatment x Sex	2.82	0.09	2.52	0.12
Day 14				
Treatment	**45.04**	**<0.001**	**18.83**	**<0.001**
Sex	0.8	0.37	2.84	0.09
Treatment x Sex	1.28	0.26	1.08	0.3

Table II: Linear regression exploring the link between growth parameters and treatment, sex and their interaction, computed between day 16 and day 48.

	Maximum growth rate		Asymptotic value	
Explanatory variables	**F-value**	**P-value**	**F-value**	**P-value**
Model: Body mass				
Treatment	**8.75**	**0.005**	**6.54**	**0.014**
Sex	1.68	0.2	0.76	0.38
Treatment x Sex	0.42	0.51	0.18	0.67
Model: Forearm length				
Treatment	11.17	0.001	3.27	0.08
Sex	2.18	0.14	**5.83**	**0.02**
Treatment x Sex	0.09	0.75	0.06	0.8

Physiological consequences of a bad start

Redox profile

Regarding the oxidative damage, there was a significant interaction between the treatment and the sampling day (cf Fig. 5, Table III). Precisely, after the treatment, on day 14, control individuals had higher oxidative damage than restricted individuals (t= 2.57, P-value= 0.01), but there were no differences anymore on day 48. Moreover, there was a significant difference between oxidative damage on day 14 and 48 for restricted individuals, with higher levels on day 48 (t=-3.8, P-value= 0.001). There was no difference according to the sex, regardless of the sampling day (cf Table III).

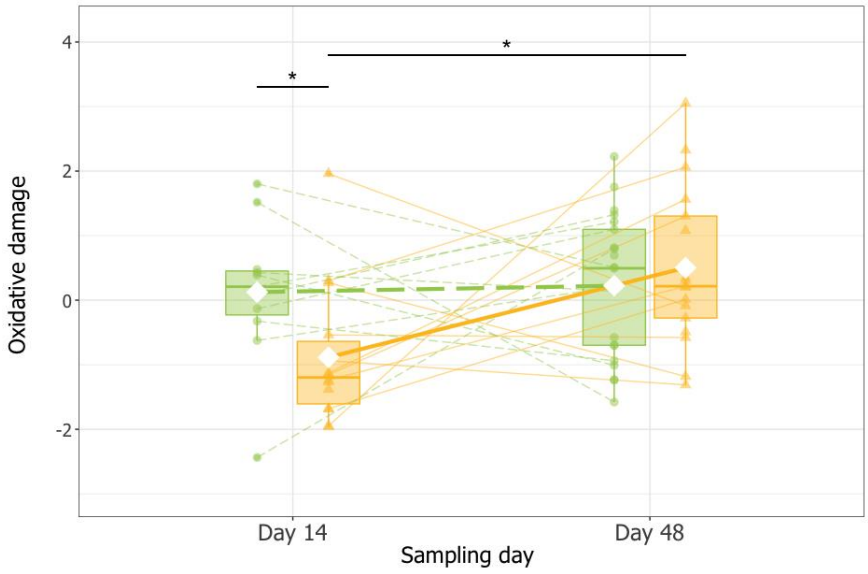


Figure 5: Oxidative damage according to the treatment. Points represent the distribution of the data, and the white diamonds the group means. Green represents control individuals, yellow the restricted individuals. Horizontal bars and asterisks indicate statistical differences between groups (*p-value≤0.05)

Table III: Linear mixed-effects models exploring the link between oxidative damage, redox profile and treatment, sampling day, sex and their interactions.

	Oxidative damage		**Redox profile**	
Explanatory variables	**F-value**	**P-value**	**F-value**	**P-value**
Treatment	6.62 $_{1,38}$	0.01	1.65 $_{1,38}$	0.21
Sampling day	0.44 $_{1,18}$	0.51	1.06 $_{1,18}$	0.32
Sex	0.00009 $_{1,38}$	0.99	0.02 $_{1,38}$	0.88
Treatment x Sampling day	**4.37 $_{1,18}$**	**0.05**	1.46 $_{1,18}$	0.24

For the redox profile, there were no differences based on the treatment, sex or sampling day, or their interactions (cf Table III).

Table IV: Linear models exploring the link between cortisol, cortisone and treatment, sex and their interaction.

	Cortisol		Cortisone	
Explanatory variables	**F-value**	**P-value**	**F-value**	**P-value**
Treatment	0.47	0.49	0.08	0.77
Sex	1.35	0.25	3.07	0.08
Treatment x Sex	0.24	0.62	1.81	0.18

The level of cortisol or its inactive form, were not different depending on the treatment, the sex or their interaction (cf Table IV).

Table V: Linear models exploring the link between oxidative damage, redox profile and cortisol, cortisone and their interaction.

	Oxidative damage		Redox profile	
Explanatory variables	**F-value**	**P-value**	**F-value**	**P-value**
Day 48				
Cortisol (log)	**7.57**	**0.01**	**8.25**	**0.007**
Cortisone (log)	**11.16**	**0.002**	1.47	0.23

The amount of oxidative damage on day 48 was negatively correlated with both the level of cortisol and of cortisone that were deposited in the hairs during growth (cf Fig. 6, Table V). Moreover, the redox profile on day 48 was negatively correlated with cortisol levels, but not with cortisone levels (cf Fig. 7, Table V).

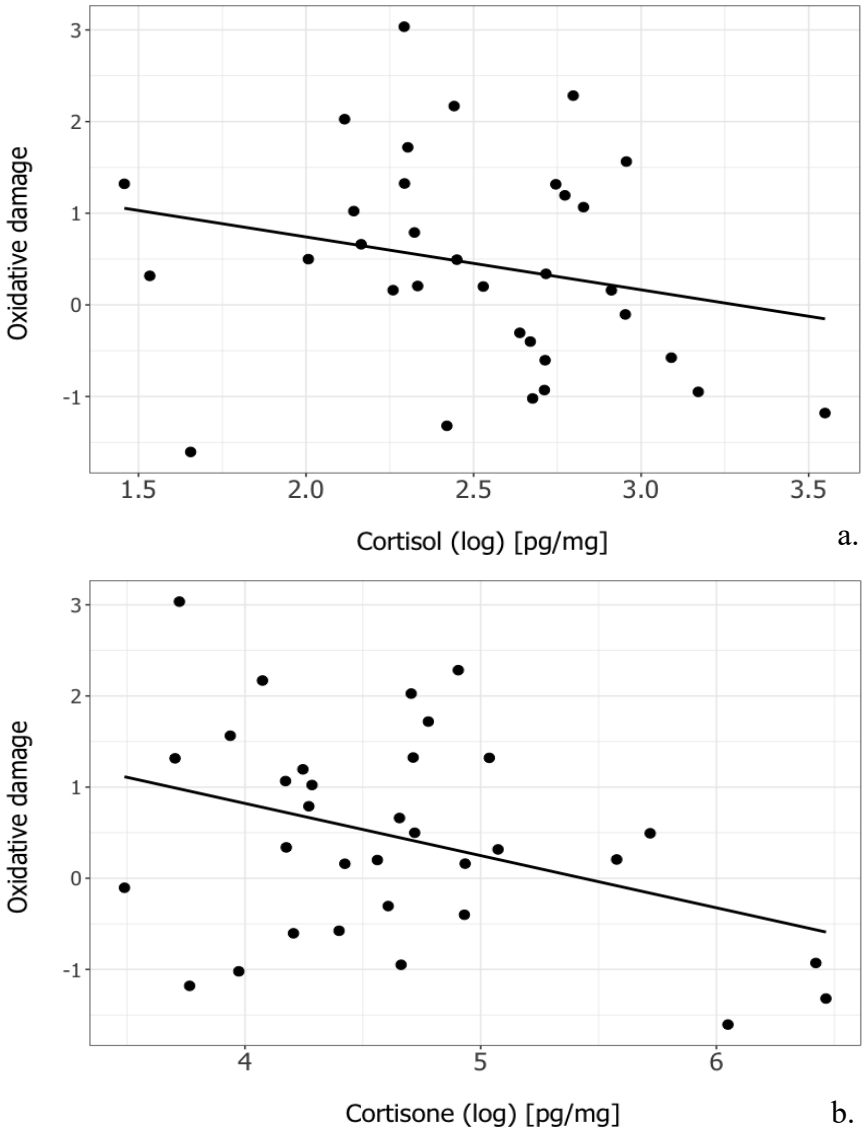


Figure 6: Correlation between oxidative damage and cortisol (a) or cortisone (b) measured at 48 days.

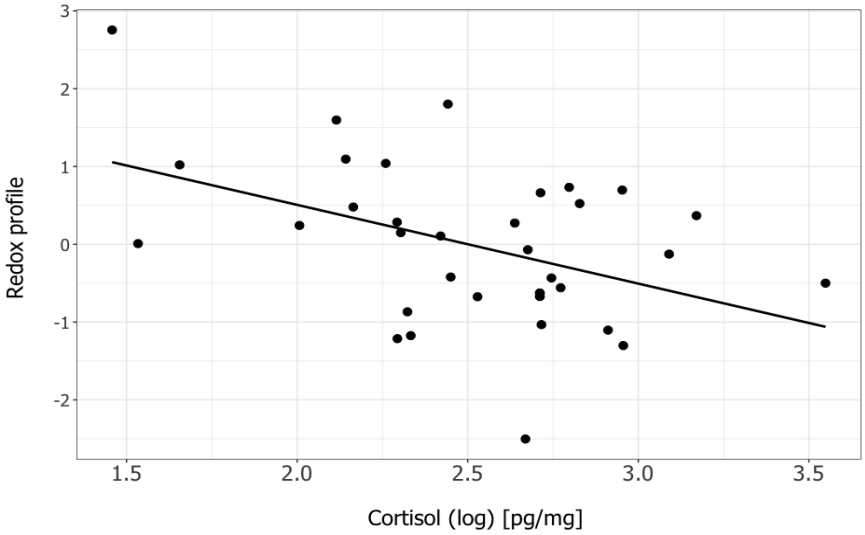


Figure 7: Correlation between redox profile and cortisol level, at day 48. Points represent the distribution of the data. The black line is the regression line of the model.

Survival

The survival after a year, was not explained by treatment, sex, or their interaction (cf Fig. ,8 Table VI). Moreover, neither the oxidative balance at day 48, not the glucocorticoids accumulated by the end of growth explained the survival during the following year (cf Table VI).

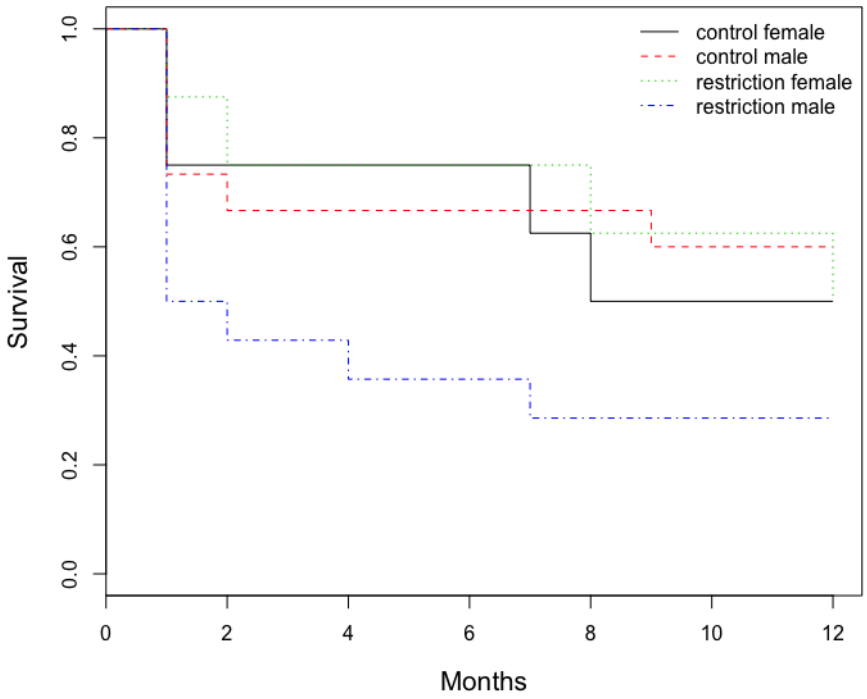


Figure 8: Survival during the first year, according to the sex and the treatment.

Table VI: General linear model exploring survival according to treatment, sex, and physiological status at the end of growth.

	Survival	
Explanatory variables	**LR Chisq**	**P-value**
Model: Treatment		
Treatment	1.87	0.17
Sex	0.13	0.72
Treatment x Sex	1.07	0.29
Model: Oxidative balance		
Redox profile	0.0001	0.99
Oxidative damage	1.02	0.31
Model: Glucocorticoids		
Cortisol (log)	0.002	0.96
Cortisone (log)	0.08	0.77

4. Discussion

We induced early adverse conditions by food restricting mothers of unweaned pups for 10 days, followed by *ad libitum* feeding. After a post-natal episode of food restriction, individuals were able to fully compensate their size delay. We found a difference in size between males and females, with the latter exhibiting longer forearm at the end of growth. This result could arise from different growth pattern between males and females, since, as adults, there is no sex difference in size or body mass in the wild (Ulian and Rossi, 2017), nor in our population (unpublished data). Alternatively, it could suggest a selective disappearance of smaller males. Unfortunately, our low sample size does not allow us to test the correlation between survival and the interaction with treatment, sex and size. Contrary to size, body mass was not fully compensated, as previously restricted individuals were lighter than controls at the end of growth. Indeed, at day 48, all individuals were still gaining weight daily. Strikingly, for all species of bats for which the information was available, the period of linear growth was restricted to the first 35 days, as we observed in *C. perspicillata,* whereas patterns of weight intake varied between species

(*Myotis lucifugus* Baptista et al., 2000, *Eptesicus fuscus* Burnett and Kunz, 1982, *Myotis myotis* (De Paz, 2009), *Phyllostomus hastatus* Stern and Kunz, 1998, *Pipistrellus subflavus* Hoying and Kunz, 1998 , *Tadarida Brasiliensis Mexicana* Kunz and Robson, 1995). This inter-specific similarity suggests strong evolutionary constraints on size, maybe in order to start flying. Indeed, in our experiment, the treatment delayed the first flight by an average of 2 days compared to control individuals that first flew at the age of 24 days, despite a significantly higher growth rate to compensate for the growth delay.

We then looked at the short-term effects of growth of the redox balance. At the end of the treatment (day 14), control individuals had higher oxidative damage than restricted individuals, as the latter grew very slowly during the restriction. This result highlights the oxidative cost of normal growth (*eg:* Soay sheeps: Nussey et al., 2009). The idea that higher metabolic rate increases ROS production, which might in turn lead to oxidative stress, has been challenged by the uncoupling to survive hypothesis (Brand, 2000). Mitochondrial uncoupling, *i.e.* dissipation of the proton gradient to produce heat, is proposed to mitigate the negative consequences of an increased metabolism (Speakman et al., 2004), as shown in the short-tailed field vole *Microtus agrestis* (Selman et al., 2008). However, our results do not support this hypothesis, as we show that higher growth rate leads to more oxidative damage. During the compensation phase, previously restricted individuals significantly increased their level of oxidative damage, and exhibited similar levels of oxidative damage compared to control individuals. This finding indicates that compensatory growth, *i.e.* growing at a faster rate than the optimal rate, did not elicit higher level of oxidative stress than normal growth, contrary to what we predicted, and has been reported before (Alonso-Alvarez et al., 2007; De Block and Stoks, 2008). However, the last blood sampling occurred approximately 18 days after the most intense compensation phase. It is possible that higher levels of oxidative stress were present then, but dissipated by the time the sampling took place. Moreover, it is possible that the *ad libitum* food regime allowed individuals to efficiently mitigate the predicted short-term costs of compensatory growth. Indeed, (Beaulieu et al., 2014) showed that when facing a thermal stress, Gouldian finches *(Erythrura gouldiae)* increased their consumption of dietary antioxidants to efficiently mitigate oxidative stress, resulting in reduced oxidative damage. Furthermore, a comparative study conducted on tropical bats showed that frugivorous bats were less prone to oxidative stress compared to bats with other diets (Schneeberger et al., 2014).

Overall, we found pups' "redox profile", *i.e.* the second component of the PCA (a higher score indicating less oxidatively stressed red blood cells and more antioxidant defences), to be

unaffected by growth. Similarly, a meta-analysis linking growth rate and oxidative stress found that there were oxidative costs to growing faster, without reporting an effect on the antioxidant defense (Smith et al., 2016). Studies have shown that faster growth can be associated with an increase (De Block and Stoks, 2008), an absence of change (Stier et al., 2014) or a decrease in antioxidant defenses (Carney Almroth et al., 2012). All those results could indicate an oxidative challenge (Smith et al., 2016), highlighting the importance of measuring both oxidative damage and antioxidant defenses to get an accurate estimate of the oxidative balance (Monaghan et al., 2009).

We did not find a difference in glucocorticoids levels accumulated during growth between control and previously restricted individuals. Therefore, experiencing early life adverse conditions did not seem to lead to higher deposition of glucocorticoids in the hair, contrary to our predictions and to previous findings (Monaghan and Haussmann, 2015). This result could be explained in three non-mutually exclusive ways. It is possible that detention in cages and our manipulations stressed all the pups more than the treatment, and therefore masked any differences between the groups. Alternatively, as maximum hair growth occurred after the treatment, it is likely that glucocorticoids measured in the hair at day 48 mostly reflects the stress level during the compensation phase, rather than the period of food restriction. However, the food restriction period is expected to correspond to maximal glucocorticoids production. Indeed, it has been repeatedly shown that food restriction leads to higher glucocorticoids production both during early life (Kitaysky et al., 2001), and as adults (Levay et al., 2010). Moreover, in order to perform a compensatory growth, it might be adaptive to maintain low levels of glucocorticoids when conditions improve, as stress hormones have long been recognized to inhibit growth (Grace et al., 2017; Mosier, 1971). Indeed, an vitro study conducted on bone cells collected from rats 8 days after birth showed that exposure to synthetic glucocorticoids inhibited growth, and that prolonged exposure could permanently prevent catch-up growth to occur after glucocorticoids exposure stopped (Chagin et al., 2010). Similarly, in humans, it was shown that children born with low body mass were less likely to catch up if their cortisol level was high (Cianfarani et al., 2002). In order to mitigate the negative consequences associated with high levels of glucocorticoids during early life, at least two mechanisms are known to exist. First, the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) that converts self-produced as well as maternally derived cortisol into inactive cortisone is particularly active during early-life (reviewed in Wyrwoll et al., 2011). Second, early life is a stress hyporesponsive period, during which the stress response is reduced both in intensity and duration to avoid detrimental effects of high glucocorticoids level

caused by external stressors. This hyporesponsiveness has been shown both for the mother during pregnancy and lactation (Slattery and Neumann, 2008) and for the young during the first weeks of life (Quillfeldt et al., 2009; Sapolsky and Meaney, 1986). Therefore, our results could indicate that once conditions improved after the food restriction, previously restricted individuals were able to maintain similar levels of glucocorticoids as control individuals, in order to efficiently compensate for their growth delay.

As predicted, the "redox profile" at the end of growth and the level of glucocorticoid accumulated during growth were correlated. Individuals displaying higher levels of cortisol had lower antioxidant defenses. It is possible that individuals with low antioxidant defenses are in lower condition. They would hence be the most susceptible to negative environmental conditions and consequently produce more glucocorticoids. In support to this hypothesis, a study conducted on Soay sheep has shown that individuals with higher levels of SOD (an enzymatic antioxidant), as young exhibited higher survival (Christensen et al., 2016). Alternatively, the correlation we found could reflect a causal relationship between glucocorticoids and oxidative stress, as glucocorticoids can generate higher levels of ROS, while decreasing antioxidant defenses (Sato et al., 2010; You et al., 2009; Zafir and Banu, 2009). Surprisingly, oxidative damage was negatively correlated with both the level of cortisol and cortisone. In order to get better insights into the complex interaction between growth, oxidative stress and glucocorticoids, we would require quantification of the glucocorticoids at specific times to associate baseline levels of glucocorticoids with oxidative balance at the same time points. However, this manipulation was considered too invasive to be conducted on the pups, as blood sample sizes were very limited, and urine collection would have required long time apart from their mothers.

At the end of the experiment, all mother-pup pairs were released in the dome within the bigger colony. Most of the individuals (N=38 out of 49, *i.e.* 77%) were re-sighted at least once within the first month of being released. Therefore, we excluded the inability to adjust to the dome as a factor explaining the mortality rate. Moreover, the mortality rate in the dome population is identical to the one for the individual of the experiment (unpublished data). Despite not being statistically significant, we found that restricted males tended to show higher mortality rate than control males after a year. The absence of significant difference could be explained by our small sample size and a low statistical power. That result would support the predicted growth versus lifespan trade-off (reviewed in Metcalfe, 2003).

In conclusion, our results show that although early life is associated with increased level of oxidative damages, compensatory growth did not entail physiological costs on the short-term.

Therefore, individuals were able to efficiently mitigate the consequences of adverse early life conditions, both morphologically and physiologically. However, the costs of early adverse conditions might arise on the long-term.

Acknowledgements

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Data availability

Data will be uploaded into a dryad digital repository upon acceptance of the manuscript.

Author Contributions statement

MM, NF and FH conceived the ideas and designed methodology;
MM, DH, MS, MR, EK collected the data;
MM and DH performed the lab analyses;
MM, NF and FH analyzed the data;
MM, NF and FH wrote the manuscript.
All authors gave final approval for publication

Chapter 2: Experimental manipulation of reproductive tactics in Seba's short-tailed bats: consequences on sperm quality and oxidative status

Magali Meniri[1], Florence Gohon[1], Ophélie Gning[1], Armelle J. Vallat-Michel[2], Gaëtan Glauser[2], Nicolas Fasel*[3] and Fabrice Helfenstein*[1]

1: Laboratory of Evolutionary Ecophysiology, Institute of Biology, University of Neuchâtel, Switzerland

2: Neuchatel Platform of Analytical Chemistry, Institute of Chemistry, University of Neuchâtel, Switzerland

3: Leibniz Institute for Zoo and Wildlife Research, Berlin, Germany

*: Shared last authorship

Abstract

To reproduce, males have to fertilize the female's eggs, sometimes in competition with ejaculates of other males. Sneakers are expected to invest relatively more resources towards sperm quality compared to dominant males, which should invest more towards the soma. Sperm cells are especially vulnerable to oxidative stress, which reduces male fertility. Therefore, antioxidant resources are expected to mediate sperm quality, and to be allocated differently between reproductive tactics. *Carollia perspicillata* exhibit a species with male alternative reproductive tactics whereby harem males have privileged access to females and face low sperm competition risk, whereas sneaker males always experience sperm competition.

To test the link between reproductive tactics, redox profile and sperm quality, we experimentally induced changes in the reproductive tactics. We monitored the blood and ejaculate oxidative balance, and the sperm quality before, 7 days and 21 days after the manipulation of reproductive tactic. Sperm quality only varied between tactics shortly after the manipulation of reproductive tactics, and was not explained by changes in the redox profile, although ejaculates' oxidative damage was negatively related to sperm velocity. When males remained in a similar social environment for three weeks (before and 21 days after the manipulation), differences between reproductive tactics were no longer detectable.

To conclude, males generally did not adjust their sperm of similar quality according to their reproductive tactic, possibly as some constraints were lifted in the experimental cages. Furthermore, our results suggest that, in Seba's short-tailed bats, the expression of alternative reproductive tactics is not subjected to strong oxidative constraints.

Key-words: *Oxidative stress, sperm competition, alternative reproductive tactics, Carollia perspicillata*

1. Introduction

A male's ability to reproduce depends both on its capacity to acquire mates, determined by pre-copulatory traits (Andersson, 1994; Clutton-Brock, 2007), and to fertilize the eggs, determined by post-copulatory traits such as sperm quality (Simmons and Fitzpatrick, 2012). Sperm competition models assume (i) costs to both pre- and post-copulatory traits, (ii) limited resources, and (iii) allocation trade-offs of these resources to both types of traits. They predict that males are selected to strategically allocate resources to pre- and post-copulatory traits depending on their ability to access females (Parker, 1990; Parker et al., 2013). Specifically, these models predict that more sexually competitive males have a greater fitness return when investing more resources to pre-copulatory traits *i.e.* to acquire mates, whereas less sexually competitive males will increase their fitness by expending more resources in post-copulatory traits, *i.e.* to improve their fertilization ability. Empirical support for these sperm competition models has been found repeatedly in several taxa (*bats:* Fasel et al., 2017; *birds:* Froman et al., 2002; *reptiles:* Kahrl et al., 2016; *beetles:* Simmons and Emlen, 2006; *fishes:* Young et al., 2013). Yet, several studies have reported a positive relationship between pre- and post-copulatory selected traits, with more attractive males having higher fertilization abilities (Locatello et al., 2006; Malo et al., 2005b; Peters et al., 2004). These results provide empirical support to the phenotype-linked fertility hypothesis (Sheldon, 1994), which states that a male's attractiveness is an honest signal of its fertility. Recently, a comparative analysis by Lüpold et al. (2014) has reconciled sperm competition models and the phenotype-linked fertility hypothesis by showing that the conditions for a trade-off between pre- vs. post-copulatory traits are found when reproductive success is mainly determined by the males' ability to monopolize females. Variation in female monopolization abilities among males may lead to alternative reproductive tactics (ARTs), whereby a few males monopolize fertile females, while most males are forced to sneak copulations and consequently inevitably face sperm competition (Engqvist and Taborsky, 2016; Oliveira et al., 2008a).

Many nutrients may be provided to organisms in limited amounts, leading to the trade-offs predicted by the sperm competition models. This may be the case for resources involved in the antioxidant defenses used to protect cells from the deleterious effect of reactive oxygen species (ROS). ROS play key roles in cellular processes (Baker and Aitken, 2004; Halliwell and Gutteridge, 2007), and all organisms have evolved an antioxidant system to keep ROS under control. However, when ROS production overwhelms the antioxidant machinery, it leads to damage to all biological molecules, a condition referred to as oxidative stress (Halliwell and

Gutteridge, 2007). Due to the many circumstances under which ROS may be produced in excess, oxidative stress has been proposed to be an important constraint shaping the evolution of physiological mechanisms underlying major life-history trade-offs (Metcalfe and Alonso-Alvarez, 2010; Monaghan et al., 2009). Sperm cells are especially vulnerable to oxidative stress (Agarwal et al., 2014), as the high content in polyunsaturated fatty acids (PUFAs) of the sperm membrane makes them susceptible to lipid peroxidation, and the condensed structure of their chromatin strongly limits any repair of DNA damage. Consequently, oxidative stress has a major negative impact on sperm quality (Cocchia et al., 2011) and male fertility (Aitken et al., 2014; Wright et al., 2014).

Seba's short-tailed bats (*Carollia perspicillata*) exhibit three alternative reproductive tactics (Fasel et al., 2016), with harem males monopolizing the access to females on their territory, by opposition to bachelor that sneak copulations, and a minority of peripheral males that are faithful to a site close to a harem. To defend their territories, harem males are frequently involved in agonistic interactions (Fernandez et al., 2014), and likely have reduced foraging and resting time, leading to higher cellular oxidative stress in the blood, whereas sneaker males produce faster and longer-lived sperm than harem males (Fasel et al., 2017).

To test the interplay between reproductive tactics, sperm quality and redox profile, we experimentally manipulated male reproductive tactics. We aimed at (i) experimentally testing the significance of the correlation between reproductive tactics and sperm quality that was found previously; (ii) testing for predicted trade-offs between the antioxidant protection of the soma vs. the ejaculate depending on the reproductive tactics; (iii) investigating the role of antioxidant protection to the ejaculate as a potential mechanism explaining differences in sperm quality. To do so, we experimentally induced males of known reproductive tactics to switch to a different tactic by shuffling males across cages. We monitored the males' redox profiles in both blood and ejaculate through a set of biomarkers: an endogenous antioxidant (Superoxide dismutase: SOD), an exogenous antioxidant (tocopherol), a marker of cellular oxidative stress (the ratio of oxidized over reduced glutathione) and a marker of oxidative damage to the lipids (malondialdehyde: MDA). To assess sperm quality, we analyzed sperm velocity and the percentage of motile sperm, two traits commonly linked to fertilizing abilities (Gasparini et al., 2010; Pusch, 1987).

As sneaker males face higher level of sperm competition, we predicted that they should privilege the antioxidant protection of their ejaculates, therefore showing lower sperm oxidative damage, and higher sperm quality. Harem males were predicted to favor the antioxidant protection of their soma at the expense of their germline, leading to lower oxidative damage in the blood and

potentially higher oxidation levels of their sperm cells. More importantly, we predicted that when forced to switch tactic, males would modify their investment in the antioxidant protection of their soma and their sperm cells so that sneakers becoming harem males would exhibit lower oxidative damage in their soma, but produce more oxidized and lower quality sperm than before the switch. Conversely, harem males becoming sneakers would exhibit greater oxidative damage in their soma, but would produce less oxidized and better quality sperm than before the switch.

2. Material and Methods

2.1 Model species and studied population

The study was conducted using a captive colony of Seba's short tailed bats (*Carollia perspicillata)*, hosted in the Papiliorama, a tropical zoo located in Switzerland. Individuals can fly freely under a 40m-diameter dome open to the public and are fed *ad-libitum* with a fruit-based mixture.

2.2 Timeline of the experiment

We conducted this experiment using five cages at a time, for one run. We carried out four runs of the experiment from October 2014 until April 2015. Each run lasted about 45 days (+/- 3 days for hierarchy establishment). Each cage measured 1m x 2m x 1m. Each cage was constituted with 3 males of known tactics from the colony: 1 harem male with 2 females belonging to his harem, and 2 sneaker males. Individuals were trapped from the colony during their sleep using a hand net. An individual was considered to be a harem male when caught in a harem and reproductive (*i.e.* with scrotal testes). For each harem male, two non-lactating females from the same harem were trapped and transferred to the cages. By having a harem male in a cage with females of his harem, we expected him to maintain his reproductive tactic despite the stress induced by the changes of spatial and social environment. The next day, sneaker males were trapped from bachelor groups in the colony and attributed randomly to the cages. During the acclimation period, the reproductive tactic of the males was determined via daily monitoring of the bats; the one male roosting with the females for two consecutive days was considered to be the harem male. Once the hierarchy was established, we never observed a change in the social structure.

After three weeks of acclimation period, we performed the first blood and ejaculate sampling (see below for details on the procedure). Immediately upon sampling, we transferred each male to a different cage, in order to induce changes in some males' reproductive tactics. Every male was in a novel cage, with individuals they had not yet encountered during the experiment. For each experimental run, we formed three types of groups, to maximize the number of reproductive tactics changes. In one control cage, we combined one harem male with two sneaker males and two females. In two cages, we combined two harem males and one sneaker male with two females. In the remaining two cages, we combined three sneaker males with two females.

The different cage composition resulted in 4 categories of reproductive tactic changes: Sneaker males that remained Sneakers (SS), Sneakers that became Harem males (SH), Harem males that became Sneakers (HS) and Harem males that remained Harem males (HH). After the manipulation of reproductive tactics, it took between 3 to 6 days for the social environment to become stable. Seven days and three weeks after the stabilization of the new hierarchy, we performed the second and third blood and ejaculate samplings, respectively.

A total of 60 males were sampled initially, but several individuals were not included in the analysis. First, 15 males switched their reproductive tactics during the acclimation period. These individuals could not be included in the analysis as their first blood and ejaculate sampling would not provide basal measures. Then, males from 4 cages did not exhibit a clear reproductive tactic; the group remained together without individuals becoming either harem or bachelor males. After a week, those individuals (12 males) were released and excluded from the analysis, in order to keep the time spent in the cages comparable, as it might impact the physiology of individuals. Finally, our sample size for statistical analysis was of 39 males before the manipulation, and 33 males after. Specifically, for the first sampling before the manipulation, there was 23 sneakers and 13 harem males: For the 2nd and 3rd sampling, there was: SS:14; SH: 7; HS: 6; HH: 5. However, we did not have all physiological measures for all males, due to limitations in blood and ejaculates samples.

2.3 Blood collection

For each individual, the blood was collected within 5 min after trapping. Blood samples were drawn from the antebrachial wing vein by puncturing with a sterile needle, and collecting the droplet of blood using a Microvette (CB 300 Hep-Li, Sarstedt). Samples were stored on ice until

transportation to the lab. Back to the lab, blood samples were centrifuged at 2'000 G for 5 minutes at 4°C to separate cells from plasma. The plasma was collected and aliquoted for later analyses. Samples were stored at –80°C.

2.4 Ejaculate collection

Ejaculates were collected using electro-ejaculation (Fasel et al., 2015). In summary, males were laid dorsally on a warming pad. During the procedure, males were anesthetized using isoflurane. Anesthesia was induced with 5% isoflurane mixed with oxygen for about 5 s, and then was decreased to 1 to 2% isoflurane. Oxygen was provided at a rate of 0.8 l/min. A probe covered with aqueous lubricant was inserted in the rectum approximately 1cm deep. Electric stimulations were transmitted using two electrodes situated at the distal end of the probe (ICSB, USA). The electrode was linked to an audio amplifier (JVC A-X2) generating three series of regular and increasing electric stimulations (maximally 4 mA). Electrical current was continuously monitored with a milli-ampere meter (Fluke 77 multimeter). After the stimulation, oxygen was provided alone until awareness. The ejaculate collected was transferred into a microcentrifuge tube containing 10 µl of PBS to avoid desiccation. According to the estimated volume of ejaculate, PBS was added to obtain a 1:2 dilution. An aliquot of 3 µl was immediately taken for mobility analysis. The remaining of the sample was stored on ice until transportation to the lab, and then kept at -80°C until analysis.

2.5 Sperm mobility traits analysis

The 3 µl aliquot was mixed with 15 µl of pre-warmed at 37°C Earle's balanced salt solution (SpermWash Cryos, Denmark) and gently mixed. Within 10 min of collection, 3 µl of this mix was loaded in a swimming chamber (SC 20-01-04-B, Leja, Nieuw-Vennep, Netherlands), and sperm mobility was recorded in the swimming chamber under an Olympus XK41 microscope with dark-field condition, mounted with a Kappa CF 8/5 camera with a 20x magnification objective and a 10x magnification C-mount adaptor. Several 2-s videos (median 8, min 3, max 15) of 25 frames/s with a median number of 15 sperm track (min 0, max 224) were then analyzed for each ejaculate using a CASA plug-in in ImageJ 1.47v (Wilson-Leedy and Ingermann, 2007) to obtain estimates of eight sperm swimming parameters: motility (proportion of motile sperm), curvilinear velocity (VCL, µm/s), velocity average path (VAP), velocity straight line (VSL),

linearity (VSL/VAP), wobble (VAP/VCL), progression (average distance of the sperm from its origin on the average path during all frames analysed) and beat cross frequency (BCF, frequency at which VCL crosses VAP, Hz). Sperm cells swimming with a higher velocity (VCL > 6 μm/s) than non-sperm particles in the sample were considered as motile.

As found previously (Fasel et al., 2015), a principal component analysis (PCA) on all sperm swimming parameters (excluding the percentage of motile sperm, analysed separately), identified a first principal component explaining 53.19% of the variance, which was positively loaded with VCL, VAP, VSL but negatively loaded with BCF, and uncorrelated with the wobble, the number of sperm tracked, and linearity. Hence, males with high scores along this first PC produced fast swimming sperm. Results obtained using VCL or PC1 scores were qualitatively similar. Thus, for the sake of comparison with our previous work, and because it might be more intuitive to the readers, we only report analyses with VCL.

2.6 Redox markers

Lab analyses were performed by MM, blindly with respect to sample identity and the experimental design. All steps were conducted on ice to reduce oxidation. All chemicals were HPLC grade, and chemical solutions were prepared using ultra pure water H_2O MQ (Milli-Q Synthesis; Millipore, Watford, UK).

Blood sample preparation

A homogenate of red blood cells was prepared by diluting 10 μl of cell pellet into 10 μl of phosphate buffer saline (PBS). The mixture was homogenized with four glass beads for 1 minute at 30 Hz using a mixer mill, immersed in an ice-cold bath and sonicated for 5 minutes, and centrifuged for 5 minutes at 11'200 G at 4°C. Of the supernatant 10 μl of RBC homogenate were transferred in an Eppendorf tube for MDA quantification. Another 2 μl were diluted with 748 μl of PBS, vortex-mixed and centrifuged for 15 minutes at 10'000 G and 4°C (final dilution RBC 1:750 v:v) for SOD colorimetric assays. Finally, 2 μl were used immediately to quantify the reduced (GSH, ng/ml) and oxidized (glutathione disulfide GSSG, ng/ml) forms of glutathione (see glutathione quantification). We also quantified α, δ, γ-tocopherol using 10 μl of plasma, and MDA in 15 μl of plasma.

Ejaculate sample preparation

The ejaculate was homogenized with two glass beads for 1 minute at 30 Hz using a mixer mill, then immersed in an ice-cold bath and sonicated for 5 minutes. Of the supernatant 10 µl of ejaculate were transferred in an Eppendorf tube for MDA quantification. Another 2.5 µl of ejaculate were diluted with 37.5 µl of PBS, vortex-mixed and centrifuged for 15 minutes at 10'000 G and 4°C (final dilution ejaculate 1:48 v:v) for SOD colorimetric assays. Finally, 3.5 µl were used immediately to quantify glutathione.

Quantification of redox markers

MDA quantification

We assessed MDA (nmol/ml) by its reaction with 2-thiobarbituric acid (TBA) to produce a pink derivate quantifiable by ultra-high performance liquid chromatography with fluorescence detection (UHPLC-FD), using a method adapted from (Losdat et al., 2014). Details about the method can be found in Annex.

SOD activity

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.

Glutathione quantification

The reduced (GSH, ng/ml) and oxidized (glutathione disulfide 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. Further details about the method can be found in Annex .

Tocopherol quantification

We quantified α, δ, γ-tocopherol in the plasma by ultra-high performance liquid chromatography tandem mass spectrophotometry (UHPLC-MS/MS). More details about the method can be found in Annex.

2.7 Statistical analysis

To test the prediction that males would adjust their redox profile and their sperm quality to match their reproductive tactic, we ran a first set of models. Separate linear mixed-effects models were run for each sampling event, *i.e.* before the manipulation, 7 days after the manipulation and 21 days after the manipulation. Our response variables were MDA concentration, SOD activity and the GSSG/GSH ratio (log-transformed) in red blood cells and ejaculates, and MDA and α, δ, γ-tocopherol in the plasma on one hand, and sperm swimming velocity and the percentage of motile sperm (logit-transformed) on the other hand. For traits measured before manipulating male tactics, the fixed factor was the initial reproductive tactic. For traits measured 7 days or 21 days after the manipulation, the fixed factor was the type of change in reproductive tactic (SS, SH, HS or HH). For each of these models, we included the cage number at the time of sampling (*i.e.* cage number before or after the manipulation of reproductive tactic) as a random factor.

To test the prediction that sperm quality was correlated with the individual redox markers we built a second set of models where the percentage of motile sperm (logit-transformed) or sperm velocity were included as response variables, and ejaculate redox markers (MDA, SOD or GSH/GSSG ratio) were used as explanatory variables. For this set of models, individual identity was used as a random factor to account for the repeated measures taken on the same male.

Data were analysed using R, version 3.3.3. The "nlme" package was used for the linear mixed-effects models, and we used the "lsmeans" package to run post-hoc tests based on least-square means with Tukey correction for test multiplicity. The significance level was set at 0.05, and the false discovery rate procedure was applied to account for the multiplicity of tests (Benjamini and Hochberg, 1995). As we did not have measures for all redox markers and sperm quality for each male, we chose not to apply linear mixed-effect models with repeated measures and individual ID as a random factor.

2.8 Ethical statement

This study was performed under the authorization 2014_44_FR, delivered by the local authorities from the Fribourg canton (Switzerland) after examination by its ethical committee.

3. Results

Initial reproductive tactics

To characterize the redox profile and the sperm quality of the males before the manipulation of their reproductive tactics, we sampled blood and ejaculate of harem and sneaker males from the colony, after they had spent three weeks of acclimation in cages. We monitored four redox markers both in the ejaculate and the blood: activity of the enzymatic antioxidant SOD, concentration of α, δ and γ-tocopherol (plasma only), the ratio of oxidized over reduced glutathione (GSSG/GSH ratio; red blood cells only), and the level of lipid peroxidation via MDA. The levels of these markers did not differ between the two initial reproductive tactics (cf Table I). Additionally, neither sperm velocity nor the percentage of motile sperm differed between the harem and the sneaker reproductive tactics (cf Table I).

Table I: Models investigating whether blood and sperm redox markers as well as sperm quality traits differed between initial male reproductive tactics (Harem or Sneaker) before the manipulation, or among tactic change categories (SS, SH, HS and HH) 7 days and 21 days after the manipulation. All linear mixed-effects models included the cage as a random factor. P-value highlighted in bold does not remain significant after correction using the false discovery rate procedure.

	Before manipulation			7 days after			21 days after		
Response variable	**N**	**F-value**	**P-value**	**N**	**F-value**	**P-value**	**N**	**F-value**	**P-value**
Redox markers blood									
MDA RBC	33	$0.10_{1,15}$	0.76	28	$1.31_{3,10}$	0.32	25	$1.11_{3,7}$	0.40
MDA plasma (sqrt)	34	$0.09_{1,16}$	0.76	26	$0.25_{3,10}$	0.85	26	$1.80_{3,8}$	0.22
Ratio GSSG/GSH blood (log)	28	$0.09_{1,10}$	0.77	23	$0.04_{3,7}$	0.98	28	$0.15_{3,12}$	0.93
SOD blood	24	$0.12_{1,11}$	0.74	25	$2.13_{3,7}$	0.18	27	$0.79_{3,11}$	0.52
α -tocopherol	21	$0.16_{1,10}$	0.69	15	$0.59_{3,5}$	0.64	-	-	-
δ -tocopherol	20	$1.05_{1,9}$	0.33	15	$1.99_{3,5}$	0.23	-	-	-
γ-tocopherol	19	$3.27_{1,9}$	0.10	14	$1.81_{3,4}$	0.28	-	-	-
Redox markers ejaculate									
MDA ejaculate (sqrt)	24	$0.78_{1,10}$	0.39	21	$0.52_{3,5}$	0.68	22	$0.33_{3,8}$	0.80
SOD ejaculate	26	$0.39_{1,9}$	0.54	25	$0.99_{3,4}$	0.43	25	$0.50_{3,8}$	0.69
Sperm quality traits									
Sperm velocity	36	$0.56_{1,20}$	0.46	**31**	**$3.92_{3,13}$**	**0.03**	30	$2.71_{3,13}$	0.09
% motile sperm	36	$0.06_{1,20}$	0.81	31	$2.84_{3,13}$	0.08	30	$0.71_{3,13}$	0.56

After the experimental manipulation of the reproductive tactics

To test the link between reproductive tactics, blood and sperm redox balance and sperm quality, we experimentally induced some males to change their reproductive tactics by shuffling the males across cages. Therefore, some males maintained their tactics, while others switched, resulting in 4 categories of males: originally Sneaker and remained Sneaker (SS), Sneaker to Harem (SH), Harem to Sneaker (HS) and Harem remaining Harem (HH).

Seven days after the experimental manipulation of the reproductive tactics, none of the redox markers investigated covaried with the changes in reproductive tactics (cf. Table I). However, we found differences in sperm velocity between the 4 categories of males ($F_{3,13} = 3.92$; p-value=0.03), with respectively SS males (Tukey post-hoc test; $t_{13} = 2.86$; p-value 0.05), SH

(t_{13}=3.15; p-value = 0.03) and HS males having higher velocity than HH males (t_{13} =2.96; p-value=0.04) (cf Fig. 1, Table I).

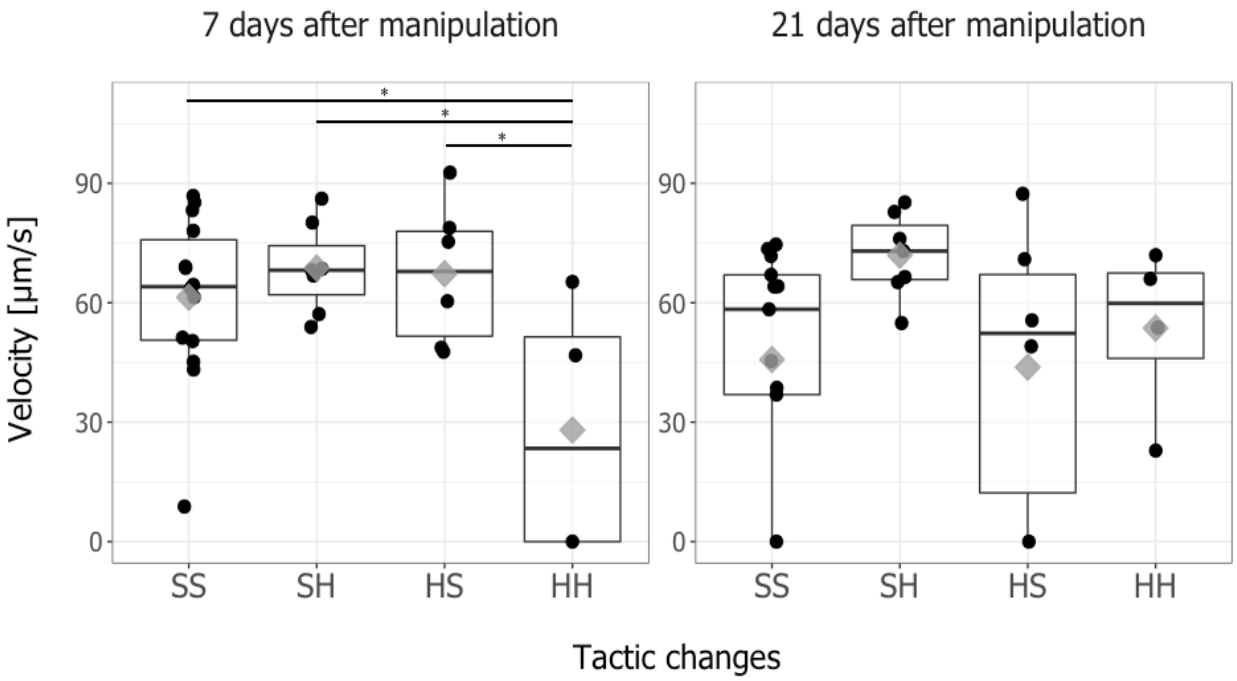

Figure 1: Sperm velocity according to the changes in reproductive tactics 7 and 21 days after the manipulation. Black dots represent the distribution of the data, and the grey diamonds the group means. Horizontal bars and asterisks indicate statistical differences between groups (*p-value≤0.05).

After 21 days, none of the blood and sperm redox markers differed across changes in reproductive tactics (cf Table I). Similarly, there were no differences in sperm mobility traits among reproductive tactics (cf Table I).

Link between redox markers and sperm mobility traits

Finally, we investigated the link between sperm mobility traits and markers of ejaculate redox balance. Sperm velocity was negatively correlated with the level of MDA in the ejaculate ($F_{1,22}$=7.16; p-value=0.01; cf Fig. 2). The percentage of motile sperm was not correlated with ejaculate MDA level. Sperm mobility traits did not correlate with SOD activity in the ejaculate, nor with the GSSG/GSH ratio (cf Table II).

Table II: Sperm mobility traits and redox markers. Linear mixed-effects models, with individual identity as a random effect. P-values highlighted in bold remained significant after correction using the false discovery rate procedure.

	Sperm velocity			% motile sperm (logit)		
Explanatory variable	**N**	**F-value**	**P-value**	**N**	**F-value**	**P-value**
MDA ejaculate	**54**	**$7.16_{1,22}$**	**0.01**	63	$0.01_{1,29}$	0.90
Ratio GSSG/GSH ejaculate (log)	31	$0.29_{1,9}$	0.60	33	$2.09_{1,10}$	0.18
SOD ejaculate	60	$0.05_{1,26}$	0.83	71	$0.32_{1,35}$	0.57

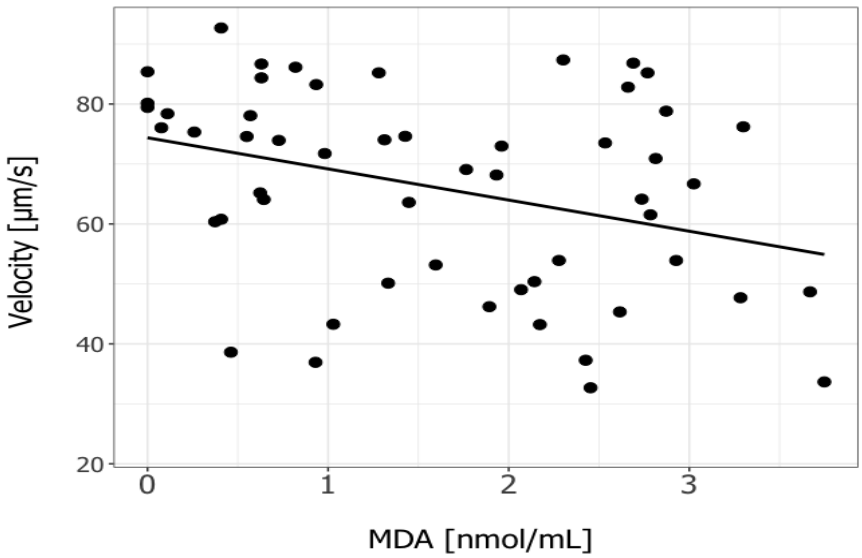

Figure 2: Correlation between sperm velocity and levels of MDA in the ejaculate. The black line is the regression line of the model. 2 values of velocity <10 were removed from the analysis. If they are included, the correlation is no longer significant ($F_{1,23} = 2.02$, p-value = 0.17).

4. Discussion

This study aimed at experimentally testing the link between male reproductive tactics, sperm quality and both blood and sperm redox profiles. To investigate these relationships, we manipulated male reproductive tactics, and sampled blood and ejaculate before the manipulation, 7 days and 21 days after the manipulation. We estimated sperm quality using the percentage of motile sperm and sperm swimming velocity, as these traits have been found to predict the fertilizing efficiency of the ejaculate in various species (Simmons and Fitzpatrick, 2012).

Our manipulation led to four categories of males, with males that remained in their initial tactic, and males that switched to the opposite tactic. Seven days after the manipulation, we found that harem males that retained a harem showed a lower sperm quality compared to all other categories of males. Although based on a small sample size, this result accords well with the predictions of the sperm competition models (Parker et al., 2013), namely that males of different reproductive tactics invest differently in their ejaculates, with males with a favored access to females being predicted to invest relatively less compared to sneakers. Our result suggests that harem males adjusted their sperm quality to their tactic, but it appears to be transient as this difference was not detected prior to the manipulation and faded away 14 days later. Numerous studies in various taxa have found that in species with alternative reproductive tactics, sneaker males exhibit higher sperm quality than males with a favored access to females (*birds:* Froman et al., 2002; Rowe et al., 2010; *insects:* Kelly, 2008; Simmons and Emlen, 2006; Yamane et al., 2010; *fishes:* Fu et al., 2001; Haugland et al., 2009; Makiguchi et al., 2016; Smith and Ryan, 2010; Vladić and Järvi, 2001; Young et al., 2013; *squid:* Hirohashi et al., 2016). In mammals however, results are more contrasted. Some studies have found the predicted trade-off between pre-copulatory expenditure and ejaculate quality (Fitzpatrick et al., 2012; Simmons et al., 2011; Stockley and Purvis, 1993), but others did not find a relationship between pre-copulatory expenditure and ejaculate quality according to the reproductive tactic (Kruczek and Styrna, 2009; Lemaître et al., 2012; Schradin et al., 2012), or even found a positive relationship (Malo et al., 2005; Preston et al., 2003). Notably, it was previously found in our model species that sneakers have higher sperm quality compared to harem males (Fasel et al., 2017). The question then arises as to why we only found a difference that was transient at best, and more generally, why males did not adjust their sperm quality according to their reproductive tactics. We can think of several non-mutually exclusive explanations to our results. First, the “dear enemy effect” proposes that territorial males exhibit less agonistic interactions towards familiar males (Temeles, 1994). A

reduction of energetically costly agonistic interactions, once the new tactics were settled, may have allowed males to allocate more resources to sperm quality. This may explain why a difference in sperm quality was detected 7 days after the manipulation and then later disappeared. Second, past a transient adaptation period during which harem males possibly needed to invest into harem acquisition and produced lower quality sperm, *ad libitum* feeding may have then enabled all males to produce sperm of similarly good quality (Catoni et al., 2008; Tuomi et al., 1983). Third, with only two females per cage, although we did not monitor copulation rates, copulations were likely less frequent compared to what males experience in the colony, where a harem can comprise up to 18 females (Fleming, 1988). Indeed, Wesseling et al. (2016) showed that the sperm quality of harem males improves after three days of sexual abstinence to reach a quality similar to that of sneaker males. Therefore, it is not surprising that, overall, we did not find a difference in sperm quality between harem males and sneakers in our experimental cages, contrary to what was found before. Fourth, Lüpold et al. (2014) suggested that how efficiently males control the access to fertile females ranges from mating systems where some males manage to monopolize females and greatly reduce the risk of sperm competition to mating systems where males apply less efficient mate-guarding and territorial tactics and where sperm competition risk is more evenly distributed among all males. In the former case dominant males are expected to invest relatively less in their sperm quality than sneakers who always have to compensate for a greater risk of sperm competition. In the latter case, males are expected to exhibit more tenuous differences in sperm quality as all males may be exposed to a similar risk of sperm competition. This pattern was highlighted in a comparative study conducted on mammals, where smaller, subordinate males have relatively bigger testes than dominant males in continuous but not seasonally breeding species. In the latter species, females' monopolization is incomplete, and thus sperm competition level may be comparable for every male (Stockley and Purvis, 1993). In our model species, it was shown that 40% of the pups in the population are sired by sneakers (Fasel et al., 2016). As harem males defend the access to females on their small territory, but do not guard females outside of it, females could freely explore and mate with other males. Thus, females' monopolization might be incomplete, which may lead harem males to invest both in somatic maintenance and ejaculate quality to optimize their reproductive success, a strategy implying that harem males are "higher quality" individuals (the "big house, big car" effect; Reznick et al., 2000). Harem males may also pay the cost later in life, as suggested by their decreased probability of maintaining their tactic as they get older (Fasel et al., 2016).

In this study, we also aimed at investigating whether allocation of resources in the antioxidant protection of the soma vs. the germline may underlie differences in sperm quality and may differ between harem and sneaker males. Although we found a negative correlation between the level of oxidative damage in the sperm and sperm velocity, the difference in sperm quality observed 7 days after the manipulation was not echoed by a difference in allocation of antioxidant resources. Therefore, antioxidant allocation does not seem to be the main proximate driver of variation in sperm velocity. Seminal fluids composition (Perry et al., 2013) or copulation frequency and abstinence (Wesseling et al., 2016) may play greater roles in explaining the difference in sperm velocity found at day 7. Moreover, we found no differences in somatic or ejaculate redox profile between individuals exhibiting different alternative reproductive tactics. Thus, and contrary to recent findings in other species (Mora et al., 2017; Tomášek et al., 2017), oxidative stress does not seem to constraint the expression of reproductive tactics in Seba's short-tailed bats, and thus does not lead to different redox profile according to male tactics (harem vs. sneaker). A comparative study conducted on tropical bats found that frugivorous bats showed both lower levels of oxidative damage and higher antioxidant level compared to species with other diets (Schneeberger et al., 2014). Therefore, Seba's short-tailed bats may be able to acquire enough antioxidant from their diet to avoid oxidative stress, at least in our experimental cages.

Half of our initial males had to be removed from the analysis, as some did not form a hierarchy in the cages. These males represents yet another example of how stress induced by detention can impact an individual's behavior, which can limit and even prevent the study of some behaviors (McCobb et al., 2005; Tauson, 1998; Uetake et al., 2013). For other males, the reproductive tactics that they exhibited in the colony changed during the acclimation period. It highlights the plasticity of these alternative reproductive tactics, and the fact that males may not be able to retain their territorial status when the environmental conditions change, *i.e.* when put in captivity with a different social surrounding. It also shows that some males can readily take over the harem position, as soon as they are given the opportunity.

To conclude, we found only transient differences in sperm quality according to reproductive tactics. We propose that a number of energetic constraints may have been lifted in the experimental cages, possibly allowing all males to exhibit sperm of similar quality regardless of their reproductive tactic. Furthermore, our results suggest that, in Seba's short-tailed bats, the expression of alternative reproductive tactics is not subjected to strong oxidative constraints, possibly due to their antioxidant rich diet.

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Data availability

Data will be uploaded into a dryad digital repository upon acceptance of the manuscript.

Author Contributions statement

NF and FH conceived the ideas and designed methodology;
MM, FG and NF collected the data;
OG, GG, AV assisted with lab analyses;
MM, NF and FH analyzed the data;
MM, NF and FH wrote the manuscript.
All authors gave final approval for publication.

Chapter 3: Sperm morphology in relation to sperm swimming performance and alternative reproductive tactics in Seba's short-tailed bats *(Carollia perspicillata)*

Magali Meniri[1], Julie Penneteau[1], Nicolas Fasel*[3] and Fabrice Helfenstein*[1]

*: Shared last authorship

1: Laboratory of Evolutionary Ecophysiology, Institute of Biology, University of Neuchâtel, Switzerland

2: Neuchatel Platform of Analytical Chemistry, Institute of Chemistry, University of Neuchâtel, Switzerland

3: Leibniz Institute for Zoo and Wildlife Research, Berlin, Germany

Abstract

Sperm competition is a potent force acting on those sperm traits that determine male fertilizing ability. It has been proposed that longer sperm cells should swim faster, thus providing a fertilizing advantage in a competitive context. Across species, sperm length has been shown to increase with increasing levels of sperm competition, while within-ejaculate variation in sperm morphology has been shown to decrease with increasing levels of sperm competition. Yet, in both cases the evidence is very equivocal within-species. With this study, we aimed at investigating (1) whether males exposed to contrasted risks of sperm competition produce spermatozoa with distinct morphology, (2) whether sperm morphology is related to sperm swimming performance, and (3) whether males exposed to greater sperm competition risk produce ejaculates with less morphologically variable spermatozoa. We used Seba's short-tailed bats (*Carollia perspicillata*), a species with male alternative reproductive tactics whereby harem males have privileged access to females and face low sperm competition risk, whereas sneaker males always experience sperm competition. We experimentally manipulated the reproductive tactics of males and sampled ejaculates before the change, and 7 days and 21 days after the change. We measured sperm morphology and sperm swimming performance for each ejaculate. Contrary to our expectations, we found no difference in sperm morphology according to the reproductive tactic. We also found no robust correlation between sperm morphology and sperm swimming performance. Lastly, regardless of the reproductive tactic, we found surprisingly high levels of within-ejaculate variation for a species with overall high levels of sperm competition.

Our results add up to the growing number of studies in various taxa that question the functional link between sperm morphology and sperm swimming performance, or at least its relevance compared to other determinants of sperm swimming performance such as the seminal fluids or compared to the constraint exerted by the female reproductive tract. Finally, we propose that high within-ejaculate variation may result from a "gametic bet-hedging" strategy whereby males would optimize their fertilization success throughout various levels of sperm competition risk.

Key words: *sperm morphology, sperm competition, alternative reproductive tactics, sperm swimming performance*

1. Introduction

Sperm competition, *i.e.* the competition between ejaculates of different males to fertilize a given set of ova (Parker, 1970), is a potent force shaping traits that influence the fertilizing abilities of the ejaculate. Sperm morphology has been shown to impact sperm fertilization abilities (*birds*: Bennison et al., 2016; but see Cramer et al., 2015; *ungulates:* Malo et al., 2006), and is therefore expected to evolve under sperm competition. Indeed, several comparative studies have shown that sperm length increases with the level of sperm competition across taxa (*frogs:* Byrne et al., 2003; *fishes:* Fitzpatrick et al., 2009; *butterflies:* Gage, 1994; *mammals:* Gomendio and Roldan, 1991; *snakes*: Tourmente et al., 2009), although other studies failed to find such a relationship (*bats:* Hosken, 1997; *birds:* Kleven et al., 2009). However, what links sperm length to sperm competitive ability and thus why sperm length evolves in a context of sperm competition is not clear. One hypothesis, that has been explored over the past three decades, postulates that longer sperm swim faster (Gomendio and Roldan, 1991). Indeed, longer mid-piece may contain more mitochondria to power the sperm cell, and a longer flagellum would increase the propelling power. Ultimately, an ejaculate containing sperm swimming relatively faster should lead to higher fertilizing efficiency under sperm competition (reviewed in Fitzpatrick and Lüpold, 2014; Simmons and Fitzpatrick, 2012).

Although a functional relationship between sperm swimming abilities and sperm morphology is theoretically appealing, empirical evidence is rather inconsistent. At the inter-specific level, most studies have shown a positive link between sperm swimming abilities and sperm length (*fishes:* Fitzpatrick et al., 2009; *mammals:* Gomendio and Roldan, 1991; Tourmente et al., 2011), although with some incongruences (*birds:* Lüpold et al., 2009). At the intra-specific level, evidence for a link between morphology and sperm swimming abilities is even more mixed. Some studies showed a positive correlation between the length of sperm cells' components and velocity in birds (Lifjeld et al., 2012; Losdat and Helfenstein, 2018; Mossman et al., 2009). However, Cramer et al. (2015) showed a negative relationship in two species of sparrows, where a positive relationship was previously found (Helfenstein et al., 2010), and no relationship was found in mallards *Anas platyrhynchos* (Denk et al., 2005). In the red deer, *Cervus elaphus,* sperm cells with longer head and shorter mid-piece swim faster (Malo et al., 2006) while in house mice *Mus domesticus,* sperm velocity increases with mid-piece length (Firman and Simmons, 2010).

The contradictory results reported between studies might stem from several causes. First, Humphries et al. (2008) suggested that longer is not always better, as a larger head could create

more drag. Hence, selection may occur on an overall sperm morphology, and we should focus on ratios between sperm components rather than on absolute length. Second, the idea that longer mid-piece leads to higher ATP production is not always supported (Bennison et al., 2016). Indeed, oxidative phosphorylation by the mitochondria is not the only source of ATP, as it can also be produced in the flagellum via glycolysis (reviewed in Turner, 2003). The relative importance of the two metabolic pathways for energy production differs across species, which might blur the relationship between mid-piece length and ATP production. Third, the environments where sperm cells compete differ widely between taxa, as for example between species with external vs. internal fertilization (Simpson et al., 2014). This may influence the link between sperm morphology and sperm swimming velocity, as the physical constraints experienced by the sperm cells are different. For all the aforementioned reasons, whether sperm morphology influences sperm swimming abilities and thus evolves via sperm competition remains unclear.

Sperm competition is further predicted to act on within-ejaculate variation in sperm morphology. Under the assumption that sperm morphology influences fertilizing abilities and if the production of longer sperm is costly (Godwin et al., 2017), males facing sperm competition are predicted to produce sperm cells with an optimal sperm morphology and to exhibit little within-ejaculate variation in sperm morphology (reviewed in Simmons and Fitzpatrick, 2012). On the contrary, in a non-competitive environment, where sperm competition is relaxed, a high within-ejaculate variance may be expected due to a reduction in the costly control of sperm production and the occurrence of random developmental errors (Hunter and Birkhead, 2002). In agreement with this idea, two comparative studies on passerine birds showed that intra-male variation in sperm design decreases with increasing level of sperm competition (Calhim et al., 2007; Immler et al., 2008), and a similar pattern was found in rodents (Varea-Sánchez et al., 2014). Yet, high levels of within-ejaculate variance have been reported in species with high levels of sperm competition (Blengini et al., 2014; Calhim et al., 2011).

Overall, whether and how variations in sperm competition levels underlie the relationship between sperm morphology and sperm swimming ability or the maintenance of within-ejaculate variation in sperm morphology remains to be clarified. In this regard, experiments in species where males face contrasting levels of sperm competition may provide useful insights. Seba's short tailed bats *(Carollia perspicillata)* are especially appropriate to study whether and how sperm competition influences sperm morphology. Indeed, males exhibit alternative reproductive tactics (ARTs), with harem-holding males defending the access to females on their territory, while a majority of males have to sneak copulations. Although a minority of harem-holding males sire

a majority of the offspring in the population, sneaker males are still able to sire 40% of the offspring, indicating overall high levels of sperm competition in the population (Fasel et al., 2016). Notably, males face very contrasted risks of sperm competition depending on their tactic: while harem-holding males only rarely face sperm competition, sneakers males always do so. Hence, based on sperm competition models (Parker, 1990; Parker et al., 2013), sneaker males are expected to compensate for a lower access to females and to invest relatively more in their sperm quality compared to harem males. Accordingly, sperm velocity was shown to be higher in sneaker males (Fasel et al., 2017). These alternative reproductive tactics, facing contrasted levels of sperm competition, may select for different optimal sperm morphology in each ART, as this has been shown in the bluegill sunfish *Lepomis macrochirus* (Burness et al., 2004), where sneaker males have longer sperm cells. Such mating system may also impact the level of intra-male, intra-ejaculate variation, with sneaker males potentially having lower intra-ejaculate variation, since they are predicted to invest relatively more in the production of high quality ejaculates.

To understand the role of sperm competition in shaping sperm morphology, and to test the functional link between sperm swimming abilities and sperm morphology, we experimentally induced changes in the reproductive tactics of the males by shuffling males of known reproductive tactic across cages. This manipulation led to four categories of males: males that were initially sneakers that remained sneakers (SS), males that were initially sneakers that became harem males (SH), harem males that became sneakers (HS), and harem males that remained harem males (HH). We sampled ejaculates from males of known reproductive tactics before the manipulation, 7 days after, and 21 days after, and measured sperm morphology and sperm swimming abilities.

We predicted that (i) males exposed to contrasted risks of sperm competition would produce spermatozoa with distinct morphology (ii) changes in sperm morphology should mirror experimental changes in reproductive tactics, except for the sampling occurring 7 days after the manipulation, as it happens before males could produce new sperm cells adjusted to the tactic; and (iii) harem males should exhibit higher within-ejaculate variance in sperm morphology due to relaxed selection on sperm quality control. Lastly, (iv) we investigated the relationship between morphology and sperm swimming performance, because, if these two types of traits are functionally related, correlations should exist between them.

2. Material and Methods

2.1 Model species and studied population

The study was conducted with males of Seba's short tailed bats (*Carollia perspicillata)* captured from a captive colony, hosted in the Papiliorama, a tropical zoo located in Switzerland. Individuals can fly freely under a 40m-diameter dome open to the public, and fed with a fruit based mixture.

2.2 Timeline of the experiment

The experiment was conducted between October 2014 and April 2015. Individuals were trapped from the colony during their resting phase using a hand net and transferred to experimental cages (1x2x2m). Each cage was constituted with 3 males of known tactics from the colony: 1 harem male with 2 sneaker males and 2 females. After three weeks of acclimation period, we performed the first ejaculate sampling (see below for details on the procedure). Immediately upon sampling, we transferred each male to a different cage, in order to induce changes in reproductive tactics of some of the males. Seven days and three weeks after the stabilization of the new hierarchy, we performed the second and third ejaculate sampling, respectively. Spermatogenesis lasts about 2 weeks in a related species, *Sturnira lilium* (Morais et al., 2013), a duration likely to be similar in *C. perspicillata,* allowing for a new cycle of spermatogenesis to occur during the experiment. Moreover, the duration of sperm maturation in the epididymis has not been quantified for bats, but is likely to be short, as it only lasts a few for other mammals: 3-5 days in humans (Johnson and Varner, 1988), 5-8 days in rats *Rattus norvegicus* (Bellentani et al., 2011). As our aim was to experimentally induce a change in the tactic used by the males, we decided to only include in our analyses the males that did not change reproductive tactics during the acclimation period (from the colony to the pre-manipulation sampling), and that exhibited a clear role, *i.e.* harem or sneaker, after the manipulation of the reproductive tactics (N = 39 males before the manipulation, N=33 males after the manipulation).

2.3 Ejaculate collection

Ejaculates were collected using electro-ejaculation (Fasel et al., 2015). Males were laid dorsally on a warming pad. During the procedure, males were anesthetized using isoflurane. Anaesthesia was induced with 5% isoflurane mixed with oxygen for about 5 s, and then was decreased to 1 to 2% isoflurane. Oxygen was provided at a rate of 0.8 l/min. A probe covered with aqueous lubricant was inserted in the rectum approximately 1cm deep. Electric stimulations were transmitted using two electrodes situated at the distal end of the probe (ICSB, USA). The electrode was linked to an audio amplifier (JVC A-X2) generating three series of regular and increasing electric stimulations (maximally 4 mA). Electrical current was continuously monitored with a milli-ampere meter (Fluke 77 multimeter). After the stimulation, oxygen was provided alone until awareness. The ejaculate collected was transferred into a microcentrifuge tube containing 10 µl of PBS to avoid desiccation. According to the estimated volume of ejaculate, PBS was added to obtain a 1:2 dilution. An aliquot of 3 µl was immediately taken for mobility analysis. An aliquot of 1 µl was gently mixed with 5 µl of formaline on a microscope slide, and smeared using a cover slip. The slide was air-dried and stored at room temperature until analysis.

2.4 Sperm mobility traits analysis

The 3 µl aliquot was gently mixed with 15 µl of pre-warmed at 37°C Earle's balanced salt solution (SpermWash Cryos, Denmark) and gently mixed. Within 10 min of collection, 3 µl of this mix was loaded in a swimming chamber (SC 20-01-04-B, Leja, Nieuw-Vennep, Netherlands), and sperm movements was recorded in the swimming chamber under an Olympus XK41 microscope with dark-field condition, mounted with a Kappa CF 8/5 camera with a 20x magnification objective and a 10x magnification C-mount adaptor. Several 2-s videos (median 8, min 3, max 15) of 25 frames/s with a median number of 15 sperm track (min 0, max 224) were then analysed for each ejaculate using a CASA plug-in in ImageJ 1.47v (Wilson-Leedy and Ingermann, 2007) to obtain estimates of eight sperm swimming parameters. The following eight sperm swimming parameters were estimated: motility (percentage of motile sperm), curvilinear velocity (VCL, µm/s), velocity average path (VAP), velocity straight line (VSL), linearity (VSL/VAP), wobble (VAP/VCL), progression (average distance of the sperm from its origin on the average path during all frames analysed) and beat cross frequency (frequency at which VCL crosses VAP, Hz). Sperm

cells swimming with a higher velocity (VCL > 6 μm/s) than non-sperm particles in the sample were considered as motile.

As found previously (Fasel et al., 2015), a principal component analysis (PCA) on all sperm swimming parameters (excluding the percentage of motile sperm, analysed separately), identified a first principal component explaining 53.19% of the variance, which was positively loaded with VCL, VAP, VSL but negatively loaded with BCF, and uncorrelated with the wobble, the number of sperm tracked, and linearity. Hence, males with high scores along this first PC produced fast swimming sperm. Results obtained using VCL or PC1 scores were qualitatively similar. Thus, for the sake of comparison with our own work and with other studies, and because it might be more intuitive to the readers, we only report analyses with VCL and motility.

2.5 Sperm morphology traits analysis

Slides were rinsed using distilled water to remove crystals of formalin, and air-dried. Pictures of sperm cells were taken with a Leica DMR microscope with dark phase contrast, mounted with a Nikon DMXM1200 camera. Twenty sperm cells were selected for each slide (min=2, median=19, max=20), but only normal sperm cells were used in the statistical analysis (min=1, median=11, max= 20). We considered sperm cells to be abnormal if any part was broken, or if the flagellum was folded. For each sperm cell, head length, head width, mid-piece length and flagellum length were measured using the software ImageJ. Out of the 1481 normal sperm cells, 77 were measured again at the end to compute the coefficient of variation (CV length head: 3.7%, CV width head: 3.7%, CV mid-piece length: 3.9%, CV flagellum length: 5.3%). All measurements were done by JP, blindly with respect to male identity.

To characterize sperm morphology using information on all the different sperm components, we computed a PCA using head length and width, mid-piece length and flagellum length for each sperm cell. Sperm cells with extreme values (mid piece length <5μm and >10μm; head length < 3.5μm and >7μm; head width <3.5 μm and >6 μm) were not considered for the PCA, as they are likely to be measurement errors or abnormal sperm, and thus disproportionately weighed in the PCA. These variables were segregated along two principal components axis. We applied a varimax rotation and used the scores from the first two axes in subsequent analysis (cumulative percentage of variance explained: 59%; lambda for rotated component 1:1.23; lambda for rotated component 2: 1.14). The first rotated component (RC1), explaining 30 % of the total variance, was positively correlated with mid-piece length ($r = 0.76$, $t = 45.2$, p-value<0.001), and

negatively correlated with flagellum length (r = -0.78, t = -48.11, p-value<0.001). It was interpreted as a mid-piece-to-flagellum ratio, with higher scores along this RC1 describing sperm cells with a long mid-piece and a short flagellum. The second rotated component (RC2), explained 29% of the variance, and was positively correlated to head length (r = 0.76, t = 44.86, p-value<0.001) and head width (r = 0.75, t = 43.22, p-value<0.001). It was interpreted as head size, with higher scores describing sperm cells with a longer and larger head.

For each ejaculate, we computed the mean and the standard deviation for the mid-piece-to-flagellum ratio and for the head size. To decide the minimum number of sperm cells per ejaculate required for an accurate estimation of the mean and standard deviation, we used a resampling procedure. We randomly sampled without replacement from 1 to 15 sperm cells per ejaculate and computed the associated mean and standard deviation (supplementary Fig. S2). As visually assessed on the plot, a minimum of 5 sperm cells per ejaculate for the mean, and minimum of 8 sperm cells per ejaculate for the standard deviation allow an accurate estimation of the parameters, without drastically reducing our sample size. Therefore, in the statistical analyses, we used ejaculates with a minimum of 5 and 8 normal sperm cells to compute mean values and within-ejaculate standard deviation, respectively.

2.6 Statistical analysis

Reproductive tactics and sperm morphology

We first tested whether, before the manipulation of the reproductive tactic, harem males and sneaker males produced morphologically different sperm by running two linear mixed-effects models with the ejaculate mean mid-piece-to-flagellum ratio or the ejaculate mean head size as the response variables and the male tactic (harem vs. sneaker) as the explanatory factor. The experimental cage was declared as a random factor.

Then, we examined whether males modified the morphology of their sperm when changing tactic. To do so, we ran two sets of models. The first one used data collected 7 days after the experimental manipulation and included the ejaculate mean mid-piece-to-flagellum ratio or the ejaculate mean head size as the response variables and changes in reproductive tactics (SS, SH, HS and HH) as the explanatory factor. The experimental cage was declared as a random factor. The second set used data collected 21 days after our manipulation and shared the exact same structure as the first one.

Variation in sperm morphology

First, we examined how much of the overall variation in sperm morphology was due to variation within ejaculates, to variation within males and across ejaculates and to variation across males. To obtain the percentage of variation for each level, we ran random models (no fixed effect) including all measures (all sperm from all ejaculates from all males) for the mid-piece-to-flagellum ratio and the head size as response variables and ejaculate identity nested within male identity as random effects, to account for multiple sperm cells per ejaculate, and for the three ejaculates per male. Additionally, we also computed coefficients of variation for the mean mid-piece-to-flagellum ratio and the mean head size across all males.

Next, we examined whether within-ejaculate variation in sperm morphology varied according to male tactic. A first set of models used data collected before the manipulation of reproductive tactic and included the within-ejaculate standard deviation in mid-piece-to-flagellum ratio or the within-ejaculate standard deviation in head size as the response variables and the male reproductive tactic (harem vs. sneaker) as the explanatory factor. The experimental cage was declared as a random factor.

Then, we examined whether changing tactic impacted the within-ejaculate variation in sperm morphology by running two sets of models. The first one used data collected 7 days after the experimental manipulation and included the within-ejaculate standard deviation in mid-piece-to-flagellum ratio or the within-ejaculate standard deviation in head size as the response variables and changes in reproductive tactics (SS, SH, HS and HH) as the explanatory factor. The experimental cage was declared as a random factor. The second set used data collected 21 days after our manipulation and shared the exact same structure as the first one.

Sperm mobility traits and sperm morphology

To test if sperm mobility traits were related to sperm morphology we ran three types of models. A first set of models used data collected before the manipulation of reproductive tactic, and included sperm velocity (VCL) or the percentage of motile sperm (logit-transformed) as the response variables, with either the mean mid-piece-to-flagellum ratio or the ejaculate mean head size as explanatory variables. A second and a third set of models with the same structure were run using data collected respectively 7 days and 21 days after the manipulation of reproductive tactic.

Data were analysed using R, version 3.3.3. The nlme package was used for the linear mixed models. The significance level was set at 0.05, and the false discovery rate procedure was applied to account for the multiplicity of tests (Benjamini and Hochberg, 1995).

3. Results

3.1 Reproductive tactics and sperm morphology

We tested whether males of different reproductive tactics produced ejaculates with different morphology, before the manipulation of reproductive tactics (harem vs sneakers males). Neither the mean mid-piece-to-flagellum ratio, nor the mean head size differed between the tactics (cf Table I). We then tested whether the sperm morphology varied based on their initial, final tactic and their interaction, 7 and 21 days after the manipulation, without finding any differences (cf Table I).

Table I: Models investigating whether sperm morphology traits differed between initial male reproductive tactics (Harem or Sneaker) before the manipulation, or among tactic change categories (SS, SH, HS and HH) 7 days and 21 days after the manipulation. All linear mixed-effects models included the cage as a random factor.

	Before manipulation			7 days after			21 days after		
Response variable	**N**	**F-value**	**P-value**	**N**	**F-value**	**P-value**	**N**	**F-value**	**P-value**
Mean mid-piece-to-flagellum ratio	26	$0.19_{1,9}$	0.67	24	$0.12_{3,7}$	0.94	24	$0.12_{3,7}$	0.94
Mean head size	26	$2.35_{1,9}$	0.16	23	$0.58_{3,6}$	0.65	24	$1.63_{3,7}$	0.27
Sd mid-piece-to-flagellum ratio	21	$0.87_{1,6}$	0.39	18	$0.68_{3,3}$	0.62	23	$1.11_{3,6}$	0.41
Sd head size	20	$0.49_{1,6}$	0.51	17	$0.94_{3,2}$	0.55	21	$0.22_{3,6}$	0.88

Variation in sperm morphology

We partitioned the variance for each sperm morphological trait at the male and ejaculate level. For the mid-piece-to-flagellum ratio, the proportion of variance due to differences across males was 8.6%, the proportion of variance due to differences within males and across ejaculates was of 18%, and 73.3 % of the variance was within males and within ejaculates. This means that, while mid-piece-to-flagellum ratio was consistent across ejaculates of a given male, most of the variation emerged within the ejaculates rather than across males. Similarly, for head size most of the variance was within-male, within-ejaculate (86.7%), whereas the variation across males or the

variation within-male and across ejaculates represented only 7.2%, and 6.1%, meaning that, while head size was consistent across ejaculates of a given male, most of the variation in sperm head size emerged within the ejaculates rather than across males. The coefficient of variation across males for the ejaculate's mean mid-piece-to-flagellum ratio was 57% and 47% for the ejaculate's mean head size.

Then, we studied whether the amount of variation differed according to the reproductive tactics before the manipulation, or according to the change in reproductive tactics 7 and 21 days after the manipulation. We found no differences in the standard deviation for the mid-piece-to-flagellum ratio, nor in the standard deviation for the head size, at any sampling time (cf Table I).

Sperm mobility traits and sperm morphology

Before the manipulation of reproductive tactics, and 7 days after, we found no correlations between sperm velocity or the percentage of motile sperm and the mid-piece-to-flagellum ratio or sperm head size. 21 days after the manipulation of reproductive tactics, we found that the percentage of motile sperm was negatively correlated with head size (F-value=5.66, P-value=0.03) (cf. Fig. 1). The mid-piece-to-flagellum ratio was not correlated with velocity or percentage of motile sperm (cf Table II).

Table II: Linear mixed-effects models investigating whether sperm swimming abilities correlate with sperm morphology traits before the manipulation, 7 days and 21 days after the manipulation. P-values highlighted in bold does not remain significant after correction using the false discovery rate procedure.

	Velocity		% of motile sperm	
Explanatory variable	**F-value**	**P-value**	**F-value**	**P-value**
Before manipulation				
Mean mid-piece-to-flagellum ratio	0.006	0.93	0.2	0.66
Mean head size	0.003	0.96	0.91	0.35
7 days after				
Mean mid-piece-to-flagellum ratio	1.06	0.31	0	0.99
Mean head size	0.3	0.59	0.96	0.34
21 days after				
Mean mid-piece-to-flagellum ratio	0.2	0.66	1.9	0.18
Mean head size	0.47	0.5	**5.66**	**0.03**

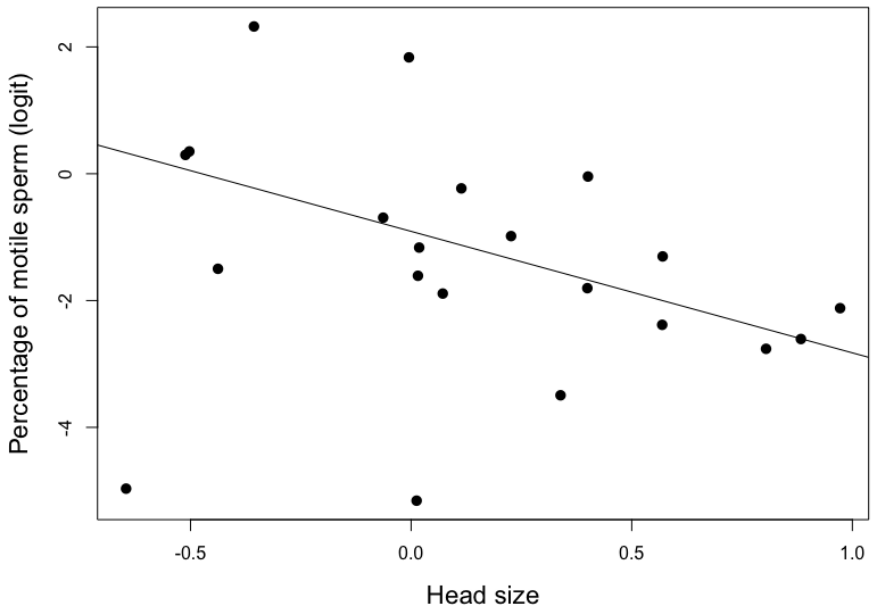


Figure 1: Negative correlation between percentage of motile sperm and head size. Black line represents the regression line of the model, when the point of coordinates (x=-0.64, y= -4.96) is excluded. This point is not an outlier, but weights a lot in the regression according to the Cook's distance diagnostic plot. If the point is added, the relationship is no longer significant (F-value=1.18, P-value=0.29).

4. Discussion

To investigate the role of sperm competition in shaping sperm morphology and to test whether sperm morphology and sperm swimming abilities are functionally linked, we experimentally manipulated male reproductive tactics. Contrary to our predictions, we did not find differences in sperm morphology between male of different reproductive tactics experiencing different levels of sperm competition. Interestingly, several studies conducted on species of fish exhibiting alternative reproductive tactics (ARTs) have also reported that males produce similar sperm morphology whatever their tactic (Leach and Montgomerie, 2000; Locatello et al., 2007; Smith and Ryan, 2010). Moreover, experimental evolution studies with lines evolving under contrasting levels of sperm competition have rarely found an impact on sperm morphology (Crudgington et al., 2009; Firman and Simmons, 2010; Gay et al., 2009; Godwin et al., 2017; Pitnick et al., 2001). Two studies have however experimentally showed that when facing higher levels of sperm competition, individuals produced longer sperm cells that they previously did (Crean and Marshall, 2008; Immler et al., 2010). Overall, a positive correlation between the level of sperm

competition and sperm length has been mostly detected inter-specifically (*frogs:* Byrne et al., 2003; *fishes:* Fitzpatrick et al., 2009; *butterflies:* Gage, 1994; *mammals:* Gomendio and Roldan, 1991; *snakes:* Tourmente et al., 2009). This suggests that intra-specifically, sperm morphology might be constrained by other factors than solely the need for speed. Indeed, beside sperm competition, multiple mating by females provides opportunity for female cryptic choice (Eberhard, 1996), and might strongly influence the evolution of sperm morphology. For example, sperm length is positively correlated with the length of female sperm storage tubules in birds (Briskie and Montgomerie, 1993). Our results highlight that intra-specifically, the link between sperm competition risk and sperm morphology is more complex than suggested by findings from inter-specific comparative studies.

Furthermore, we did not find evidence for a correlation between sperm swimming ability and sperm morphology. The percentage of motile sperm and sperm head size were negatively correlated 21 days after the manipulation in reproductive tactics. That relationship was not robust to the inclusion of one point. Therefore, our results question the existence of a functional link between sperm swimming performance and sperm morphology in *Carollia perspicillata*. In our model species as well as in many other species, other factors such as seminal fluid composition (Perry et al., 2013), antioxidant allocation (Mora et al., 2017), or sexual abstinence (Wesseling et al., 2016) are likely to have a greater impact on sperm swimming performance than sperm morphology. Indeed, across taxa, several studies have reported changes in sperm velocity that were not associated with changes in sperm morphology. Notably, in the Chinook salmon *Oncorhynchus tshawytscha*, males of different alternative reproductive tactics exhibited similar sperm morphology, while sperm velocity was higher for sneaker males (Flannery et al., 2013). Similarly, in an experimental evolution study conducted on mice *Mus musculus*, males from polygamous lines exhibited higher sperm swimming abilities than males from monogamous lines, without changes on sperm morphology. However, high within-ejaculate variation might hide the link between sperm morphology and sperm velocity. Measuring sperm velocity and sperm morphology from a single cell would allow a precise estimation of the potential correlation (Fitzpatrick et al., 2010).

Sperm competition is predicted to select for an optimal sperm morphology, and hence should lead to low within ejaculate variation (Calhim et al., 2007; Immler et al., 2008; Varea-Sánchez et al., 2014). We found high within-ejaculate variation, despite significant sperm competition level in our model species. This is a puzzling result that has also been reported in other studies (Blengini et al., 2014; Calhim et al., 2011). Moreover, the degree of within-ejaculate

variance did not correlate with the reproductive tactics, contrary to our predictions, and contrary to what has been found in another species (Rojas Mora et al. 2017). On the one hand, the high variation that we report could reflect males' inability to maintain efficient sperm "quality control", maybe as a consequence of the stress induced by the experiment. Alternatively, the high level of variation in sperm design could be a form of "gametic bet-hedging" strategy in response to sperm competition. By producing ejaculates of morphologically diverse sperm males would "hedge their bets" to optimize their fertilizing abilities and their chance of paternity whatever the social context and the sperm competition risk. Indeed, a number of factors are likely to impact fertilization success, such as mating role, number of competing ejaculates, time since ovulation, number of copulations, number of sperm allocated to the ejaculate, number of future matings. These factors can be combined in an infinite number of ways, for each of which a different optimal sperm morphology could potentially exist. In the superb fairy wren *Malurus cyaneus*, a highly promiscuous bird species, Calhim et al. (2011) showed that sperm cells with different phenotypes can perform better depending on the male mating role, shorter sperm leading to higher extra-pair fertilization success, whereas longer sperm achieved higher within-pair success.

In conclusion, our results do not support the hypothesis that sperm competition exerts a strong selective pressure on sperm morphology at the intra-specific level. Interactions with the female reproductive tract are likely to strongly limit the evolution of sperm morphology within a species. Moreover, our results question the existence of a functional link between sperm morphology and sperm swimming ability, or at least its relevance compared to other mechanisms impacting sperm quality, such as the composition of the seminal fluid. Finally, as found in another species with high sperm competition levels but contrary to what sperm competition theory predicts, males exhibited a large intra-ejaculate variance in sperm morphology. This phenomenon may result from a strategic "gametic bet-hedging" to optimize fertilization success under various sperm competition regimes, a hypothesis deserving to be properly tested.

Acknowledgements

We are very thankful to the Papiliorama for allowing us to work with their bat colony under excellent conditions. This study was supported by a grant from the Swiss National Science Foundation n°PP00P3_139011 to FH. No competing interests declared.

Data availability

Data will be uploaded into a dryad digital repository upon acceptance of the manuscript.

Author Contributions statement

MM, NF and FH conceived the ideas and designed methodology;

MM, JP and NF collected the data;

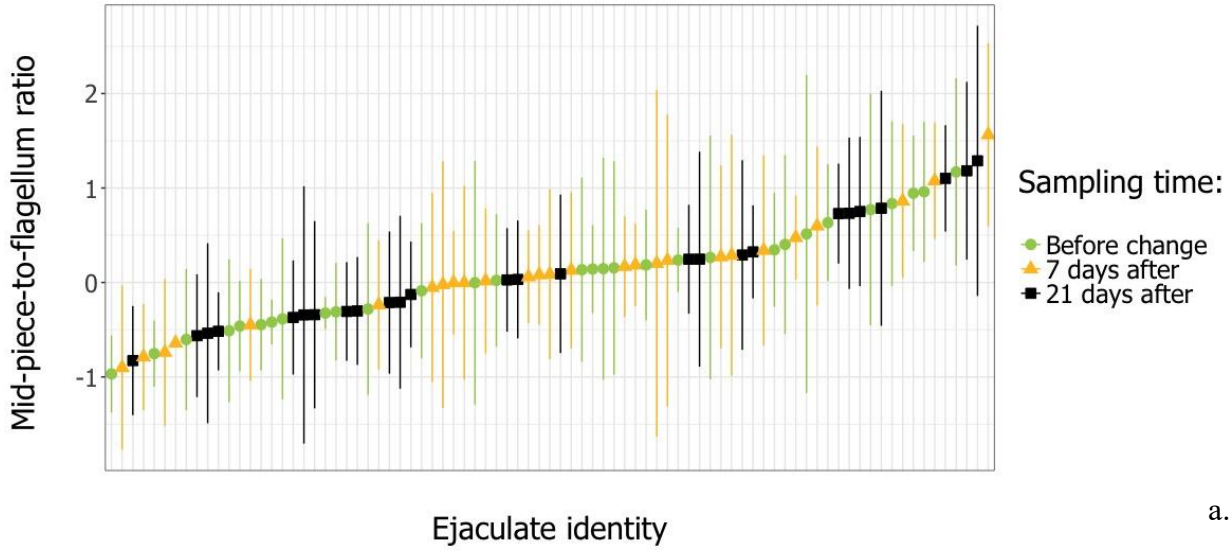
JP measured the sperm cells ;

MM, NF and FH analysed the data;

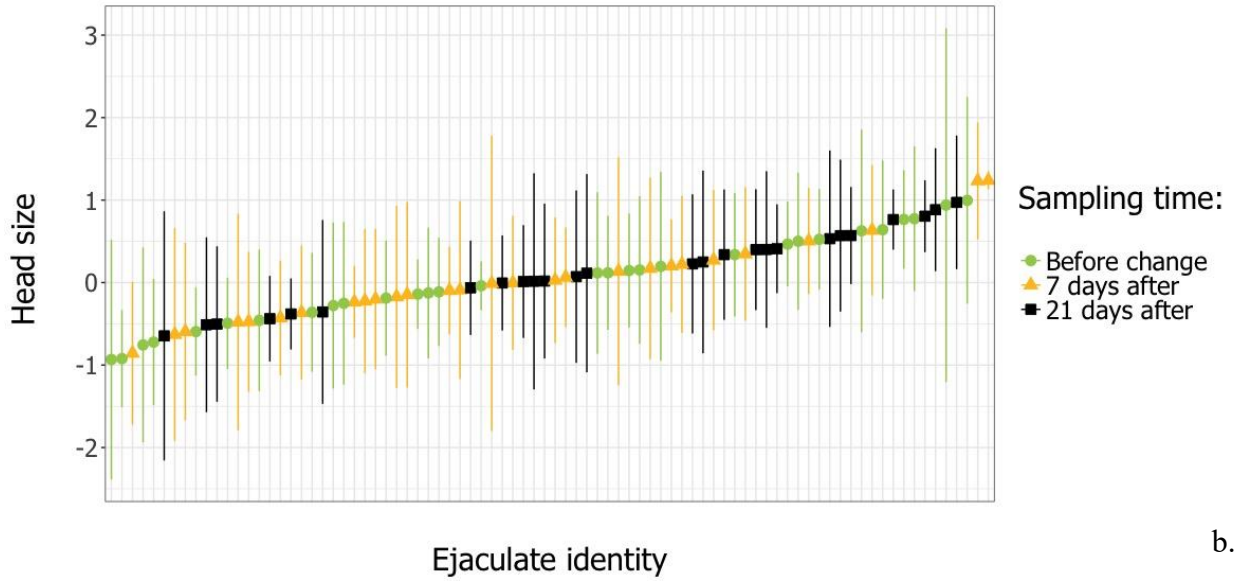
MM, NF and FH wrote the manuscript.

All authors gave final approval for publication.

Supplementary documents



a.



b.

Figure S1: Data distribution, for mid-piece-to-flagellum ratio (a) and head size (b). Dots represent mean value for ejaculates, vertical bars represent the standard deviation for the ejaculate.

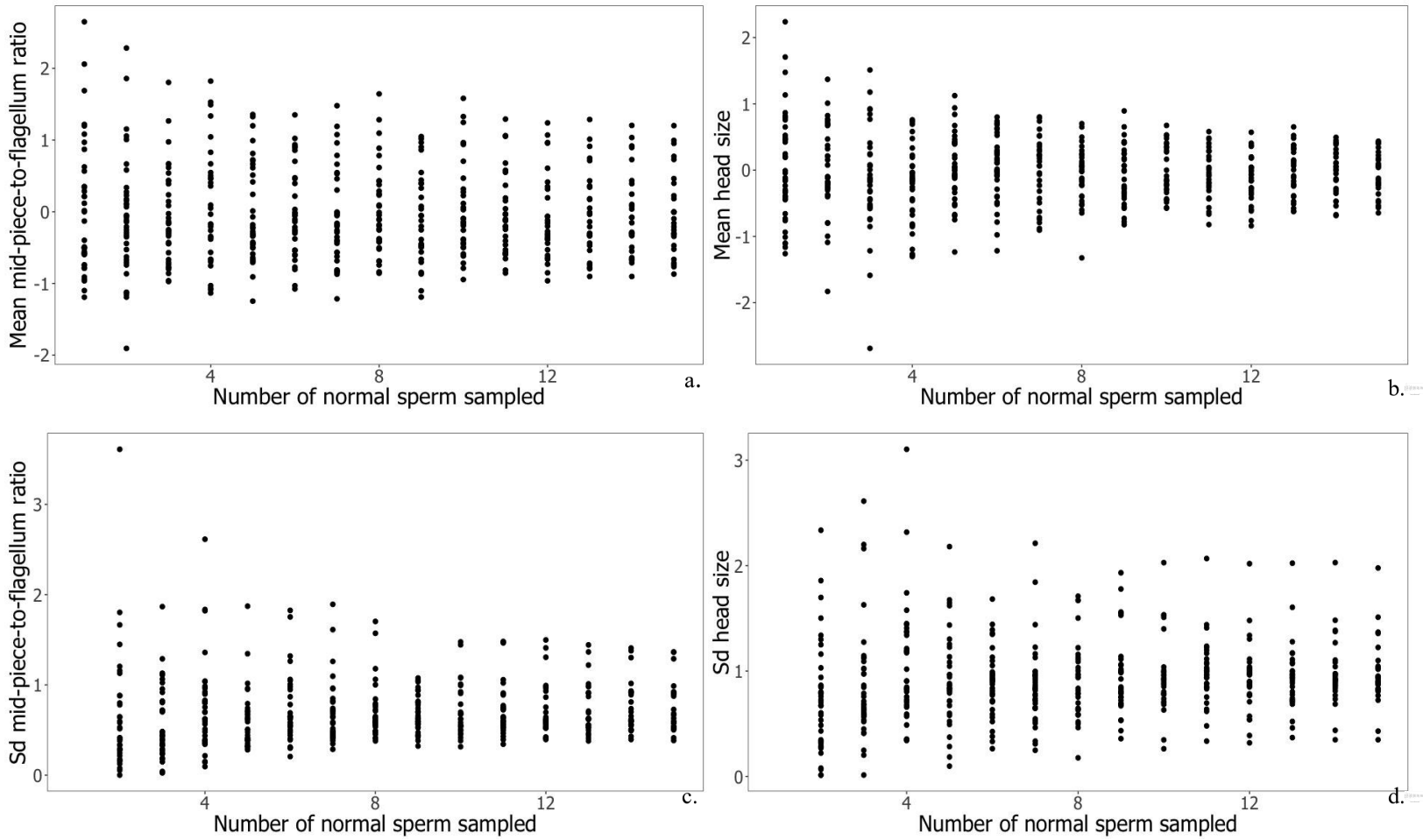


Figure S2: Plots of the resampling procedure for the mean mid-piece-to-flagellum ratio (a) and mean head size (b), and for the mid-piece-to-flagellum ratio standard deviation (c), and head size standard deviation (d).

Chapter 4: A guide for ecologists to build a low-cost selective trap using Radio Frequency Identification Detection

Magali Meniri[1], Anthony Farley[1], Fabrice Helfenstein[1]*, Nicolas Fasel[2]*

*: shared last authorship

1: Laboratory of Evolutionary Ecophysiology, Institute of Biology, University of Neuchâtel, Switzerland

2: Leibniz Institute for Zoo and Wildlife Research, Germany

Abstract

Behavioral studies often aim to perform specific actions on focal individuals and could benefit from automated procedures. With the current available technology, they are expending possibilities. With this paper, our goal is to demonstrate to ecologists that building a selective, automated device triggered by Radio Frequency Identification Detection (RFID) running on a battery is easy and affordable (~100 dollars). We provide a step-by-step description of how to build such an RFID triggered trap for small animals. We built and tested our selective traps in a colony of 300 captive bats, flying in a 40m-diameter dome. Our device proved successful in trapping focal individuals using RFID identification while recording every single visit to the trap-feeder. Our guide not only provides information for building RFID-triggered traps, but also offers a general framework for building any device triggered by RFID and can thus help build tailored setups matching specific studies requirement. Home-made selective device using RFID detection have a great potential in opening-up exciting new possibilities for a wide range of studies on animals, ranging from trapping specific individuals, to automatically monitoring activities at the nest-box, or supplementing specific individuals in a population.

Key-words: *open-source, Raspberry pi, Carollia perspicillata, selective trap, RFID*

1. Introduction

Behavioral and ecological studies often require specific actions to be performed on focal individuals, such as selective supplementation where only one group of individuals should be supplemented (Demeyrier et al., 2017), an audio playback to be played to specific individuals (Hinde, 2006), and/or the trapping of specific individuals. Moreover, long-term monitoring of a population often involves regular opportunistic trapping in order to collect data such as presence/absence, or body mass. The automation of data recording would be more efficient in terms of time, impact and resources invested.

Thanks to technological advances, it is now relatively easy to build inexpensive devices equipped with sensors that can be modulated and customized to fit specific studies requirements (Lendvai et al., 2015; Whytock and Christie, 2017). Using a single-board computer, it is possible to assemble devices that can automatically perform pre-programmed actions when triggered by specific events, for example the detection of an individual carrying a given RFID (Radio Frequency Identification) tag. RFID technology, where a PIT-tag (Passive Integrated Transponder) holding a unique identification number can be read using an RFID reader, is widely used to identify pets, but also to monitor wildlife (Aplin et al., 2013). However, although PIT-tags are rather low cost, standard ready-to-use commercially available RFID readers can be prohibitively expensive. Luckily, inexpensive RFID reader circuit boards equipped with an antenna are now available for about 50 dollars or euros (as compared to several hundreds of euros/dollars for a traditional reader). These systems can be readily connected to a small computer to record the tags detected.

Raspberry Pi (Rpi) are credit-card sized single-board computers, which are low-cost, reliable, and easy to program even for neophytes. Although such technology may be intimidating for people with little or no knowledge in electronics and/or programming skills, Rpi are actually easy to handle and customize. Rpi were first released in 2012 and rapidly became very popular, for example for home robotic project, and increasingly so for research studies (Ambrož, 2017; Pasquali et al., 2017). Rpi are equipped with generic metal pins allowing to physically connect different devices such as RFID readers, LEDs or diverse sensors using wires. By connecting a Raspberry Pi, an RFID identification system and a small device that can quickly release a sliding door when triggered (in our case, an electromagnet-solenoid), one can build a low-cost selective trap. In this study, our goal was to selectively trap individuals from a captive population of Seba's

short tailed bats (*Carollia perspicillata*), while recording visits at the homemade selective traps-feeders.

2. Material and methods

2.1 Model species and studied population

We study a captive colony of Seba's short tailed bats *(Carollia perspicillata)* of about 300 individuals, flying freely under a 40m-diameter dome, in a tropical zoo (Papiliorama, Switzerland). All the bats are equipped with RFID PIT-tags inserted between the scapulas. They are fed with a fruit-based mixture, twice a day, from 5 feeding stations located inside the dome.

2.2 Set-up

Feeder

The feeders were shaped as an octagon, with the antenna circling the entrance of the feeder located 10 cm away from the sliding door, to avoid hurting the focal bat as the door closes.

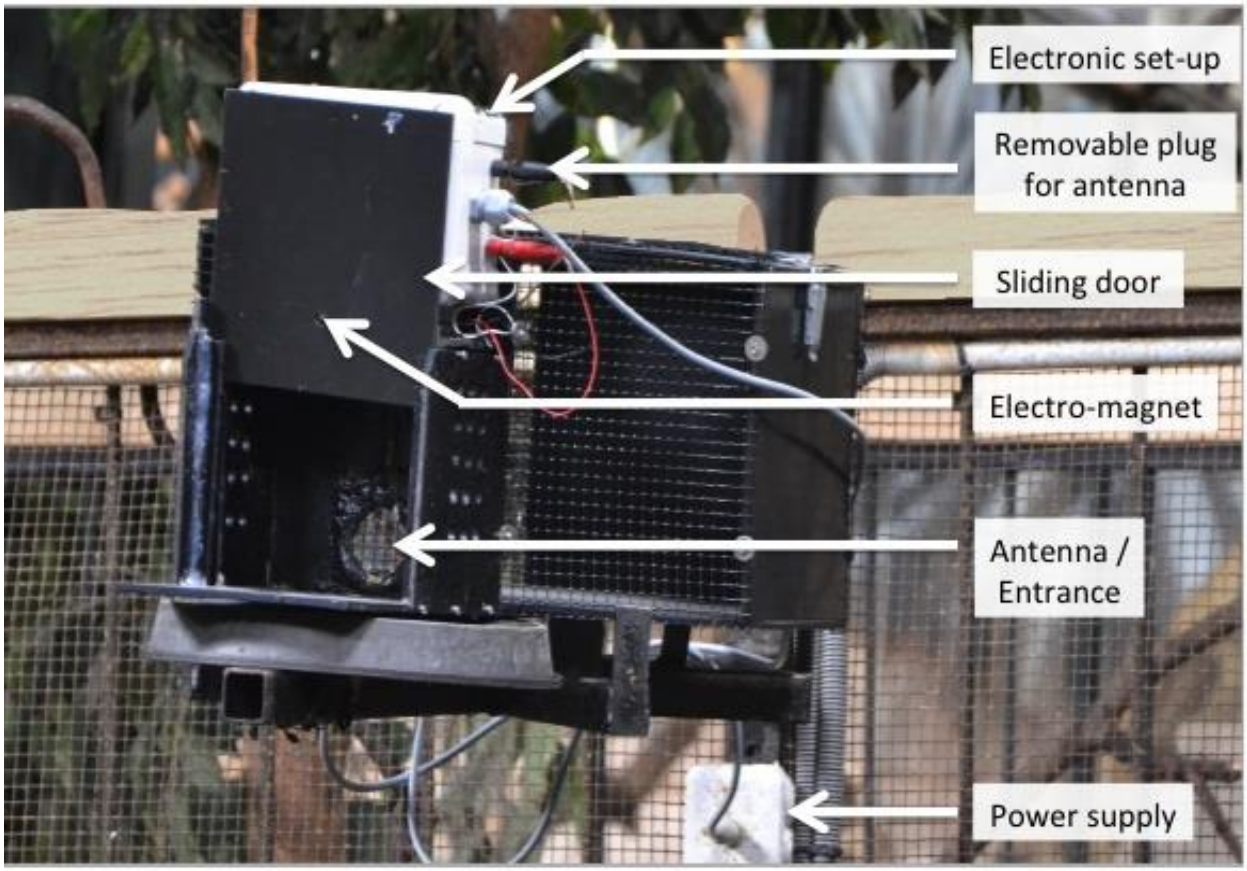


Figure 1: Pictures of trap-feeder

Electronic set-up

We connected a Raspberry Pi 3 model b to an RFID reader (connected to the antenna) and a relay board acting as an on/off switch for an electromagnet-solenoid (powered separately either by a battery or using a charger of the appropriate voltage). We then programmed the Rpi to trigger the electromagnet-solenoid and close the sliding door, when one of the pre-defined focal individuals was detected. The electronic set-up was assembled in a waterproof plastic container, which could easily be removed from the feeder-trap (Fig. 2.a).

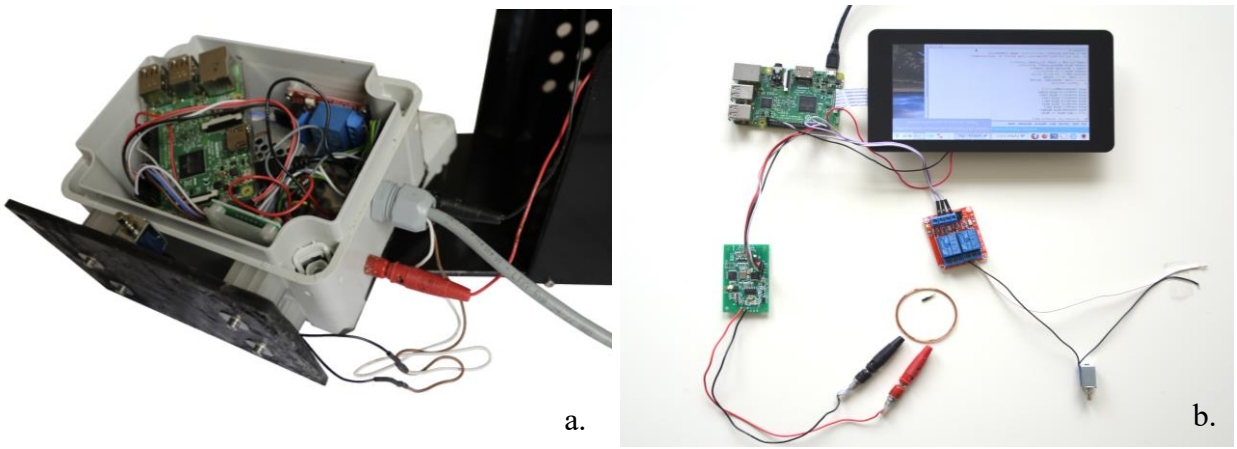


Figure 2: Pictures of the electronic set-up: a. Assembled in the waterproof container. b. With a compatible screen

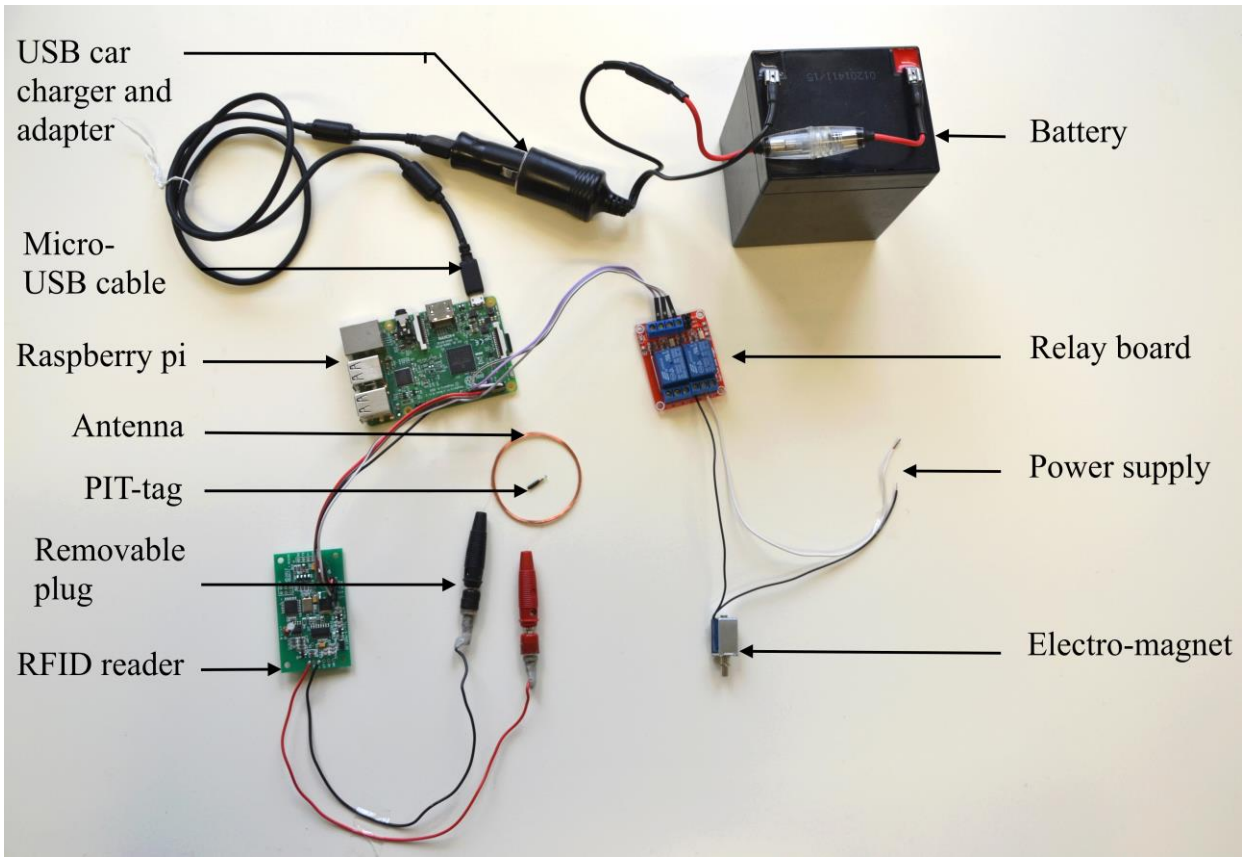


Figure 3: Detailed picture of the electronic set-up. Details about individual components can be found in the text.

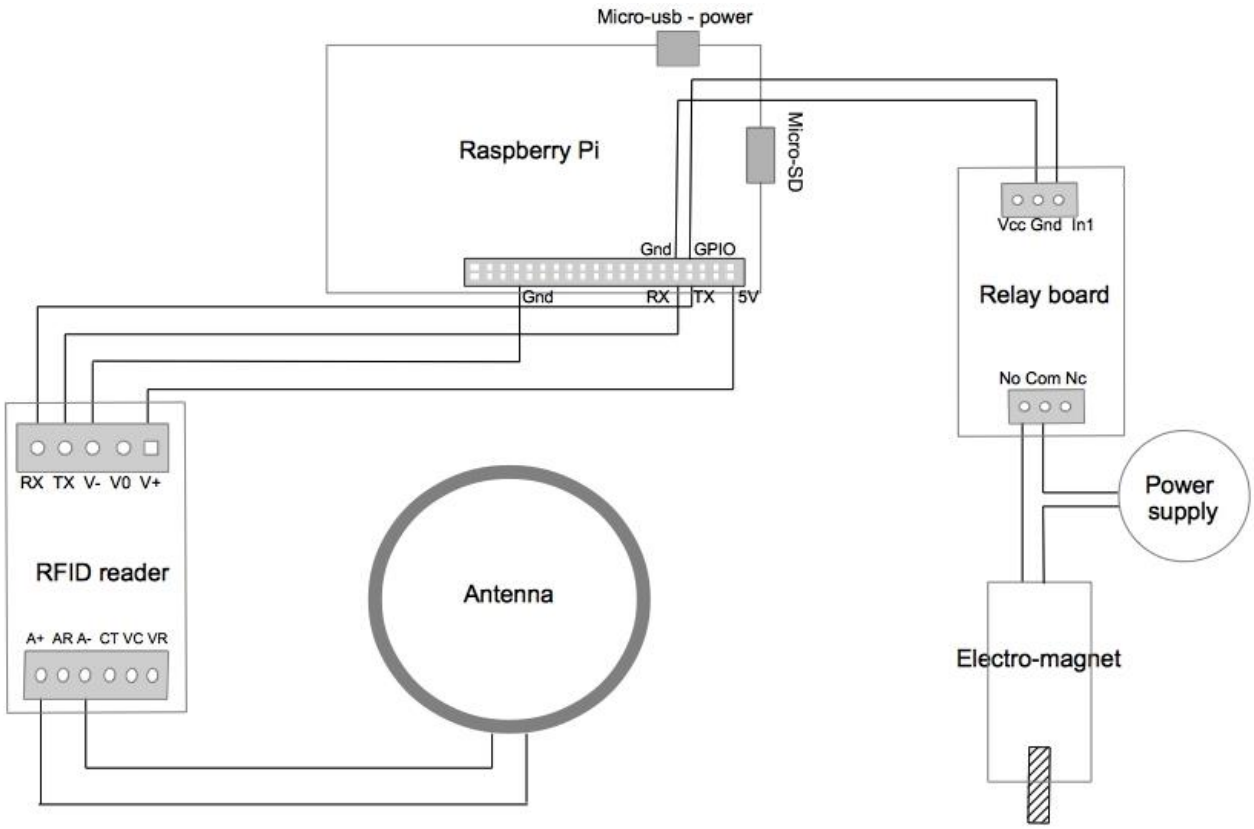


Figure 4: Schematic of electronic set-up. Black lines represent wires, which are plugged/soldered to the appropriate connectors, represented by the small white circles/squares. Relevant abbreviations for the connectors are reported as they appear on the circuit boards.

- *Raspberry pi – single board computer*

A Raspberry Pi can be considered as a small system unit from a standard computer. As such, it requires peripherals, such as a mouse, a keyboard that can be directly plugged in the USB ports and a computer screen that can be connected using the HDMI port without previous configuration. Compatible touch screens that can be powered using the Rpi GPIO are also available, making manipulations in the field easier (Fig. 2b). The Rpi is powered via a mini-USB port, using an alimentation able to provide 5 Volts Direct Current (VDC) and a minimum of 1 Ampere (e.g. phone charger).

Upon first use, it is also necessary to download an operating system for the Rpi onto a micro SD memory card. The micro SD card can then be inserted in the Rpi, and the power supply plugged in to turn on the Rpi. Once the Rpi is started, it can be used exactly like a standard computer.

The Rpi holds a row of 40 metal pins (Fig. 5), of which 28 are GPIO (General Purpose Input/Output Ports), which can be used as input (to read information from environmental sensors) or output (to control motors, LEDs, or send signals) pins. Other pins are composed of power supply pins (5 VDC or 3.3 VDC) and ground pins (0 VDC).

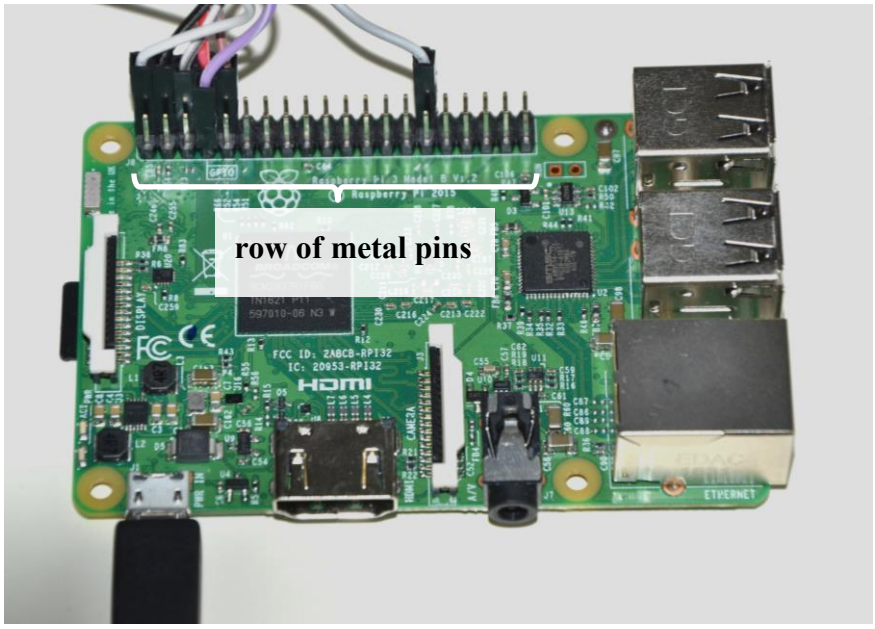


Figure 5: Picture of a Raspberry Pi 3 model b

- *RFID reader*

In order to transmit the identity of PIT-tags detected by the RFID reader to the Rpi, one needs to physically connect them using wires. By using male to female jumper wires, the RFID reader board "TX" (Transmission) connector can be connected to the "RX" (Reception) of the Rpi, and the "RX" to the "TX", *i.e.* to the GPIO 14 and 15 of the Rpi, also known as serial port.

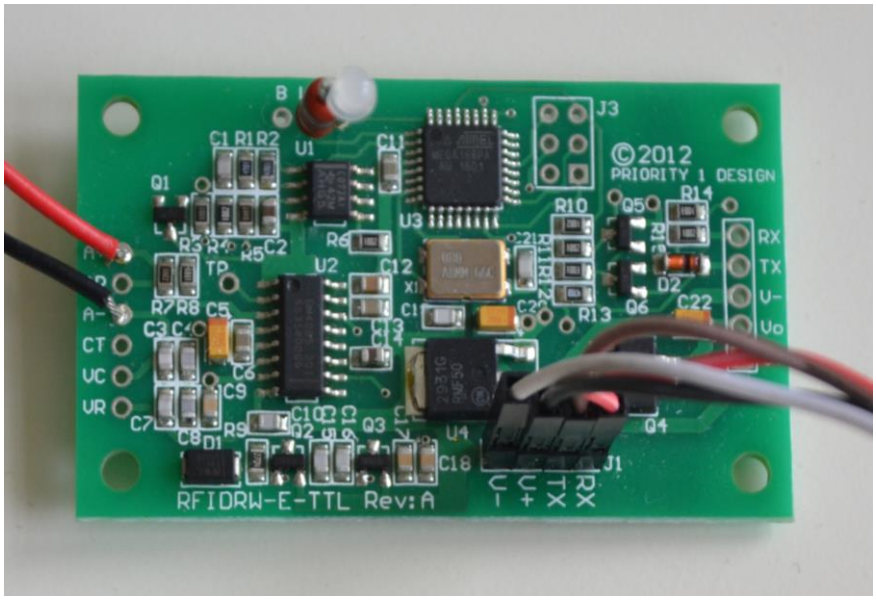


Figure 6: Picture of an RFID reader board

- *Electromagnet/solenoid – Relay board*

The Rpi could not power our electromagnet using a Rpi's GPIO since it required more than 3.3 VDC. Therefore, the Rpi could not directly control whether the electromagnet got switched on or off, so we had to use a relay board, which acts as an on/off switch. Using jumper wires, we connected the relay board to the Rpi: the relay board "In1" connector to a Rpi's 5 VDC pin and the relay board "Gnd" connector to a Rpi ground pin. We then connected one wire of the electromagnet to the relay board "No" (Normally Open) connector, and the other one to one wire of an external power supply, from which we had previously cut off the connector at the tip, and stripped the isolation off the cable to have access to the two wires. We then connected the relay board "Com" (Common) connector to the other wire of the power supply.

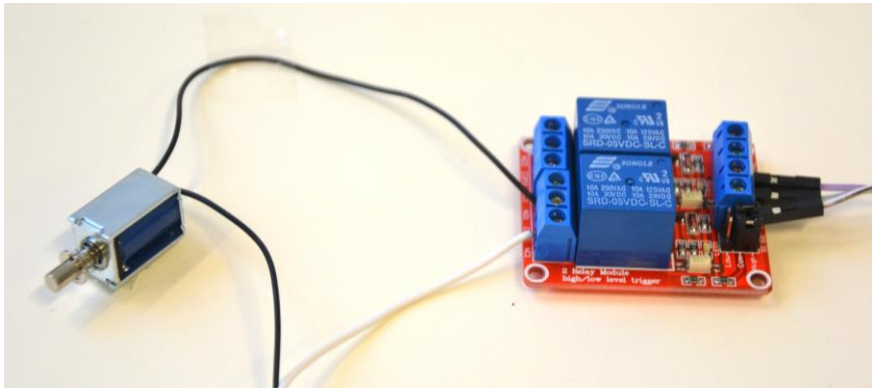


Figure 7: Picture of a relay-board connected to an electromagnet/solenoid

In our set-up, when switched off, the bar inside the electromagnet is holding the sliding door up. When triggered by a pre-programmed event (here the detection of a focal PIT-tag), the relay switches on, allowing electricity through the electromagnet, which automatically leads the metal bar inside the electromagnet to be pulled back, hence releasing the sliding door, and therefore closing the trap.

Table 1: Table with costs and material. Items highlighted in bold are essential to build a selective trap

Component	Price	Model	Website	Function
Raspberry Pi	38 €	Raspberry Pi 3 model b	https://www.raspberrypi.org	Single board computer
Micro USB power supply	15 €	-	Generic	Power Rpi, 5VC, minimum of 1 A
RFID reader + antenna	48 €	FDX-B/HDX RFID Reader Writer with TTL serial port + RFID coil antenna 49 mm	http://www.priority1design.com.au	RFID reader board and antenna to detect RFID tags
RFID PIT-tags	1 €	ISO 11784 certified PIT tags (134.2 kHz)	http://www.loligosystems.com	RFID tags for the animals
Relay board	10 €	1 channel 5 VDC	Generic	Electrically operated switch, if solenoid not powered by RPi
Micro SD memory card	10 €	Class 10, 16 GB minimum	Generic	To store operating system and acquired data
Solenoid electromagnet push/pull	5 €	Force: 50g minimum Stroke 6 mm	Generic	Release the sliding door when activated
Waterproof plastic container	4 €	Electric junction box	Generic	Hold and keep set-up dry
PiFace clock	10 €	PiFace real time clock	http://www.piface.org.uk	Keep track of real time
LEDs + resistor	0.50 €	-	Generic	Provide visual signals
Banana connectors, 2 males and 2 females	2 €	-	Generic	Allow to connect disconnect antenna to rest of the set-up
Raspberry Pi Touch Display	70 €	-	https://www.raspberrypi.org	Screen to allow easy visualization on the field as Rpi doesn't include peripherals

- Tools

Some of the material cited in Table 1 might require some soldering using a soldering iron. Diverse items such as a drill, glue, heat shrink sleeve, jumper wires and standard wire might also be necessary.

Programming

The Rpi can be programmed using multiple languages, including Python. Commands can be issued either directly on the terminal, or using Python IDLE, which may be easier to use as it provides a graphical user interface. With the operating system Raspbian, Python IDLE is available from the desktop menu under *Programming*. It combines an interactive interface ("Shell") to run single commands, or script using the combined built-in file editor. The program we used for this

project can be found in the supplementary document (S1). In our program, we also recorded all the PIT-tags that were detected in a text file, along with the time and date of detection.

3. Results

The selective traps functioned very efficiently, and they allowed us to trap all the focal individuals needed, while additionally recording information when individuals came to feed. We installed 5 traps in the bat dome during 2.5 months. After filtering the data for individuals that remained too close to the antenna and were detected several times during a single visit, *i.e.* within 5 minutes, we recorded a total of 16035 visits to the feeders.

There was a high variability both in the number of visits in the traps per individual (min: 1 visit, max: 210 visits, median: 63.50), and also in the number of different traps visited per individual (min: 0 trap, max: all 5 traps, median: 3 traps). It took about one month for most individuals to learn how to get inside the traps. The selective traps were not the only food dispensers, which lengthened the learning process.

4. Discussion

The selective traps we built proved to be efficient, reliable and affordable. They allowed us to trap all of our focal individuals, including individuals that had been avoiding our standard opportunistic trapping for years. Moreover, thanks to the information we collected during the visits at the feeder-trap, we can address a wide range of questions about their foraging habits.

RFID based set-up can be implemented in a wide variety of research projects. Our set-up could also be fitted to nest-boxes to trap individuals or to monitor activities at the nest, such as parental provisioning rate, or even record visits from prospecting or extra-pair conspecifics. Social learning could be studied, with a precise monitoring of which individuals triggered the set-up and when. It could be used to collect information on social networks, if individuals are regularly detected at similar locations at the same time. It can also be used to perform cognitive trials, without requiring isolation of the individuals or constant monitoring from the researcher to control which individual performed the action. In captivity, it could also be used to restrict food access or

to supplement focal individuals while remaining in the same enclosure as the control group. In some cases, if individuals are habituated and come readily inside the set-up, it could even allow for experiments to take place in the natural environment of the animals, without restraining them in cages. To fit specific needs, it is possible to customize the set-up with devices such as temperature sensor, audio recorder, camera recorder, a scale, etc. Notably, it is possible to fit servo-motors to perform rotating or linear actions.

The main limitation of our selective traps was linked to the detection range of the antenna, about 5 cm using 7 mm long PIT-tags. Since tags were inserted between the scapulas of the bats, to optimize detection bats had to crawl inside the antenna, which circled the entrance. As they were previously used to just taking a quick mouthful of food while flying above the open-air feeder, and this species is not used to crawl, it required time for the bats to get accustomed to the modified feeders. This may be less of a problem in other species, such as passerine birds, where the PIT-tag can be attached to an identification ring, then allowing the bird to be detected when it steps on the antenna.

It is preferable to avoid models of RFID reader equipped with a USB port if detection range is an issue, because USB connection can be "noisy". Also, a classic cable, rather than a coaxial cable, will ensure a better connection between the antenna and the reader.

Power consumption was not a concern for us so we used the Raspberry Pi 3 model b, but other models of single board computer have lower power consumption, such as Raspberry Pi A+. If standard electricity is not available, it is possible to power the Rpi using a car battery, fitted for example with a USB car charger. Using a standard car battery of 50 Ah, (*i.e.* able to deliver 1 ampere for 50 hours), the set-up could run for about 10 days continuously using a Raspberry Pi A+, consuming approximately 80 mA (0.4 W).

Our set-up offers numerous possibilities for improvements, to make it more functional. For example, it is possible to send a text or e-mail when the traps get triggered, or for any pre-determined event. One could also use LEDs to signal when a tag is being detected, or whether the program is still running, and hence the trap is in function.

With reasonable costs and efforts, we were able to build open-source, programmable selective traps, which can be customized for very specifics needs, which may not be the case for commercially available set-ups. Therefore, when planning for future experiments, it should be taken into account that building a selective set-up tailored for a study can be achieved in a fair time, for a low cost, and without previous background knowledge.

Acknowledgements

We are very thankful to the Papiliorama, for allowing us to study their bat colony under such excellent research conditions. We are especially thankful to the technical team, for installing electricity and helping with installing the traps, Michel Ruegger, Tiago Marques and Erland Mühlheim. We warmly thank Idonus, Paul Buckley and Robert Accardi, from Priority 1 design. We also thank Lucie Masseboeuf for her help with the bats. This study was supported by a grant from the Swiss National Science Foundation n°PP00P3_139011 to FH.

Authors' contribution

MM, AF, NF and FH conceived the ideas
MM and AF designed the methodology
MM and AF build the set-up
MM wrote the manuscript, with contributions from all authors.
All authors accepted the final version of the manuscript.

General discussion

In this thesis, I tested the role of oxidative stress as a constraint for life history traits at two key moments in an individual's life: during early life and reproduction. I used the Seba's short-tailed bats *(Carollia perspicillata)* as a model species. First, I studied the role of oxidative stress as a constraint during early life (chapter 1). Then, I tested the role of oxidative stress in mediating the pre- vs post-copulatory trade-off, and monitored the potential consequences on sperm quality (chapter 2). I also looked at the sperm morphology and within-ejaculate variation in sperm morphology depending on the reproductive tactic, and at the potential functional link between sperm swimming performance and sperm morphology (chapter 3). Finally, I present a home-made selective trap that we designed to study our colony of Seba's short-tailed bats, and which can find applications in many study designs (chapter 4).

1. Early life adverse conditions

1.1 Short-term consequences

First, I looked at the effect of oxidative stress during early life when facing adverse conditions, as presented in chapter 1. Adverse conditions were created by imposing a food restriction for 10 days on mothers of unweaned pups, followed by *ad libitum* feeding. During the food restriction, we observed a decrease in growth rate, both for size and body mass. The food restriction also impacted the shape of the wing at the end of the treatment (unpublished results). That delay was fully compensated in terms of size, thanks to an increase in growth rate. However, the difference in body mass was not fully compensated. We did not find an impact of the treatment on the wing morphology at the end of growth. We found that growth induced an oxidative cost, as evidenced by the lesser oxidative damage accumulated by the food-restricted, slow-growing pups compared to the unrestricted pups exhibiting linear growth, and by the faster accumulation of oxidative damage associated to the increased growth rate of previously food-restricted individuals. Therefore, oxidative stress could represent a threat constraining growth rate, which could explain why individuals generally do not grow at maximal rate (Arendt, 1997). However, compensatory

growth was not found to induce higher oxidative costs than normal growth, contrary to our predictions, and to previous findings (Alonso-Alvarez et al., 2007; De Block and Stoks, 2008; Tarry-Adkins et al., 2008, 2009). Moreover, we found that glucocorticoids level, although not associated with adverse conditions, were correlated with oxidative stress level, as previously found (Haussmann et al., 2012; Stojiljković et al., 2009; You et al., 2009). Therefore, oxidative stress could represent a proximal mechanism of the negative physiological consequences associated with elevated glucocorticoid level (Zafir and Banu, 2009). Evidence suggests that oxidative stress might be linked to impaired cognitive abilities (Baierle et al., 2015; Fukui et al., 2006; Revel et al., 2015). Moreover, in zebra finches *Taeniopygia guttata*, compensatory growth was found to affect ability to learn and solve tasks (Fisher et al., 2006). Therefore, we looked at pups' exploratory behavior and probability to find their mother in a crawling maze at two different times, after the compensatory growth and at the end of growth (unpublished results). Preliminary results show that exploratory behavior of the pups was not directly impacted by the treatment. However, the how likely the pups were to find their mother in the maze tended to be lower for restricted individuals, although this difference did not reach statistical significance. Finally, we monitored survival during the first year. Although the survival for previously restricted males was two times lower compared to control males after a year, the difference was not statistically significant. It is possible that our low sample size did not provide enough statistical power to reveal a potential long-term survival cost of compensatory growth.

To conclude, we found that growth was associated with elevated oxidative damage on the short-term, suggesting that oxidative stress could represent a constraint during early life. However, we did not find evidence for physiological costs (oxidative stress or glucocorticoids level) of a compensatory growth on the short-term. In conclusion, our results show that individuals were able to efficiently mitigate the short-term consequences of adverse early life conditions.

1.2. Long-term consequences

Initially, we aimed to conduct a follow-up study one year after the food restriction experiment to monitor the long-term consequences on sperm quality and physiological traits, and to test the competitive abilities of males in a social challenge. However, because of the high mortality of the individuals from the experiment, we could not conduct the social challenge tests between one restricted and one control individual. We could only sample sperm and blood from a few surviving males (N=12), but these data have not been analyzed at the time of redaction. So far, very few

studies have linked early life conditions and sperm quality. One study recently showed that early life stress reduced the sperm levels of several mi-RNAs in both mice and humans (Dickson et al., 2018), which has been correlated to decreased sperm quality in humans (Abu-Halima et al., 2013). Interestingly, the reduction of mi-RNAs remained in the embryos, and thus might be linked to the epigenetic transgenerational transmission of early life stress (Dickson et al., 2018). Therefore, our data will give us precious insights on the long-term effect of early life adverse conditions on sperm quality. Although we did not find a difference in glucocorticoids levels accumulated during early life, our treatment might have affected the Hypothalamus-Pituitary-Adrenal axis, and thus individuals might exhibit differences in their baseline stress levels, or in their stress response as adults (van Bodegom et al., 2017). Finally, early life conditions have been shown to impact lifespan (Lee et al., 2013b; Ozanne and Hales, 2005), which remains to be investigated in our individuals.

In the Seba's short-tailed bats, males exhibit three alternative reproductive tactics, and reproductive success is highly skewed towards a few males, suggesting large opportunity for selection. As early life conditions might have major consequences on an individual's future phenotype, they could predict which tactic is to be adopted by the males. In females' meerkats *Suricata suricatta,* early growth determined dominance acquisition, with females growing faster being more likely to become dominant (English et al., 2013). Since tactics acquisition is not determined by age or size in our model species, which are often the typical determinants of switch between alternative reproductive tactics, early life conditions could play a major role. Therefore, it will be interesting to monitor the individuals from our experiment to study the potential impact of early life adverse conditions on dominance acquisition in the colony, and thus the effect of on reproductive success.

2. Reproduction and oxidative stress

We looked at the role of oxidative stress as a constraint for reproduction, and specifically as a mediator of the pre- vs. post-copulatory trade-off (chapter 2). Based on the predictions of the sperm competition models (Parker, 1990; Parker et al., 2013), we proposed that harem males might invest more antioxidant resources toward the protection of their soma, whereas sneaker males were expected to favor the protection of their ejaculates to compensate for a lower access to females. Indeed, a previous study in our model species showed that sneakers exhibit higher

sperm quality than harem males (Fasel et al., 2017). To experimentally test this hypothesis, we manipulated male reproductive tactics, by shuffling males across cages. We monitored the redox profile in the blood and the ejaculates, and sperm quality before the manipulation of reproductive tactics, and 7 days and 21 days.

2.2 Sperm swimming performance

Our experiment showed that individuals exhibited similar sperm swimming performance regardless of their reproductive tactics, with one noticeable exception. Indeed, shortly (*i.e*. 7 days) after the manipulation, in accordance with the sperm competition models' predictions (Parker, 1990; Parker et al., 2013), harem males that retained their harem exhibited the lowest sperm quality compared to all other categories of males. This difference disappeared by the next sampling, two weeks later. Overall, we did not find strong evidence for adjustment of sperm quality based on the reproductive tactics, contrary to our predictions. This result contrasts with a study in our model species, showing that sneakers exhibited a higher sperm quality compared to harem males (Fasel et al., 2017), and to evidence found in other species with alternative reproductive tactics (*birds:* Froman et al., 2002; Rowe et al., 2010; *insects:* Kelly, 2008; Simmons and Emlen, 2006; Yamane et al., 2010; *fishes:* Fu et al., 2001; Haugland et al., 2009; Makiguchi et al., 2016; Smith and Ryan, 2010; Vladić and Järvi, 2001; Young et al., 2013; *squid:* Hirohashi et al., 2016). However, a number of constraints were lifted in the cages compared to the situation in the colony: reduction of the number of agonistic interactions, food available *ad libitum*, lower copulation rate. In a previous study on our model species, the difference in sperm quality between harem and sneaker males disappeared after males spent 3 days spend in the cages without females, which was interpreted as a positive effect of sexual abstinence on sperm quality (Wesseling et al., 2016). Therefore, the conditions in the cages may have allowed all males to exhibit a similar sperm quality, which might explain why we did not find the predicted difference in sperm quality between reproductive tactics.

2.3 Sperm morphology

We also looked at sperm morphology in relation to the reproductive tactics and explored how sperm morphology relates to sperm swimming performance (chapter 3). Inter-specifically, sperm morphometry (particularly sperm length) has been shown to covary with sperm competition levels (*frogs:* Byrne et al., 2003; *fishes:* Fitzpatrick et al., 2009; *butterflies:* Gage, 1994; mammals: Gomendio and Roldan, 1991; *snakes:* Tourmente et al., 2009). Therefore, we postulated that sperm morphology might differ between reproductive tactics, as males experience different levels of sperm competition: sneaker males always experience sperm competition compared to harem males, which might not always face it. However, we did not find differences in sperm morphology between the reproductive tactics. Interestingly, other studies conducted on fish's species with alternative reproductive tactics also did not find differences (Leach and Montgomerie, 2000; Locatello et al., 2007; Smith and Ryan, 2010). Also, we did not find convincing evidence for a link between sperm morphology and sperm swimming performance. Our results add to several studies investigating such relationship and for which evidence is very equivocal (*positive correlation:* Lifjeld et al., 2012; Losdat and Helfenstein, 2018; Malo et al., 2006; Mossman et al., 2009; Firman and Simmons, 2010; *negative correlation*: Cramer et al., 2015; *no correlation:* Denk et al., 2005) Altogether, these results lead us to question the existence of a functional link between sperm morphology and sperm swimming performance in the Seba's short-tailed bats, and possibly in many other taxa. We also looked at the level of within-ejaculate variation in sperm morphology, which was surprisingly high for a species with sperm competition, contrary to our prediction and to previous findings (Calhim et al., 2007; Immler et al., 2008; Varea-Sánchez et al., 2014). However, other studies have reported a high level of within ejaculate variation despite sperm competition (Blengini et al., 2014; Calhim et al., 2011). Moreover, contrary to our predictions, the level of within-ejaculate variation did not differ between reproductive tactics. We propose that high within-ejaculate variation could be a form of "gametic bet-hedging", whereby males, by producing morphologically diverse sperm cells, would optimize their fertilizing abilities across different social contexts and sperm competition risks. Indeed, multiple factors can impact fertilization success: time since ovulation, number of competing ejaculates, number of copulations, number of sperm allocated to the ejaculate, position in the mating order, number of future mating, etc. The combination of those different factors creates an infinite list of possible situations, for each of which a different optimal sperm morphology could potentially exist. In species with high level of sperm competition, by producing morphologically different sperm cells

and thus maintaining high within-ejaculate variation, males could thus increase their fertilization success. For example, in the highly promiscuous superb fairy wren *Malurus cyaneus*, it was shom that shorter sperm cells lead to higher extrapair fertilization success, whereas longer sperms leader to higher within-pair fertilization success (Calhim et al. 2011).

In conclusion, it is likely that factors such as interactions with the female's tract might constraint sperm morphology intra-specifically, thus limiting the selective pressure of sperm competition. Additionally, sperm swimming performance might be more strongly influenced by other factors, such as the seminal fluids, than by sperm morphology. Moreover, we suggest that a form of "gametic bet-hedging" might exist, therefore explaining the high within-ejaculate variation found in species with high sperm competition risk.

2.4 Then… are males attractive, have good ejaculates, or both ?

Overall, we did not find major differences in sperm swimming performance (chapter 2), or in sperm morphology (chapter 3) between males exhibiting different reproductive tactics. The similarity in sperm quality that we reported between males with different reproductive tactics might be a consequence of the conditions specific to the experimental cages, such as food *ad libitum*, low copulation rate and reduced number of agonistic interactions. We will address this issue in the future by conducting experiments directly in the colony, without restraining individuals in cages, as allowed by our selective traps (chapter 4). However, a previous study has found a difference in sperm quality between sneakers and harem males (Fasel et al., 2017), which disappeared after males spent 3 days in the cages without females (Wesseling et al., 2016). Thus, it suggests that the lower quality exhibited by harem males resulted from differences in copulation rates and constraints exerted on accessory glands, rather than an adaptation to sperm competition from sneakers (Wesseling et al., 2016). Therefore, despite methodological issues, our results are likely to highlight a relevant pattern for the species, *i.e.* the intrinsic similitude in sperm quality between males of different reproductive tactics.

In mammals, studies conducted on species with alternative reproductive tactics have yielded conflicting results regarding a trade-off between pre and post-copulatory traits. Some studies have found the predicted trade-off between pre-copulatory expenditure and ejaculate quality (Fitzpatrick et al., 2012; Simmons, 2011; Stockley and Purvis, 1993), but others did not find a relationship between pre-copulatory expenditure and ejaculate quality according to the reproductive tactic (Kruczek and Styrna, 2009; Lemaître et al., 2012; Schradin et al., 2012), or

even found a positive relationship (Malo et al., 2005b; Preston et al., 2003). Lüpold et al. (2014) recently proposed that the level of female monopolization might mediate the relationship between pre and post-copulatory traits. In a comparative study, they showed that if males are able to fully monopolize the females, therefore reducing the risk of sperm competition, they invest relatively more towards pre-copulatory traits. However, if the monopolization is incomplete, males invest in both types of traits, as sperm competition risk is distributed more evenly among males, regardless of their reproductive tactic. Similar results were found in a comparative study conducted by (Stockley and Purvis, 1993), who showed that in mammalian continuous breeders, a trade-off is found between pre- and post-copulatory selected traits, with subordinate males exhibiting relatively bigger testes compared to dominant males. However, in seasonal breeders, dominant and subordinate males exhibit testes of similar size, as dominant males might be overwhelmed by the number of simultaneously fertile females, and thus unable to monopolize females efficiently.

In the Seba's short-tailed bats, harem males exhibit resource defense polygyny, meaning that they defend a small territory on which they have access to females, but do not guard females when the latter are outside of their territory. Harem males are also forced to leave their territory to forage, leaving their females unattended. Therefore, females are free to explore other territories, but also to mate with sneaker males away from the harem males' territory. Moreover, females are not faithful to a single harem male during their life, but have been found to move often between harems (Fleming, 1988). Although the harem-holding tactic has the highest pay-off, since 60% of the pups are sired by the 20% of harem males, 40 % of the pups are sired by sneakers (Fasel et al., 2016). Moreover, these estimates come from our captive population where reproduction is not synchronized among females. It is likely that in the wild, where reproduction occurs during two annual peaks, harem males may suffer from sperm depletion (Preston et al., 2001; Wesseling et al., 2016), and thus loose out more fertilization opportunities to sneakers. Overall, the mating system, the females' behavior and the high proportion of pups sired by sneakers suggest that in the Seba's short-tailed bats, females' monopolization might be incomplete. Therefore, I propose that contrary to our initial predictions, harem males might invest heavily in both pre- and post-copulatory traits, to both attract females and secure fertilizations. As suggested by the "big house, big car" effect, harem males might be higher quality individuals which would enable them to invest in both types of traits (Reznick et al., 2000). Harem males could also pay the cost later in life, as suggested by the decreased probability of maintaining their tactic as they get older (Fasel et al., 2016).

3. Oxidative stress as a life history constraint?

We studied the role of oxidative stress as a constraint for life history traits. First, although we found that growth leads to oxidative damage, we did not find oxidative costs to compensatory growth on the short-term (chapter 1). We also found that oxidative stress has a negative impact on sperm quality (chapter 2). However, we did not find differences in the redox profile for the soma or the ejaculates between the reproductive tactics. Moreover, a previous sampling conducted on individuals from the colony only reported a difference in a marker of cellular oxidative stress in the blood, and no differences in the ejaculates of males exhibiting different reproductive tactics (Fasel et al., 2017). Therefore, our results are cannot solely arise as a consequence of the conditions in the experimental cages. Overall, this suggests that, in Seba's short-tailed bats, alternative reproductive tactics are not associated with differences in the redox profile, neither of the soma or the germline, and oxidative stress does not seem to act as a strong physiological constraint in the trade-off between pre and post-copulatory functions that is typically associated with alternative reproductive tactics.

4. Selective trap-feeders

In chapter 4, I presented a selective trap based on radio frequency identification that we custom designed for the study of the colony. Indeed, I am convinced that more researchers could benefit from that type of selective device, and that our paper could bring new perspectives and ideas to implement in their own studies. The technology has recently become available, at a low cost and easy to use, but very few ecologists are aware of its existence, and even less would fill comfortable assembling a device without specialized support from a technical team. We designed this trap in order to recapture individuals at precise time from the experiment presented in chapter 1. Moreover, it will allow us to perform experiments directly in the colony without restraining the bats in cages, while being able to recapture the individuals when needed, for example to perform sampling. Thus, the selective traps will greatly improve future experimentations, as it will remove the stress of captivity, and allow individuals to maintain their normal behavior of territory defense, foraging, and sneaking.

5. Perspectives

5.1 Foraging habits

With the selective traps, we were able to record the identity of every individual visiting the feeders. These data could be used to study the foraging habits of individuals based on their reproductive tactic. Indeed, we suspect that harem males' foraging might be constrained by their territory defense strategy and vice-versa. Therefore, analyzing these data could allow us to answer a number of questions such as whether individuals of different reproductive tactics come to feed at similar rate; whether harem males choose feeding station closer to their territory; whether they have shorter forging bouts compared to sneakers, etc. Overall, we will get a better understanding of the constraints inherent with each tactic in terms of foraging habits.

5.2 Seminal fluid

Contrary to our predictions, we did not find differences in sperm swimming performance (chapter 2), or in sperm morphology (chapter 3) between the reproductive tactics. However, it is possible that differences in ejaculate fertilization abilities might arise from differences in seminal fluid, as suggested by an increasing body of evidence (Perry et al., 2013; Poiani, 2006). Indeed, seminal fluid has been shown to impact sperm swimming performance, for example allowing for rapid changes in sperm velocity in response to changes in sperm competition risk (Bartlett et al., 2017). It can also impact females, by modifying their remating behavior (Chapman and Davies, 2004) or their reproductive tract physiology to increase fertilization success (Robertson, 2007; Robertson and Sharkey, 2016).

Sneakers and harem males may invest differently in the seminal fluid. As sneakers are expected to always face sperm competition, they may take advantage from harem males' investment in seminal fluid. Indeed, in the grass goby *Zosterisessor ophiocephalus*, it was experimentally shown that sperm velocity of sneakers is increased when combined with the seminal fluid of territorial males compared to sneakers' seminal fluid, whereas the velocity was decreased for territorial males' sperm combined with sneakers' seminal fluid (Locatello et al., 2013). Alternatively, sneakers may be predicted to invest relatively more in the seminal fluid, in order to optimize their ejaculate quality. Therefore, it will be important to investigate seminal fluid composition in the future. For example, we could try to replicate the experimental design

from the study of Locatello et al. (2013), by mixing sperm cells of males of one tactic with the seminal fluid from males of the other tactic. Alternatively, we could investigate the proteomic profile from the ejaculates of males exhibiting different reproductive tactics, to test whether proteins expression profile is similar between the two tactics.

5.3 Female choice

During my thesis, I focused on males. However, some harem males are able to attract more females that others. Ultimately, female choice is likely to have a strong impact on male reproductive success, as forced copulations are unlikely to occur in our model species. Indeed, males and females have similar size and weight (unpublished results), and females have been observed to fly off when solicited for unwanted copulations (personal observations). Studying female's social network could shed some light on female choice in the Seba's short-tailed bats. Indeed, as of now, we have very little knowledge about females' movement between harem groups, and a lot remains to be discovered. For instance, a master's work that I co-supervised suggests that individuals avoid inbreeding, as the probability of siring a pup decreases as relatedness between the parents increases. Since we work with a captive population, the mechanism leading to inbreeding avoidance is unlikely to be dispersal and might be based on female choice. Moreover, a previous study on the Seba's short-tailed bats has shown the potential for individual discrimination based on acoustic communication, as individuals exhibit individual vocal signature that can be detected statistically (Knörnschild et al., 2014). In another neotropical species of bats, the Greater sac-winged bat *Saccopteryx bilineata* also exhibiting territory defense polygyny, individuals have been shown to recognize their conspecifics individually (Knörnschild, personal communication). Individual discrimination could play a role in mate choice (Cheetham et al., 2008).

5.4 Comparison with wild individuals

This thesis is based on experiments conducted on captive individuals, although they could fly freely within a 40m diameter dome and had an artificial cave to roost. So far, from the information that are available, the colony that we study presents comparable characteristics to what has been reported from the wild: same sex-ratio biased towards males at birth and balanced in adult age-

classes; similar juvenile mortality despite lower predation and absence of external parasites; similar proportion of harem males (*wild*: Fleming, 1988, *captive colony*: unpublished results).

However, the constant environmental conditions allow individuals to breed all year around, whereas in the wild reproduction is limited to two synchronized reproductive peaks. Indeed, the same very energetic food is provided all year around, which might influence reproduction. Moreover, it might have an impact on the level of oxidative stress, as an antioxidant rich diet might lower the risk of oxidative stress (Schneeberger et al., 2014). Therefore, a comparison with wild individuals is required before drawing strong conclusions at the species level.

6. Conclusion

To conclude, we found that early life was associated with elevated levels of oxidative stress. Overall, individuals seemed to efficiently mitigate the short-term consequences of early life adverse conditions. Future studies will be required to determine the potential long-term costs. I suggest that oxidative stress does not represent a strong physiological constraint in Seba's short-tailed bats, a result that requires further confirmation from wild individuals who do not enjoy *ad libitum* and antioxidant-rich food all year round. Furthermore, contrary to our initial predictions, I propose that harem males do not trade-off their investment in pre- and post-copulatory traits, but rather invest simultaneously in both in order to attract females and secure fertilizations. Finally, I advocate for experimental studies to be conducted in the natural environment rather than in cages and propose a selective trap for that purpose.

Annex : Protocols

MDA quantification

Two different MDA quantification methods where used in this thesis. The method used in chapter 1 was developed after the method used in chapter 2. The method used in chapter 1 is more sensitive, and thus require smaller volume. Therefore, it was preferred to the other method in the susequent experiments. Both methods show similar repeatability, as computed on standards. Method chapter 1: coefficient of variation: 5% n= 3, in triplicates, prepared and analyzed on different days; method chapter 2: coefficient of variation: 6.7% n= 6, in triplicates, prepared and analyzed on different days.

Method chapter 1

We assessed MDA (nmol/ml) by derivatization with 2,4-dinitrophenylhydrazine (DNPH) quantified using ultra-HPLC-high-resolution MS, following (Mendonça et al., 2017).
A volume of 5 µl of homogenate or standard was added to 40 µl of NaOH 1.125 M. The mixture was heated for 30 min at 60℃, after which samples were cooled down in the fridge for 10 min and 155 µl of TCA 20% was added. Samples were vortexed, sonicated for 30 s, and centrifuged (5 min, 25000 *g*). The supernatant was collected and derivatized with DNPH (5 mM in TCA 20%; volume used was 10% of that of the supernatant collected), for 10 min at room temperature. The reaction was then stopped with 22 µl of NaOH 10 M and the resulting MDA-DNPH was extracted twice with 250 µl of a mixture of cyclohexane:toluene (1:1 v/v). Organic phases were combined and evaporated in a centrifugal evaporator (Labconco) at 25 °C for about 60 min. The dry residue was reconstituted in 100 µl of MeOH 50% and the resulting solution was sonicated, centrifuged and transferred into an HPLC vial fitted with a 250 µl conical insert.

The analysis of MDA-DNPH was performed on an Acquity UPLCTM system coupled to both an eλ PDA detector and a Synapt G2 QTOF mass spectrometer (Waters). The separation was carried out on an Acquity BEH C18 column (50x2.1 mm i.d., 1.7 µm particle size) at a flow rate of 0.4 mL min-1 in gradient mode. Mobile phases consisted of water + 0.05% formic acid (phase A) and acetonitrile + 0.05% formic acid (phase B). The gradient program started at 2% B and increased linearly to 60.8% B in 3.0 min, then increased to 100% B in 0.4 min, was held at 100%

B for 2.0 min before switching back to initial conditions and re-equilibrating for 1.5 min. The column temperature and that of the autosampler were both set to 25 °C. A volume of 2.5 µL was injected in the so-called partial loop with needle overfill mode into the column, after which the autosampler needle was washed with 700 µL of "strong" wash (ACN:MeOH:isopropanol 1:1:1, v/v) followed by 600 µL of "weak" wash (MeOH 20%). UV detection range was set from 190-400 nm with a resolution of 1.2 nm and a frequency of 20 Hz. Absorbance maxima for DNPH and MDA-DNPH were 358 nm and 305 nm, respectively. The mass spectrometer was operated in electrospray positive ionization in full scan mode over a mass range of 85-600 Da (scan time 0.4 s). The enhanced duty cycle (EDC) mode was activated and centred on m/z 235. EDC increases the quadrupole transmission at and around the selected m/z system. TOF resolution at full width half maximum (FWHM) was about 20000 at m/z 500. Source parameters were as follows: capillary voltage 2.8 kV, cone voltage 25 V, source temperature 120 °C, desolvation gas flow and temperature 800 L h-1 and 450 °C, respectively, cone gas flow 20 L h^{-1}. Exact mass measurements (< 2 ppm) were ensured by infusing a 500 ng mL^{-1} solution of leucine-enkephalin at 15 µL min^{-1} through the Lockspray™ probe. In addition, external calibration using a 0.5 mM sodium formate solution was performed every week. For MS/MS analysis, argon at a flow of 2.2 mL min^{-1} and a voltage ramp from 10-35 eV were used as collision gas and energy, respectively. The UHPLC flow was diverted from the mass spectrometer from 0.0-2.6 min and from 3.1-6.0 min. The entire system was controlled by Masslynx™ v.4.1. Peaks were automatically integrated using Quanlynx™. with a 0.1 min chromatographic window centred on the retention time of MDA-DNPH (2.87 min) and a 0.02 Da mass window centred on the (M+H) + ion of MDA-DNPH (m/z 235.0462).

Method chapter 2

We assessed MDA (nmol/ml) by its reaction with 2-thiobarbituric acid (TBA) to produce a pink derivate quantifiable by ultra-high performance liquid chromatography with fluorescence detection (UHPLC-FD), using a method adapted from (Losdat et al., 2014). For calibration, a standard curve was prepared using a TEP (1,1,3,3-tetraethoxypropane) stock solution (5 µM in H_2O MQ) serially diluted using H_2O MQ, prepared fresh, and the same volume was used as for the samples. A volume of 10 µl of homogenized RBC or ejaculate, or 15 µl of plasma, or standards was first mixed with 40 µl of TCA (Trichloroacetic acid) 5%, allowing the deproteinization of protein-bound MDA, and with 20 µl of TBA (42 mM). The TBA solution was prepared fresh by

adding 30.89 mg of 98% TBA diluted with 5ml of H_2O MQ, and dissolved on a stirring hot plate at 50°C. A volume of 150 µl of H_2O MQ was added to the mixture of RBC and ejaculates (145 µl for plasma) or standards, which was then vortexed for 5 seconds and centrifuged for 14 min at 21'913 G at 4°C. The supernatant (205 µl) was transferred into screw-top tubes and incubated for exactly 60 min at 100°C in a dry bath, allowing the acid-catalysed formation of MDA-$(TBA)_2$ adducts. Tubes were then cooled on ice for 5 min and vortexed for 10 seconds. We added 150 µl of butanol to each tube, which was then vortexed and centrifuged for 10 minutes at 21'913 G at 4°C. The epiphase was transferred into Eppendorf tubes. Another 150 µl of butanol was added to each screw-top tube, and centrifuged again for 10 minutes at 21'913 G at 4°C. The second supernatant was added to the first one. Butanol was evaporated in Speedvac for 60 min at 35°C and the dry extract re-suspended in 90 µl of 30% methanol, sonicated for 5 s and vortexed. 70 µl were transferred into HPLC vial inserts (0.250 ml capacity) and stored at -80°C until HPLC analysis. Samples (5 µl) were injected into an Ultimate 3000 RSLC (Dionex, Thermo) coupled to an Acquity UPLCOR BEH C18 column 1.7 µm, 2.1 x 50 mm, with temperature set at 30°C. Separation was achieved using gradient elution at a flow rate of 0.4 ml/min with solvent A being 0.05% acetic acid buffer at pH 6 with ammonium hydroxide and solvent B being acetonitrile. The gradient was as follows: linear increase from 5% to 100% solvent B over 5 min, followed by 100% solvent B for 1.5 min and re-equilibration at initial conditions (5% B) for 3.2 minutes. The total analysis time was 9.7 min. The auto-sampler syringe was washed with 700 µl of solvent B after each injection. Data were acquired using a fluorescence detector set at 515 nm (excitation) and 553 nm (emission). TEP standards assayed in triplicate showed high repeatability (intra-class correlation coefficient = 0.99, P-value < 0.0001, n = 12).

Glutathione quantification

We mixed 2 µl of RBC homogenate or 3.5 µl of ejaculate homogenate with 5 µl TCA 5%, 38 µl (36.5 µl for ejaculates) H_2O MQ and spiked with 5 µl of glutathione ethyl-ester as internal standard (GSHee, 20 µg/mL in TCA 0.2%) The mixture was vortex-mixed for 5 seconds, kept on ice for 5 minutes and centrifuged 14 minutes at 21'913 G and 4°C. 5 µl of supernatant were further diluted with 195 of H_2O MQ for RBC. For the ejaculates samples, 46 µl of supernatant were further diluted with 34.5 µl of H_2O MQ + 80 µl of dichloromethane. All samples were then vortexed, centrifuged for 2 min 14'000 G (final dilution, samples: 1:2000 v:v; internal standard:

1:400 v:v). 150 µl for RBC samples, or 70 µl for ejaculates were then collected into 0.250 ml capacity HPLC inserts for glutathione quantification with LC-MS/MS. All samples were stored at -80°C until analyses. The reduced (GSH, ng/ml) and oxidized (glutathione disulfide 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. Samples were injected into an Ultimate 3000 RSLC (Dionex, Thermo) fitted with API 4000 QTrap (ABSciex) coupled with an Acquity UPLCOR HSS T3 column. The solvent was acetonitrile and 0.05% formic acid, and ran isocratically over 8 min at a 0.4 ml.min-1 flow rate. 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 µg/ml to 2 ng/ml for GSH and GSSG and 50 ng/ml for GSH-ee. Peaks were automatically integrated using Analyst™.

Tocopherol quantification

Ten µL of plasma was placed in a 1.7 ml microcentrifuge tube and 80 µL of ethanol and 10 µL of α-tocopherol-D-6 at 100 ng/ml in ethanol were added to precipitate proteins. The tubes were vortexed, sonicated for about 20 s and centrifuged at 14'000 g for 3 min. The supernatant was recovered, transferred to an HPLC vial fitted with a conical insert and analyzed immediately by ultra-high performance liquid chromatography tandem mass spectrophotometry (UHPLC-MS/MS).

Five µl of ethanolic extract was injected in the UHPLC-MS/MS system consisting of a Dionex Ultimate 3000 RSLC (Thermo Scientific) coupled to a 4000 QTRAP mass spectrometer (AB Sciex). The separation was performed on an Acquity UPLC BEH column (50x2.1mm i.d., 1.7 um particle size, Waters) at a temperature of 60°C and a flow rate of 0.6 mL/min. Mobile phase A was H_2O and mobile phase B was methanol. The following gradient program was applied: 70-100% B in 4.0 min, then holding at 100% B for 2.5 min and returning to initial conditions at 70% B for 3.5 min. The flow was deviated from the mass spectrometer from 0.0-4.6 min and from 5.9-10.0 min using a 6 port Valco valve (Vici). MS detection was performed in negative APCI mode using the following parameters: nebulizer current -3 µA, desolvation temperature 550 °C, nebulizing gas flow (GS1) 45 psi, curtain gas flow 15 psi. The analysis was carried out in the multiple reaction monitoring (MRM) mode using the following transitions and parameters: α-

tocopherol, 429>163 (DP -70V, CE -36V, CXP -21V); γ-tocopherol, 415>149 (DP -45V, CE -38V, CXP -1V); and δ-tocopherol, 401>135 (DP -45V, CE -38V, CXP -1V); and α-tocopherol-D6, 435>169 (DP -86V, CE -27V, CXP -26V). Analyst 1.6.2 (AB Sciex) was employed to process the data. Tocopherols were quantified using calibration solutions prepared in 100% ethanol at 2, 5, 50, 100 and 250 ng/ml, each containing α-tocopherol-D6 as internal standard at a concentration of 10 ng/ml. The limit of quantification was estimated to 0.5 ng/l for all tocopherols.

Glucocorticoids quantification

Samples were stored in the dark at room temperature until processing. Hair samples were washed by slowly shaking them in 200 µl pure methanol on a slow rotator (Vortex Genie 2, Scientific Industries, Inc, USA), and dried in a centrifugal evaporator. 5 mg of whole hair were transferred into a 2 mL screw cap micro-tube (72.1009.001, Sarstedt, Numbrecht, Germany) and cut into 1-2 mm pieces using a pair of fine surgical scissors. The samples were pulverized (mixer mill MM400, Retsch) at 27 Hz for 6 minutes with 2 stainless steal metal beads (dia. 3mm, DOMEL, 4228, Zelezniki, Slovenia) and 10 solid borosilicate beads (dia. 1mm, Sigma-Aldrich). After crushing, each hair sample was spiked with 1.0 ml internal standard (d4-cortisol 0.8 ng/ml in pure methanol) then shaked with a mixer mill for 6 minutes at 27Hz for steroids pre-extraction. Samples were spun in a centrifuge at 14,000 rpm for 3 minutes at room temperature, of which 1 ml of the clear supernatant was pipetted into a new 2 ml micro centrifuge tube (Eppendorf, Hamburg, Germany). 0.5 ml pure methanol was added to the hair samples, and shaked at 27 Hz 6 minutes. Samples were centrifuged and 0.5 ml of the clear supernatant was added to the previous. The 1.5 ml resulting supernatant was evaporated at 40 °C into a centrifugal evaporator until samples were completely dried (approximately 90 minutes). The dry residues were reconstituted with 1 ml 5% methanol, shacked in an ultrasonic bath for 1min and extracted in solid phase extraction columns (SPE HLC 3cc 60mg Extraction Cartridges, Oasis Waters, Oasis, Ireland). First, to condition the column, 3 ml of 100% methanol was added to the SPE column and flushed, followed 3 ml of 5% methanol to balance. Then, the sample was charged in the column with 2 ml of 5% methanol and flushed. Then the sample was washed with 3 ml 5% methanol, flushed, washed with 3 ml pure hexane, flushed. Finally, the steroid hormones were eluted using 3 ml pure Acetate Ethyl collected in 13*100 mm Pyrex tubes (Sigma Aldrich, St. Louis, MO). Pyrex tubes were then dried into centrifuged evaporator for approximately 2 hour and half at 40 °C. The dried mass was then

reconstituted in 100 µl of 50% methanol, filtrated through a 1ml syringe (Eppendorf, Germany) coupled with a 4mm syringe filter (PTFE, Hydrophilic (0.22 um), BGB, USA) then transferred into HPLC-MS/MS vials with screw caps, and stored at -80 C° until analysis. 2.5 µl of each sample were used to LC-MS/MS analysis.

Samples were injected in an Acquity UPLC™ system coupled to a Xevo TQ-S triple quadrupole (Waters, Milford, MA, USA). All aspects of the system were controlled by Masslynx™ v4.1. Separation was performed at a flow rate of 0.4 ml/min on an Acquity UPLC BEH C18 column (50x2.1mm i.d., 1.7 um particle size, Waters) heated at 30°C. Mobiles phases consisted of water and formic acid 0.05% (mobile phase A) and acetonitrile and formic acid 0.05% (mobile phase B). The following gradient program was employed: 10-50% B in 6.5 min, 50-100% B in 0.5 min, holding at 100% B for 1.5 min, and reequilibrating at 10% B for 1.5 min. The injection volume was 5 µl. Detection was performed in electrospray positive ionization mode using the multiple reaction monitoring (MRM) mode, in which specific precursor to product transitions are acquired. The following MRM transitions were monitored: 363>121 (cortisol), 367>121 (cortisol-D4), 361>163 (cortisone). Cone voltages and collision energies were optimized for each MRM transition. Source parameters were as follows: capillary voltage 1.5 kV, source temperature 150 °C, desolvation gas flow and temperature 1000 L/h and 600°C, respectively. Peaks were automatically integrated using Quanlynx™ and normalized to those of the internal standards. Calibration solutions containing cortisol and cortisone at 0.1, 1, 20, 100 and 250 ng/ml, as well as cortisol-D4 at 0.8 ng/ml. The limit of quantification was estimated to 0.1 ng/ml.

SOD quantification

We assessed Superoxide Dismutase (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. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. For quantification in red blood cells, we diluted each sample 3000x, and 48x ejaculates samples.

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