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Consequences of the different social roles in a cooperatively breeding society: insights from oxidative stress

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General summary

Large differences in cooperative contributions are evident, both within and between individuals of cooperatively breeding species, where helpers forfeit their own reproduction and aid the breeders in raising their young. Understanding this variation has been a major goal for evolutionary ecologists and inclusive fitness theory has stimulated such investigation. Significant research has focused on the effects of differences in indirect fitness benefits and in relatedness between cooperating individuals, on the expression of cooperative behaviours. Yet, fewer studies have focused on the differences in direct fitness costs incurred by cooperative behaviours, the third relevant component of inclusive fitness theory. Oxidative stress represents a prominent candidate mechanism for underlying short-term costs of energetically demanding cooperative behaviours and may lead to decreased fecundity and survival, thereby affecting an individual's direct fitness. In this thesis, I investigate the short-term oxidative costs of cooperative behaviours in a cooperatively breeding rodent, the Damaraland mole-rat (*Fukomys damarensis*).

I started by developing a new, sensitive, and selective method, to allow the quantification of oxidative damage to lipids (malondialdehyde – MDA; Chapter 2) in further work of this thesis. I then experimentally manipulated cooperative contributions in captive Damaraland mole-rats (Chapter 3) and provided the first evidence that increases in cooperative contributions induce oxidative stress in males and females. Although this did not result in the accumulation of oxidative damage in a somatic tissue (erythrocytes), it did so in the germline of male helpers (ejaculates), suggesting that these males favour the protection of somatic over germline tissues against oxidative stress. These results are indicative of a trade-off between survival and

reproduction, and helpers may experience fitness costs through decreased fecundity upon increased cooperative contributions. Lastly, I tested the possibility that glucocorticoid hormones (GCs) mediate a link between cooperative contributions and their oxidative costs (Chapter 4), due to GCs effects on the upregulation of cooperative behaviours in Damaraland mole-rats and other cooperative breeders. Using female helpers, I revealed that increased GC levels did not affect oxidative stress in erythrocytes but led to the accumulation of oxidative damage in the plasma. Although the treatment increased GC levels, cooperative contributions and oxidative stress, the different relationships between these variables suggest that the oxidative costs induced by elevated GC levels and by increased cooperative contributions may independently affect individuals' direct fitness.

In sum, my results suggest that cooperative contributions and the physiological mechanisms responsible for their regulation induce short-term oxidative costs, prone to accumulate and impact inclusive fitness in cooperatively breeding species.

Keywords: Cooperation; costs; Damaraland mole-rat; *Fukomys damarensis*; glucocorticoids; life history; oxidative stress; physiology; trade-offs.

Résumé général

De grandes différences dans l´expression des comportements coopératifs existent entre et au sein d´individus chez les espèces pratiquant la reproduction coopérative, où des aides reportent leur dispersion du groupe natal, renoncent à se reproduire et augmentent le succès reproducteur des parents. Comprendre ces différences a été un objectif majeur pour les écologistes évolutionnistes, et la théorie de la sélection de parentèle et de la valeur sélective inclusive ont apporté un éclairage important sur cette question. Des recherches importantes ont montré les rôles prépondérants des bénéfices de succès reproducteur indirects et du degré d'apparentement entre individus dans l'expression des comportements coopératifs. Cependant, peu d'études ont examiné les coûts directs encourus par les individus coopérants, alors même que ces coûts constituent la troisième composante de la sélection de parentèle. Le stress oxydant pourrait représenter un important coût à court terme des comportements coopératifs énergétiquement exigeants, car le stress oxydant peut entraîner une diminution de la fécondité et de la survie, diminuant ainsi la valeur sélective des individus. Dans cette thèse, j'étudie les coûts d'oxydation à court terme des comportements coopératifs chez un rongeur pratiquant la reproduction coopérative, le rat-taupe de Damaraland (*Fukomys damarensis*).

J'ai tout d´abord développé une nouvelle méthode, sensible et sélective, pour permettre la quantification des dommages d'oxydation aux lipides (malondialdéhyde - MDA, Chapitre 2). J'ai ensuite expérimentalement manipulé les contributions coopératives chez des rats-taupes de Damaraland en captivité (Chapitre 3) et fournis la première preuve que l'augmentation des contributions coopératives induit un stress oxydant chez les mâles et les femelles. Bien que cela n'aboutisse pas à l'accumulation de dégâts d'oxydation dans les globules rouges (un tissu

somatique), de tels dégâts sont visibles dans la lignée germinale des aides mâles (éjaculats), suggérant que ces mâles privilégient la protection antioxydant des tissus germinaux. Ces résultats suggèrent un compromis évolutif entre survie et reproduction, et suggère que les aides mâles peuvent subir des coûts en termes de valeur sélective en raison d'une diminution de fécondité causée par l'augmentation de leurs contributions coopératives. Enfin, j'ai testé la possibilité que les hormones glucocorticoïdes (GC) puissent réguler le lien entre les contributions coopératives et leurs coûts d'oxydation (Chapitre 4), en raison des effets des GC sur la régulation positive des comportements coopératifs, chez les rats-taupes de Damaraland, comme chez d'autres espèces pratiquant la reproduction coopérative. Chez les aides femelles, j´ai démontré que l'augmentation des niveaux de GC n'affecte pas le stress oxydant dans les érythrocytes, mais conduit à l'accumulation de dommages d'oxydation dans le plasma. Bien que le traitement aie conduit à l'augmentions concomitante des niveaux de GC, des contributions coopératives et du stress oxydant, les différentes relations entre ces variables suggèrent que les coûts d'oxydation induits par des niveaux élevés de GC et par des contributions coopératives accrues peuvent affecter indépendamment la valeur sélective des individus.

En résumé, mes résultats suggèrent que, chez les espèces pratiquant la reproduction coopérative, les contributions coopératives et les mécanismes physiologiques responsables de leur régulation induisent des coûts d'oxydation à court terme susceptibles de s'accumuler et d'avoir un impact sur la valeur sélective inclusive des individus.

Mots clés: Compromis; coopération; coûts; Damaraland mole-rat; *Fukomys damarensis*; glucocorticoïdes; histoire de vie; physiologie; stress oxydatif.

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Chapter 1

General Introduction

For many decades, explaining the existence of cooperation in a world where competition is predicted by natural selection, has been a priority for evolutionary biologists (Axelrod and Hamilton, 1981; Bergmüller et al., 2007; Hammerstein, 2003; Lehmann and Keller, 2006; Nowak, 2006; Sachs et al., 2004; West et al., 2007a). Inclusive fitness theory has provided a robust evolutionary framework to explain why individuals behave to the benefit of others, i.e. the evolution and maintenance of cooperative societies (Bourke, 2011; Davies et al., 2012). This theory is captured by Hamilton's Rule, which states that cooperative behaviours are favoured by natural selection if the changes in the lifetime fitness benefits to the recipient (B), weighed by the genetic relatedness (r) between the recipient and the actor, overcompensate the changes in lifetime fitness costs incurred to the actor (C), $rB - C > 0$ (Hamilton, 1964a, 1964b, West et al., 2007a, 2007b). Cooperative behaviours are defined as acts that provide fitness benefits to the recipient (positive B) and are under positive selection due to such beneficial effects (West et al., 2007b). Cooperative behaviours are mutually beneficial when C is positive, or altruistic when C is negative (West et al., 2007a).

Inclusive fitness theory has also been incredibly helpful in explaining the pronounced variation in cooperative contributions (the extent to which individuals cooperate), both within and between individuals. Such variation is striking in cooperatively breeding societies (e.g. Clutton-Brock et al., 2001a; Field et al., 2006; Hodge, 2007; Zöttl et al., 2016), where the majority of group members, commonly referred to as helpers, delay or sacrifice their reproduction and help a minority of breeding individuals in raising their young and in maintaining, defending and expanding their territories (Clutton-Brock, 2016; Koenig and Dickinson, 2016). While explaining the existence of variation in cooperative contributions, much emphasis has been placed on the influence of relatedness (r) between the actor and the recipient, and on the fitness benefits (B) of cooperative behaviours to the recipient (Kingma et

al., 2011; West et al., 2007a; Wright et al., 2010). Hamilton's Rule predicts that cooperative behaviours should be favoured when directed towards closely related individuals and some empirical studies have supported this prediction (Emlen and Wrege, 1988; Komdeur, 1994; Nam et al., 2010). The term B is often identified in the form of increased survival and reproductive output of the related breeders (Brown et al., 1982; Hodge et al., 2008; Reyer, 1984; Russell et al., 2003a; Taborsky, 1984), or increased growth rates, survival and reproductive potential of the breeders' offspring (Clutton-Brock et al., 2001b; Moehlman, 1979; Russell et al., 2007). Contrasting with the focus placed on the effect of relatedness and of the benefits of cooperative behaviours in explaining individual differences in cooperative contributions, the effects of their costs (C) have attracted less attention (Cant and Field, 2005, 2001; Cockburn, 1998; Field et al., 2006; Heinsohn and Legge, 1999).

1.1. COSTS OF COOPERATIVE CONTRIBUTIONS

Cooperative behaviours may on the one hand generate direct fitness benefits to the actor (positive C in Hamilton's Rule), such as access to group living and to direct reproductive opportunities (Cockburn, 1998; Griffin and West, 2002; Kokko et al., 2002; Solomon and French, 1997). Yet on the other hand, cooperative behaviours may also generate direct fitness costs to the actor (negative C in Hamilton's Rule), such as reduced fecundity and even survival (Clutton-Brock, 1991; Reyer, 1984; Russell et al., 2003b; Stacey and Koenig, 1990).

In the short-term, cooperative behaviours can be costly due to their energetic demands. Experimental studies in a cooperatively breeding cichlid (*Neolamprologus pulcher*) and in two cooperatively breeding mole-rats (naked mole-rat, *Heterocephalus glaber*, and Damaraland mole-rat, *Fukomys damarensis*), have shown that engaging in cooperative activities increases

energy expenditure and metabolic rate (Grantner and Taborsky, 1998; Lovegrove, 1989). Hence, greater cooperative contributions may incur higher energetic costs. These costs are well illustrated in correlative and experimental studies in several cooperatively breeding species, where increasing levels of cooperative contributions lead to reduced growth rate (Russell et al., 2003b; Taborsky, 1984), slower weight gain (Hodge, 2007; Russell et al., 2003b), loss of weight (Clutton-Brock et al., 1998; Heinsohn and Cockburn, 1994; Hodge, 2007), or reduced time spent foraging (Heinsohn et al., 1988; Price, 1992; Sánchez et al., 1999; Schradin and Anzenberger, 2001).

In the long-term, the immediate energetic costs of cooperative behaviours may accumulate and result in cascading effects that affect fecundity (Russell et al., 2003b) and survival (Reyer, 1984; Stacey and Koenig, 1990). However, evidence for such long-term costs is scarce because animals may adjust their cooperative contributions according to internal and external cues, in order to minimize the long-term costs incurred. For example, cooperative contributions are often increased when group size is small (Clutton-Brock et al., 2001a; Heinsohn and Cockburn, 1994; Heinsohn et al., 1988; Wright, 1997), food availability is high (Bell, 2010; Boland et al., 1997; Nichols et al., 2012a), or helpers forage more successively (Clutton-Brock et al., 2001a). Contrastingly, cooperative contributions decrease when individuals have previously displayed high levels of cooperation (Russell et al., 2003b; Sanderson et al., 2014), have a low body condition (Clutton-Brock et al., 2002; Gilchrist and Russell, 2007; Russell et al., 2003b), or are at the front of the cue for a reproductive position (Bergmüller et al., 2005; Cant and Field, 2005, 2001; Gilchrist and Russell, 2007).

1.2. PHYSIOLOGICAL COSTS OF COOPERATIVE BEHAVIOURS: A ROLE FOR OXIDATIVE STRESS?

The costs of cooperative contributions have largely been investigated regarding their effects on individual physical characteristics, such as body mass and growth rate. Such studies suggest that individual adjustments in cooperative contributions must be linked with appropriate tuning of metabolism and energy production (Grantner and Taborsky, 1998; Lovegrove, 1989). In this sense, oxidative stress could represent a physiological mechanism underlying the short-term costs of cooperative behaviours, also able to incur pervasive long-term costs susceptible of affecting individual lifetime fitness (Costantini, 2014; Finkel and Holbrook, 2000; Harshman and Zera, 2007; Monaghan et al., 2009).

In aerobic organisms, cellular energy is produced in the mitochondria under the form of adenosine triphosphate (ATP), through a metabolic pathway called oxidative phosphorylation. This pathway involves a flow of electrons, removed from fuel molecules (predominantly glucose), through the electron transport chain, where oxygen is the final acceptor of electrons (Halliwell and Gutteridge, 2007). This process leads to the inevitable by-product formation of pro-oxidants, the so-called reactive oxygen species (ROS; Figure 1.1), which can oxidize and therefore damage important biomolecules such as deoxyribonucleic acid (DNA), proteins and lipids (Davies et al., 1982; Halliwell and Gutteridge, 2007). To counterbalance the damaging effects of ROS, cells have evolved defence mechanisms, such as the antioxidant system. Antioxidants are molecules that neutralize ROS, preventing them from damaging important components of the cell. The cell enters a state of oxidative stress when there is an imbalance between the formation of ROS and the capacity of the antioxidant system to neutralize them (Sies, 1985). Oxidative stress may lead to an increase in the rate of accumulation of oxidative damages, which has the potential to disrupt cell and tissue functioning and thereby compromise

individuals' survival and reproduction (Costantini, 2014; Finkel and Holbrook, 2000; Harshman and Zera, 2007; Monaghan et al., 2009).

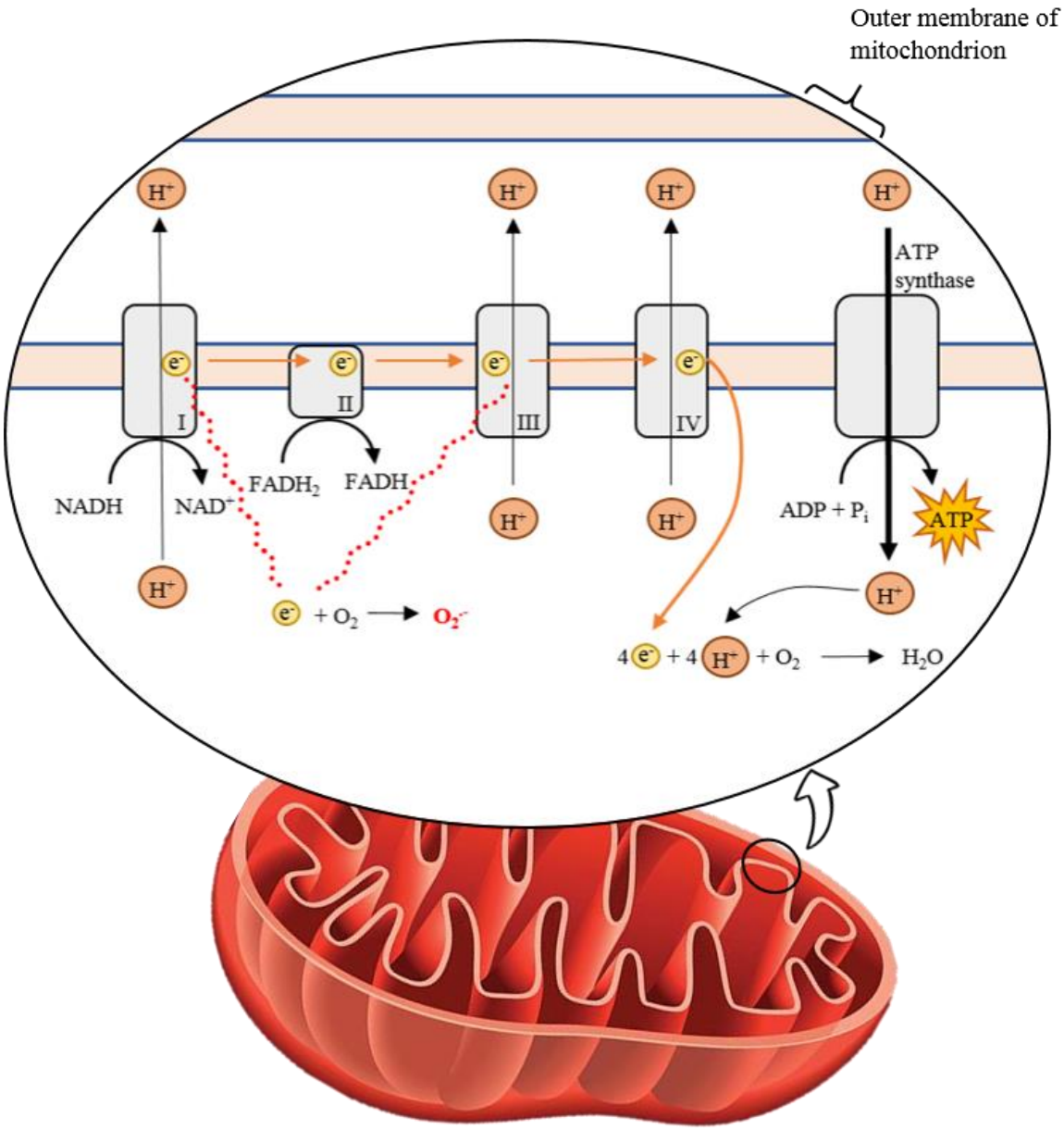

Figure 1.1 - The electron transport chain (ETC) is the major site of ROS ($O_2^{\cdot-}$) formation. NADH and $FADH_2$ are produced during the Krebs cycle and donate electrons to the protein complexes I and II, respectively, of the ETC. Electrons flow through protein complexes I, II, III and IV (orange arrows), releasing free energy that is used to pump protons (H^+) into the inter-membrane space of the mitochondrion (black upwards arrows). As H^+ gradient increases, ATP-synthase uses the flow of protons back to the matrix of the mitochondrion (black downwards arrow) to induce the formation of ATP, from adenosine diphosphate (ADP) and inorganic phosphate (P_i). At the end of the electron transport chain, electrons combine with protons and oxygen, to produce water. However, this process is not 100% efficient and electrons may leak from complexes I and III (red dots), and directly react with O_2, generating the superoxide anion ($O_2^{\cdot-}$), a potent ROS.

Intense physical activities, requiring increases in energy production, have been shown to affect the oxidative balance by raising the formation of ROS, inducing oxidative stress and resulting in the accumulation of oxidative damage (Bejma and Ji, 1999; Davies et al., 1982; Leeuwenburgh and Heinecke, 2001; Radak et al., 2008). However, this relationship may be less straightforward than initially thought as physiological adaptations may allow individuals to cope with exercise-induced oxidative stress and reduce the damaging effects of high energetic demands (Radak et al., 2008). Such adaptations include the upregulation of enzymatic antioxidant activities and oxidative damage repairing mechanisms, and alterations of the composition of cell membranes (e.g. high percentage of saturated or monounsaturated fatty acids increases resistance to oxidation compared to a high proportion of polyunsaturated ones) (Costantini, 2008; Hulbert et al., 2007; Radak et al., 2008).

In cooperative breeders, where energetically demanding cooperative contributions increase aerobic respiration (Grantner and Taborsky, 1998; Lovegrove, 1989), the effects of individual cooperative contributions on oxidative balance remain elusive. Correlative studies in only two cooperatively breeding birds have investigated the relationship between oxidative balance and social/breeding status or alloparental care. In Seychelles warblers (*Acrocephalus sechellensins*), social groups include a dominant breeding pair and subordinates of both sexes which may or may not help (helpers and non-helpers, respectively) the breeders with territory defence and rearing of the offspring (Komdeur and Richardson, 2007). Prior to the breeding season, female non-helpers had lower body mass and higher plasma levels of oxidative damage when compared to female helpers or breeders, suggesting that only those individuals in good physical and oxidative condition can afford the oxidative challenges imposed by cooperative behaviours (van de Crommenacker et al., 2011). However, it is unclear from that study whether such differences were related to the decision to help or to reproduce since the percentage of

female helpers that co-breed is relatively high (Richardson et al., 2001). In contrast, in the white-browed sparrow weaver (*Plocepasser mahali*) the oxidative balance prior to the breeding season did not affect the decision to help the breeders provisioning the nestlings, but female breeders and female helpers, who provision nestlings at higher rates, showed higher levels of oxidative damage at the end of the reproductive season (Cram et al., 2014). In a subsequent study, Cram et al. (2015b) showed that individual provisioning rate was negatively correlated with plasma total antioxidant capacity. Additionally, in small groups, individuals provisioning offspring had higher oxidative damage to lipids compared to those that were not provisioning, but the effect was not detectable in large groups (Cram et al., 2015b). Altogether, these studies suggest that variation in energetically demanding cooperative contributions could generate and/or be caused by differences in oxidative stress physiology. Whether more generous individuals face higher oxidative costs, both on the short- and on the long-term, as a consequence of the associated increases in energy expenditure, is an exciting hypothesis that remains to be experimentally tested.

1.3. GLUCOCORTICOIDS, ENERGETICALLY DEMANDING COOPERATIVE BEHAVIOURS AND OXIDATIVE STRESS

The potential oxidative costs of cooperative behaviours could be the direct consequence of their associated energetic demands and/or an indirect consequence of a physiological regulator of cooperative behaviours, such as glucocorticoid steroid hormones (GCs). Indeed, GCs, which secretion by the adrenals is modulated through the hypothalamic-pituitary-adrenal axis (HPA) (Sapolsky et al., 2000), can regulate cooperative contributions and induce oxidative stress.

In cooperatively breeding species, multiple studies have recently suggested a role of GCs in the regulation of cooperative behaviours (Bender et al., 2008; Carlson et al., 2006a, 2006b, Dantzer et al., 2017a, 2017b; Mota et al., 2006; Raynaud and Schradin, 2015; Sanderson et al., 2014; Santema et al., 2013), but not always in the predicted direction that higher GC secretion increases cooperative contributions and vice-versa. The prediction of a reciprocal relationship between GC levels and energetically demanding cooperative behaviours relies on the generally positive effects exerted by GCs on energy production and metabolism (Landys et al., 2006; McEwen and Wingfield, 2003; Sapolsky et al., 2000), and the reciprocal positive effect of energy expenditure on GC levels (Hackney and Viru, 1999; Malisch et al., 2008; Stranahan et al., 2008). In Damaraland mole-rats, a positive and reciprocal relationship between GC levels and cooperative contributions has recently been experimentally demonstrated. Indeed, burrowing contributions of female helpers is doubled in response to the administration of exogenous GCs, and GC levels tend to be increased by the experimental elevation of cooperative contributions (Vullioud et al., in preparation). In other species, positive (Carlson et al., 2006a, 2006b, Dantzer et al., 2017a, 2017b; Sanderson et al., 2014; Wright et al., 2010), null (Bender et al., 2008; Mota et al., 2006; Santema et al., 2013), and negative (Dantzer et al., 2017b; Raynaud and Schradin, 2015) associations between GC levels and cooperative contributions have been reported. Anticipating that future experimental work confirms the discrepancies in the direction of the associations highlighted by correlational studies conducted so far (Bender et al., 2008; Carlson et al., 2006b; Dantzer et al., 2017a; Mota et al., 2006; Raynaud and Schradin, 2015; Sanderson et al., 2014; Young et al., 2010), the relationship between GC secretion and cooperative contributions may be contingent on the type of cooperative activity, the species, the sex and the nutritional state of the animals.

In addition to the regulation of the expression of cooperative behaviours, increased GC levels generate oxidative stress (Costantini et al., 2011). Experimental elevations of GC levels have been reported to stimulate ROS formation (Wilson et al., 2013) and to decrease antioxidant protection (McIntosh et al., 1998; Orzechowski et al., 2002; Pereira et al., 1994; Rajashree and Puvanakrishnan, 1998; Zafir and Banu, 2009). Although such effects can be tissue and antioxidant-specific (Cote et al., 2010; McIntosh et al., 1998; Orzechowski et al., 2000; Pereira et al., 1994), increased GC levels ultimately result in fast accumulation of oxidative damage to DNA, proteins and lipids in a multitude of tissues (Caro et al., 2007; Costantini et al., 2008; Cote et al., 2010; Eid et al., 2003; Lin et al., 2004a; Pereira et al., 1994; Rajashree and Puvanakrishnan, 1998; Zafir and Banu, 2009). Furthermore, there is evidence that longer exposures to high GC levels result in greater oxidative costs compared to shorter-term elevations (Lin et al., 2004a, 2004b; Wilson et al., 2013). Thus, GC-induced oxidative stress may represent a causal mechanism underlying the costs of prolonged elevated GC secretions (Costantini et al., 2011), such as an increased susceptibility to disease (Cram et al., 2015a; Sapolsky et al., 2000), and reduced fertility (Nargund, 2015; Whirledge and Cidlowski, 2010) and survival (Bonier et al., 2009; Romero and Wikelski, 2001).

Although it is increasingly recognized that elevated GC levels induced by unpredictable and life-threatening situations can affect the oxidative balance (Costantini et al., 2011), the effects of GC fluctuations within levels associated with predictable life-history events remains poorly understood. This is particularly relevant in the context of cooperative behaviours, since their expression is not expected to be stimulated by GC levels reflecting a stressful situation. Furthermore, whether the potential oxidative costs of energetically demanding cooperative behaviours could be modulated by the associated fluctuations in GC secretion is currently unknown.

1.4. OXIDATIVE STRESS AND LIFE-HISTORY TRADE-OFFS

Life-history theory has provided an important framework for the field of evolutionary biology, acknowledging and demonstrating negative associations between life-history traits, most often explained by resource limitations (Stearns, 1976, 1989; Zera and Harshman, 2001). Under such circumstances, resources allocated to one life-history trait become unavailable to others, thereby generating trade-offs between competing demands (Reznick et al., 2000; Stearns, 1992). It has recently been proposed that oxidative stress may represent a proximate mechanism underlying life-history trade-offs (Dowling and Simmons, 2009; Metcalfe and Alonso-Alvarez, 2010; Monaghan et al., 2009; Speakman and Garratt, 2013).

The protection against the damaging effects of ROS, possibly exacerbated during the expression of energetically demanding cooperative behaviours, may be particularly relevant in mediating the universal trade-off between survival and reproduction, i.e. between somatic and germline functions (Metcalfe and Alonso-Alvarez, 2010; Stearns, 1976; Velando et al., 2008), yet this exciting possibility has remained largely overlooked. Because reproduction is costly, resources allocated to reproductive functions are traded-off against other life-history traits, such as future survival (Calow, 1979). The germline, and particularly sperm cells, are routinely exposed to ROS (Dowling and Simmons, 2009; Velando et al., 2008), to which they are highly sensitive (Tremellen, 2008). Hence, it can be hypothesised that the diversion of resources fighting ROS damaging effects towards germline functions may come at the expense of somatic protection, provided these resources are limited (Bertrand et al., 2006; Blount et al., 2001, 2000; Helfenstein et al., 2010; Losdat et al., 2011; Mora et al., 2016). Supporting this hypothesis, increased investment in reproduction is often associated with high vulnerability of somatic tissues to oxidative stress (Alonso-Alvarez et al., 2004; Blount et al., 2016; Heiss and

Schoech, 2012; Merkling et al., 2017; Wiersma et al., 2004), but such association may not be evident when antioxidants are not limiting resources (Bertrand et al., 2006).

In cooperatively breeding species, differences in current and future breeding prospects between breeders and helpers raise the possibility that the strategy used to avoid an accumulation of oxidative damage in the germline and in the soma, may vary as a function of breeding status. Indeed, optimal investment towards competing life-history traits are dependent on intrinsic and extrinsic cues that change through time, and plastic adjustments are therefore expected to maximise individuals' fitness (Drenos and Kirkwood, 2005; Perrin and Sibly, 1993). Particularly in cooperative societies with high reproductive skew, helpers may have been selected to prioritize somatic over germline protection, as long as reproductive opportunities are unlikely to arise. Breeders, who monopolize reproduction, may conversely be expected to prioritize germline over somatic protection. Alternatively, the superior phenotype of breeders may relax the constraints involved in the trade-off between somatic and germline tissues, explaining, at least partly, their generally extended longevity (Carey, 2001; Dammann and Burda, 2007, 2006).

1.5. MEASURES OF OXIDATIVE STRESS

Oxidative balance is dependent on a complex network of interactions between pro-oxidants and antioxidant molecules (Halliwell and Gutteridge, 2007; Monaghan et al., 2009). Addressing the whole complexity of this system is usually unfeasible and researchers focus on a part of it. Most importantly, ecological studies have traditionally focused on the evaluation of the antioxidant system, which alone provides little insight into the oxidative balance (Costantini and Verhulst, 2009; Hõrak and Cohen, 2010). At present, many techniques are

available to quantify ROS formation, different antioxidants, oxidative damage to different cellular components (lipids, proteins and DNA) and the activity of enzymes responsible to repair oxidative damage (Monaghan et al., 2009). Furthermore, there is a diversity of techniques available that vary in sensitivity and specificity, which researchers must carefully evaluate before selecting the appropriate method, according to the hypothesis being tested.

In this thesis, I used a suite of markers that mirror antioxidant protection, the state of cellular oxidation and the level of oxidative damage. Firstly, I measured superoxide dismutase (SOD), an enzymatic antioxidant responsible for neutralizing the superoxide anion ($O_2^{\cdot -}$; Figure 1.2), the first ROS formed during oxidative phosphorylation (Godin and Garnett, 1992). The reaction of SOD with $O_2^{\cdot -}$ results in the formation of oxygen and hydrogen peroxide (H_2O_2), which is a less harmful ROS (Halliwell and Gutteridge, 2007). The quantification of SOD activity is justified by its significant positive correlation with metabolic rate (Godin and Garnett, 1992).

Secondly, I quantified the concentration of glutathione, an endogenous antioxidant involved in the neutralization of hydrogen peroxide, thereby acting in concert with SOD. Reduced glutathione (GSH) is a tripeptide that reduces H_2O_2 into oxidized glutathione (GSSG) and water, in a reaction catalysed by the enzyme glutathione peroxidase (GPx; Figure 1.2). The recycling of GSSG back to GSH is achieved by a reaction catalysed by another enzyme, glutathione reductase (GR) (Halliwell and Gutteridge, 2007). In my work, I quantified the two forms of glutathione, and used the molar ratio between the two (GSSG/GSH) as a measure of cellular oxidation (Jones, 2006). This is a powerful tool since changes of this ratio are suggestive of ROS formation and disturbances of the oxidative balance (Flohé, 2013; Jones, 2006).

Lastly, I quantified malondialdehyde (MDA), the end-product of peroxidation of fatty acids, a frequently used metric of oxidative damage to lipids (Del Rio et al., 2005). MDA may also act as a pro-oxidant, further oxidising lipids, proteins and DNA, thus increasing the extent of oxidative damage. Yet, the reactivity of MDA *in vivo* is still a matter of discussion (Halliwell and Gutteridge, 2007).

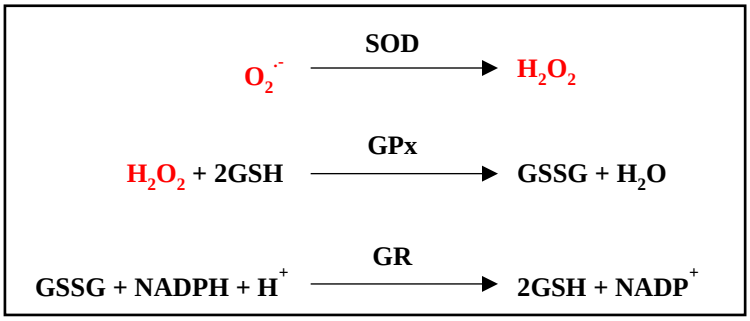

Figure 1.2 - Antioxidant reactions involving the enzymatic antioxidants superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR). SOD catalyses the dismutation of the superoxide anion ($O_2^{\cdot -}$), formed upon electron leakage from the electron transport chain (see Figure 1.1), producing hydrogen peroxide (H_2O_2). The glutathione antioxidant system is one involved in the neutralization of the pro-oxidant H_2O_2. Reduced glutathione (GSH) reacts with H_2O_2 to form oxidised glutathione (GSSG) and water, a reaction catalysed by glutathione peroxidase (GPx). Glutathione reductase (GR) is the enzyme catalysing the conversion of GSSG back to GSH (Halliwell and Gutteridge, 2007).

1.6. MODEL SPECIES

Damaraland mole-rats (*Fukomys damarensis*) belong to the family of obligatory subterranean rodents, the Bathyergidae, and are endemic to southern Africa, being found in xeric habitats, where rainfall is low and unpredictable (Bennett and Faulkes, 2000). This cooperatively breeding species, lives in colonies of 12 to 25, but up to 41 individuals (Figure 1.3), with an extreme reproductive skew (Burland et al., 2002; Jarvis et al., 1994). In each colony, one female and usually only one male monopolize reproduction, although sometimes up to three males may father offspring (Bennett and Jarvis, 1988; Burland et al., 2004, 2002). The breeding

female and male are unrelated (Burland et al., 2002) and reproduction occurs continuously with up to four litters being sired around the year (Bennett and Faulkes, 2000; Bennett and Jarvis, 1988; Young et al., 2010). The rest of the colony consists of the offspring of the breeding pair, also called helpers, who do not breed while in their natal colony. In fact, the reproduction of female helpers is physiologically suppressed, rendering these females anovulatory (Bennett, 1994; Molteno and Bennett, 2000). Contrastingly, there is no physiological suppression of reproduction in males, as helpers have similar Testosterone levels (Bennett, 1994; Maswanganye et al., 1999) and produce similar numbers and proportion of motile spermatozoa to male breeders (Faulkes et al., 1994; but see Maswanganye et al., 1999). Helpers may face breeding opportunities when the underground tunnels of two neighbouring colonies become temporarily connected (N.C. Bennett, personal observation), which may give rise to intense social instability, changes of the social hierarchy (Cooney and Bennett, 2000), and trigger dispersal (Young et al., 2010).

While in their natal colony, helpers increase the reproductive success of the breeding pair (Young et al., 2015), possibly through direct care of the young offspring (huddle, groom and carry pups away from threatening situations), colony defence and indirect food provisioning (Bennett and Faulkes, 2000; Bennett and Jarvis, 1988; Cooney, 2002; Jarvis et al., 1998). The most pronounced form of cooperation in Damaraland mole-rats relates to burrowing activities (Figure 1.4), which enable the expansion of the tunnel system and allow the mole-rats to locate and expose food sources (Figure 1.4), which are underground storage organs of geophytes (Bennett and Faulkes, 2000; Jarvis et al., 1998). Food resources are then transported and accumulated in storing areas (Figure 1.4) close to the nest (Figure 1.4) and represent a common good that benefits all group members (Jarvis et al., 1998).

Figure 1.3 - Wild-caught Damaraland mole-rats. The photo shows only a few of the colony members who were trapped on the same night. Colonies can have up to 41 individuals in the wild. Source: © Kyle Finn.

Burrowing activities are energetically demanding (Lovegrove, 1989), and more pronounced in the wet season, when the rain softens the sand and makes it easier to dig through (Jarvis et al., 1998; Young et al., 2010). All members of the colony that can burrow independently undertake these activities, yet there are great differences in cooperative contributions, both between and within individuals (Bennett, 1990; Bennett and Jarvis, 1988; Zöttl et al., 2016), making this an ideal study system to investigate the costs of individual variation in cooperative contributions.

Figure 1.4 - Underground burrowing activities of Damaraland mole-rat colonies lead to the formation of sandy mounds at the surface (top left). These activities are vital in the search for food, i.e. underground storage organs of geophytes, such as those of the gemsbok cucumber (*Acanthosicyos naudinianus*) (top right). Damaraland mole-rats transport and accumulate food sources in storing areas (bottom left), often close to the nest (bottom right). Source: © Kyle Finn.

For this thesis, I used a captive population of Damaraland mole-rats, maintained in laboratory facilities at the Kuruman River Reserve, Northern Cape, in South Africa (Figure 1.5). The colonies used were originally trapped in the surrounding farms of the reserve between February and October 2013. At the time of the experimental work presented here, the colonies were fully habituated to the laboratory conditions and successfully producing offspring (Figure 1.6) for at least one year.

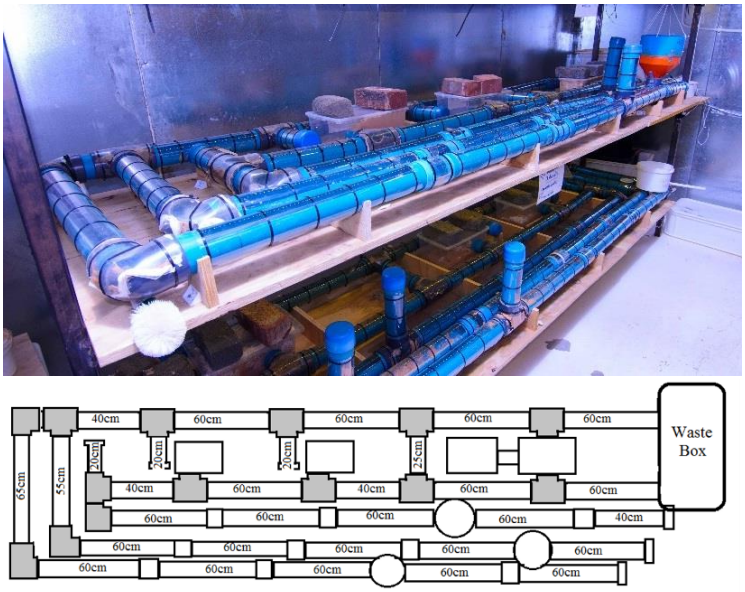

Figure 1.5 - Artificial tunnel systems used to house colonies of Damaraland mole-rats (top left) and a diagram representing a top view of the standard tunnel system (bottom left; rectangles represent transparent plastic boxes used as nesting/storage areas and circles represent the vertical sand dispensers). The captive colonies used in this thesis were housed in the laboratory facilities of the Kuruman River Reserve, South Africa (right; star shows the approximate location of the study site). Source: © Kyle Finn (top left); © Andrea Cooper (right).

Figure 1.6 - Damaraland mole-rat colonies successfully breed in laboratory conditions, with each breeding female giving birth 3 to 4 times per year. Left: new-borns found in the colonies are weighed, marked with food colorant and quickly returned to their nest. Right: a pup, two months old, starting to feed on sweet potato, showing a red food-colour mark used for individual recognition during observation sessions.

1.7. THESIS AIMS AND STRUCTURE

My thesis aims to test the hypothesis that energetically demanding cooperative behaviours are associated with physiological costs. I specifically investigated whether increased energetically demanding cooperative contributions affect oxidative balance, and whether and how individuals trade-off between somatic and germline protection against oxidative stress. Furthermore, I explored whether an internal cue regulating cooperative contributions (GCs) may be responsible for the potential oxidative costs induced by cooperative behaviours.

In Chapter 2, I developed a highly sensitive and selective methodology to quantify MDA. This work was motivated by the inability of the previously available assay to quantify MDA in our laboratory to measure lipid peroxidation in ejaculate samples, due to the small sample volumes available and their intrinsically low concentrations of MDA. This chapter has been published in the *Journal of Lipid Research,* and although it deals with plasma samples only, it can be easily adapted to the use of cell homogenates (e.g. erythrocytes and ejaculates).

In Chapter 3, I investigated whether the expression of energetically demanding cooperative behaviours induces oxidative stress. For this, I experimentally increased the expression of energetically demanding cooperative behaviours (burrowing) of entire colonies. I tested whether increased cooperative contributions affect the somatic oxidative balance of males and females, and the trade-off between somatic (erythrocytes) and germline (ejaculates) tissues in Damaraland mole-rat male helpers.

In Chapter 4, I assessed the effect of Cortisol (a glucocorticoid that increases the expression of burrowing behaviours in Damaraland mole-rats) on the oxidative balance of female Damaraland mole-rat helpers, through experimental increases of circulating Cortisol levels.

In Chapter 5, I provide a summary of the findings of this thesis and critically discuss their relevance, and application and highlight promising future research.

Chapter 2

Sensitive and selective quantification of free and total malondialdehyde in plasma using UHPLC-HRMS

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ABSTRACT

Quantification of malondialdehyde (MDA) as a marker of lipid peroxidation is relevant for many research fields. We describe a new sensitive and selective method to measure free and total plasmatic MDA, using derivatization with 2,4-dinitrophenylhydrazine (DNPH) and ultrahigh performance liquid chromatography-high resolution mass spectrometry (UHPLC-HRMS). Free and total MDA were extracted from minute sample amounts (10 µl) using acidic precipitation and alkaline hydrolysis followed by acidic precipitation, respectively. Derivatization was completed within 10 min at room temperature, and the excess DNPH discarded by liquid-liquid extraction. Quantification was achieved by internal standardization using dideuterated MDA as internal standard. The method lowest limit of quantification (LLOQ) was 100 nM and linearity spanned > 3 orders of magnitude. Intra- and inter-day precisions for total MDA were 2.9% and 3.0%, respectively, and those for free MDA were 12.8% and 24.9%, respectively. Accuracy was 101% and 107% at low and high concentrations, respectively. In human plasma, free MDA levels were 120 nM (SD 36.26) and total MDA levels were 6.7 µM (SD 0.46). In addition, we show the applicability of this method to measure MDA plasma levels from a variety of animal species, making it invaluable to scientists in various fields.

2.1. INTRODUCTION

Oxidative stress is a cellular state in which the production of reactive oxygen species (ROS) is in misbalance with the available antioxidants (Sies, 1985). Although ROS are constitutive by-products of cell metabolism, their production can be amplified in the presence of environmental perturbations (Halliwell, 1994). The presence of ROS can result in the oxidation of important biomolecules such as DNA, proteins and lipids, potentially leading to cell damage. One of the most studied oxidative injuries is that incurred to lipids due to their importance in the maintenance of cellular structure. Peroxidation of polyunsaturated fatty acids (PUFAs) results in lipid hydroperoxides, which after breakdown result in a variety of aldehydes, malondialdehyde (MDA) being the primary and best studied of these molecules (Del Rio et al., 2005).

The study and quantification of MDA as a marker of lipid peroxidation has been relevant for many research fields. In medical research, several diseases have been related to higher levels of MDA compared to healthy controls (Gönenç et al., 2001; Madazli et al., 1999; Noberasco et al., 1991). In food sciences quantification of MDA concentration has been frequently taken as a measure of quality of stored foodstuff (Marcincak et al., 2004). MDA levels have also been used as a toxicity marker of herbicides used in agriculture, such as paraquat (Bus et al., 1976). Recently, research in ecology, animal behaviour and evolution has also used MDA as a key marker of oxidative damage to investigate the physiological mechanisms underlying important evolutionary concepts such as sexual selection and life-history trade-offs (Costantini, 2008; Metcalfe and Alonso-Alvarez, 2010; Monaghan et al., 2009).

In biological matrices, MDA exists in both free and protein-bound forms, the latter representing the vast majority of total MDA (Cighetti et al., 1999; Pilz et al., 2000; Steghens et al., 2001).

Several techniques have been developed to assess either total MDA or its free form, most often based on derivatization chemistry coupled with various separation and/or detection methods including gas chromatography (GC) or liquid chromatography (LC), ultraviolet (UV) or fluorescence spectrophotometry, and mass spectrometry (MS) (Esterbauer et al., 1991; Giera et al., 2012; Kadiiska et al., 2005; Mateos and Bravo, 2007; Sobsey et al., 2016). The most widely used derivatizing agent is the thiobarbituric acid (TBA). Although this method is relatively easy to perform, its specificity is low (Knight et al., 1988) as TBA reacts with other compounds naturally available in biological samples (Baumgartner et al., 1975; Gutteridge and Tickner, 1978; Halliwell and Chirico, 1993; Knight et al., 1988). Also, because derivatization with TBA requires high temperatures (95-100 °C) under strongly acidic conditions, this method likely leads to an overestimation of MDA concentrations (Halliwell and Chirico, 1993). Finally, consistent and significant peaks in blank samples result in an increased limit of detection, precluding the analysis of samples with low levels of MDA (Agarwal and Chase, 2002). Currently, an important need is the development of a method that allows specific and accurate quantification of MDA while reducing sample volumes used. Specifically, a method that does not produce peaks in blank samples is of most importance for researchers working with limited sample volumes and/or naturally low concentrations of MDA.

To overcome the limitations of the TBA assay, more specific and reliable methods have been developed using other derivatizing agents, such as phenylhydrazine (PH), 2,4,6-trichlorophenilhydrazine (TCPH), 3-nitrophenylhydrazine, pentafluorophenylhydrazine (PFPH) and 2,4-dinitrophenylhydrazine (DNPH) (Cighetti et al., 1999; Sobsey et al., 2016; Yeo et al., 1994). A clear advantage of hydrazine-based over TBA-based derivatization is the milder conditions needed for the reaction, which can be performed at room temperature. Specifically, derivatization with DNPH is completed after 10 to 30 min (Pilz et al., 2000; Sim

et al., 2003), a much shorter time in comparison to TBA (1h), PH (3h) and TCPH (1h) methods, and yields a stable MDA-DNPH derivative (Cighetti et al., 1999; Pilz et al., 2000). Chemicals used for the MDA-DNPH derivatization have minimal costs, and accurate and specific quantification can be done using robust LC-UV (Marcincak et al., 2004; Pilz et al., 2000; Sim et al., 2003) or LC-MS (Douny et al., 2015). However, one main disadvantage of most published protocols for quantification of MDA using DNPH is the high volume of sample required - from 50 µl (Sim et al., 2003) up to 1 ml (Pilz et al., 2000), precluding analysis in studies where sample volumes are restrictively small.

Here we evaluated the use of ultra-high performance liquid chromatography – high-resolution mass spectrometry (UHPLC-HRMS) for the quantification of both free and total plasmatic MDA, using DNPH as the derivatizing agent. While HRMS was once not considered suitable for quantitative analysis, new generation HRMS instruments have been shown to provide similar quantitative performances to e.g. triple quadrupoles (Grund et al., 2016). We show that our validated method allows using minute sample volumes and can be used for quantification of MDA in plasma from different vertebrate species, including human, rodent (mole-rat, *Fukomys damarensis*), bat (*Carollia perspicillata)*, bird (*Passer domesticus)* and fish (*Scolopsis bilineatus)*, making it of great interest to scientists in various fields, from biomedicine to ecology and evolution.

2.2. MATERIALS AND METHODS

Reagents and chemicals

Malondialdehyde tetrabutylammonium salt (MDA-salt), 2,4-dinitrophenylhydrazine (DNPH), sodium hydroxide (NaOH) and methyl tert-butyl ether (MTBE) were purchased from Sigma-Aldrich. [D_2]-1,1,3,3-tetra-ethoxypropane (D_2-TEP) was obtained from Dr. Ehrenstorfer GmbH. Trichloroacetic acid (TCA), cyclohexane and toluene were obtained from Merck KGaA. Hydrochloric acid (HCl 37%), methanol (MeOH), and ethyl acetate (EtOAc) were purchased from Fisher Scientific. Hexane was purchased from Acros Organics. Acetonitrile (ACN), water and formic acid used for LC-MS analyses were purchased at Biosolve. For semi-preparative isolation, HPLC grade acetonitrile and formic acid p.a. from Sigma-Aldrich and milli-Q water were employed.

Blood collection

Peripheral human blood was collected from healthy volunteers among the authors of this study (female and male, Caucasian) into a Sarstedt Monovette ® with heparin as an anticoagulant and centrifuged straight away for 10 min at 4 ºC and 2000 *g*.

Mole-rat (*Fukomys damarensis*) blood was collected into heparinized tubes from individuals under anaesthesia from a pre-warmed hind foot by using a sterile needle to puncture a vein. Blood was centrifuged straight away at room temperature for 5 min at 2000 *g*.

Blood samples from bats (*Carollia perspicillata*) and house sparrows (*Passer domesticus*) were drawn from the antebrachial wing vein and from the alar vein, respectively, into heparinized

CB300 Sarstedt Microvette® by puncturing with a sterile needle. Samples were transported to the laboratory on ice, where they were centrifuged for 5 min at 4 ºC and 2000 *g*.

Fish (*Scolopsis bilineatus*) blood was collected from sedated individuals from the caudal vein using a sterile needle and a pre-heparinized syringe. Blood was centrifuged for 5 min at 2000 *g*.

For all species, plasma was separated from the cellular component following centrifugation and stored at -80 ºC until analysis. For method validation, human plasmas were split into 70 µl aliquots before freezing.

Human subjects provided written informed consent before sampling. Animal samples were collected as part of other ongoing studies. All animal procedures complied with Public Health Service policy on humane care and use of laboratory animals. Blood sampling procedures were approved by: the University of Pretoria Animal Ethics Committee (*Fukomys damarensis*, EC093-14); the veterinary office of the Canton Fribourg (*Carollia perspicillata*, FR_2013_46 and FR_2014_44); the veterinary office of the Canton Bern (*Passer domesticus*, BE41/12 and WTH/g-525/14) and the Australian National University animal experimentation ethics committee (*Scolopsis bilineatus*, A2015/66).

Synthesis of deuterated MDA

Deuterated MDA (D_2-MDA) was synthesized from D_2-TEP by acidic hydrolysis. D_2-TEP 30 µM was incubated in 0.1M HCl for 40 min at 40°C. The resulting D_2-MDA (30 µM in 0.1 M HCl) was used as internal standard (IS) for MDA quantification (Weber et al., 2004).

Extraction of free and total MDA

For extraction of the free fraction of MDA, 10 µl of plasma was mixed with 40 µl of water, 10 µl of IS and 150 µl of TCA 20% in a microcentrifuge tube to precipitate proteins. Samples were vortexed, sonicated for 30 s and centrifuged (5 min, 25000 *g*). The supernatant (190 µl) was collected and MDA was derivatized by incubating for 10 min with 19 µl of DNPH (5 mM in TCA 20%) at room temperature. The reaction was then stopped by adding 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 (2 times 240 µl) were combined and evaporated in a centrifugal evaporator (Labconco) at 25 °C for ca. 60 min. The 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 reconstituted solution represented a 10-fold dilution of the initial sample concentration.

For extraction of total MDA (free + bound MDA fractions) 10 µl of plasma was mixed with 40 µl of NaOH 1.25 M in a microcentrifuge tube and heated for 30 min at 60 °C. Samples were cooled down in the fridge for 10 min and 10 µl of IS and 150 µl of TCA 20% were added. The rest of the procedure was performed as described for extraction of free MDA.

UHPLC-PDA-HRMS conditions

The analysis of MDA-DNPH was performed on an Acquity UPLC^{TM} 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 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 and 450 °C, respectively, cone gas flow 20 l/h. Exact mass measurements (< 2 ppm) were ensured by infusing a 500 ng/ml solution of leucine-enkephalin at 15 µl/min 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 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).

Isolation of MDA-DNPH

Twenty mg of MDA salt and 40 mg of DNPH were dissolved into 100 ml of TCA 20%. The mixture was stirred for 30 min and partitioned twice with 100 ml hexane. Hexane phases were combined, evaporated and reconstituted in 1.2 ml of MeOH 60%. The solution was filtered through a 0.22 µm hydrophilic PTFE syringe filter and split in two equal parts. A volume of 500 µl was injected twice on a semi-preparative XTerra MS C18 (150x19 mm i.d., 5 µm particle size). The semi-preparative HPLC system was composed of a 1525 EF pump (Waters), a manual Rheodyne valve fitted with a 500 µl loop, and a dual wavelength 2487 UV detector (Waters) equipped a semi-preparative UV cell (path length 3 mm). Gradient conditions using the same mobile phases as for analytical separation (see above) were as follows: 2% B for 5 min, 2-60 % B from 5-63 min, 60-100 % B from 63-65 min, holding at 100% B for 7 min. The flow rate was 5 ml/min. Two wavelengths at 305 and 358 nm were monitored. Fractions were collected every minute in 9 ml glass tubes using a FC203B fraction collector (Gilson). Fractions containing most of the MDA-DNPH, which eluted between 57.5-59.5 min were combined and evaporated by centrifugal evaporation followed by lyophilisation of the remaining water. Under these conditions, 2.00 mg of pure MDA-DNPH was obtained. The purity (> 95%) was controlled by UV, MS and NMR.

Method validation

MDA-DNPH was quantified by internal standardization using standard curves prepared from a stock solution of MDA salt at 3.19 mM (1 mg/ml) in milli-Q water. The stock solution was further diluted in milli-Q water to obtain calibration solutions at 64.27, 16.06, 8.03, 3.21, 0.80 and 0.32 µM. Standard solutions were subjected to the same conditions as the plasma samples (see extraction process above). A linear regression weighted by 1/x was applied.

An assay validation was carried out using human plasma regarding selectivity, linearity, lowest limits of detection (LLOD) and quantification (LLOQ), intra-day and inter-day precisions, accuracy, recovery, matrix effects, process efficiency, and stability. Selectivity was determined by analysing blank samples and plasma samples spiked with a known concentration of MDA-DNPH (1.28 µM) upon reconstitution. Linearity was assessed by using 10 standard solutions of MDA-DNPH from 1 ng/ml to 5000 ng/ml in MeOH 50%. LLOD and LLOQ were defined as the concentrations that produced signals 3 and 10 times higher than the noise level, respectively. Precision, accuracy, recovery, matrix effects and process efficiency were all determined at low and high concentrations, using the "free MDA" and the "total MDA" sample preparations, respectively. Spike solutions were obtained from dilutions in milli-Q water of the MDA stock solution to yield concentrations of 240 nM (similar to physiological levels of plasma free MDA) and 12.8 µM (similar to physiological levels of plasma total MDA). For evaluation of intra-day and inter-day precisions, a plasma sample was spiked, extracted and analysed over 3 consecutive days (5 replicates per day). Precision was expressed in percentage of relative standard deviation (%RSD). Accuracy was determined by comparing non-spiked with spiked plasma samples and expressed as percentage of deviation between nominal and measured concentrations (n = 5). Extraction recovery was determined by comparing samples spiked with equivalent amounts of MDA salt and MDA-DNPH before and after extraction, respectively and the corresponding ratio calculated and expressed in % (n = 5). Matrix effects were evaluated by comparing plasma samples spiked after extraction with MDA-DNPH with a stock solution of identical concentration (n = 5). Process efficiency was calculated as the product of recovery and matrix effects. Stability of samples stored over time was assessed by comparing freshly collected samples with aliquots of the same samples stored for 1 month at -80 °C before analysis was performed.

2.3. RESULTS

Sample preparation

As an alternative to the frequently used but controversial TBA assay, derivatization of MDA with DNPH at room temperature has been shown to yield stable MDA-DNPH derivatives which can be monitored by both UV and MS detections (Manini et al., 2010; Pilz et al., 2000). However, to our knowledge, it has never been investigated whether the MDA salt is able to react quantitatively and exhaustively with DNPH (Figure 2.1). We therefore first synthesized an MDA-DNPH standard according to conditions adapted from previous reports (Pilz et al., 2000), and further isolated it by semi-preparative HPLC to yield a pure standard. All subsequent experiments were then performed relatively to the MDA-DNPH standard to determine the different yields of derivatization and extraction. At this stage of the process, UV detection at 358 and 305 nm for DNPH and MDA-DNPH, respectively, was employed. We found that an incubation time of 10 min at room temperature was sufficient for a complete reaction between the MDA salt and DNPH (added in excess). To eliminate the excess DNPH before analysis, liquid-liquid extraction (LLE) of the aqueous reaction mixture with different organic solvents was evaluated (Figure 2.2). While relatively polar solvents such as EtOAc or MTBE extracted MDA-DNPH efficiently, they were not able to selectively remove DNPH. In contrast, non-polar solvents such as hexane and cyclohexane totally prevented the extraction of DNPH, but yielded significantly lower amounts of MDA-DNPH. Finally, a 1:1 mixture of toluene:cyclohexane was found to provide excellent recovery of MDA-DNPH and complete removal of the DNPH in excess. We also tested solid phase extraction (SPE) as a potential alternative to LLE. We found that 100% wettable polymeric-based sorbents such as Oasis HLB were able to irreversibly retain low amounts of DNPH while still released MDA-DNPH under

strong acidic conditions (TCA 5%). However, they exhibited too low loading capacity to afford efficient removal of DNPH at the concentration employed for derivatization (data not shown).

Free and total MDA were extracted using acidic precipitation and alkaline hydrolysis followed by acidic precipitation, respectively. For the free fraction, TCA 20% was found to efficiently precipitate proteins while keeping MDA intact for further derivatization. For the total MDA, hydrolysis performed in 1M NaOH at 60 °C for 30 min gave the best results, in accordance with Pilz and collaborators (2000). To determine whether MDA was bound to components other than proteins, plasma samples were split into three equal parts and subjected to either i) "normal" protocol involving alkaline hydrolysis of bound MDA from intact plasma, or ii) lipid extraction of plasma using a mixture of H_2O:chloroform:MeOH (1:2:1, v/v) followed by alkaline hydrolysis, or iii) protein precipitation with acetonitrile followed by resolubilisation of the pellet in NaOH 1M and alkaline hydrolysis. Results showed that proteins and lipids account for more than 90% and less than 10% of bound MDA, respectively, which confirms previous findings (Cighetti et al., 1999; Hong et al., 2000; Pilz et al., 2000; Steghens et al., 2001).

Optimization of UHPLC-HRMS conditions

To our knowledge, no study has reportedly used UHPLC coupled to HRMS for measuring MDA in plasma samples. In the present study, we developed a very rapid gradient on an ethylene bridged hybrid C18 column which enabled the selective detection of MDA-DNPH as a sharp and single peak at a retention time (RT) of 2.87 min (Figure 2.3), well after the solvent peak (RT 0.25 min) and the DNPH peak (RT 2.35 min). Total run time including re-equilibration did not exceed 6.0 min. Detection was achieved by QTOFMS in positive electrospray mode and yielded an intense $(M+H)^+$ ion at *m/z* 235.0464 corresponding to the

molecular formula $C_9H_7N_4O_4$ (error 0.2 mDa; calculated mass 235.0462 Da) (Figure 2.3). Additional HRMS/MS data confirmed the identity of MDA-DNPH based on fragments at *m/z* 189.0539 ($C_9H_7N_3O_2$), 159.0558 ($C_9H_7N_2O$), 143.0605 ($C_9H_7N_2$), and 116.0501 (C_8H_6N) (Figure 2.3). To obtain maximal sensitivity for MDA-DNPH, several parameters of the mass spectrometer including capillary voltage (2.8 kV), cone voltage (25 V), desolvation gas flow (800 l/h) and temperature (450 °C) were optimized. The signal was further improved by using the Enhanced Duty Cycle (EDC) mode, which led to a 2-fold increase in signal-to-noise ratio as compared to the "normal" mode. To construct extracted ion chromatograms, a mass extraction window of 0.012 Da centred on *m/z* 235.0462 was selected. Using optimized conditions, a 2.5 µl injection of a 6.5 nM MDA-DNPH solution gave a signal-to-noise ratio equal to 10. Based on values from the literature for MDA levels, we anticipated that this level of sensitivity should be sufficient for quantification of both free and total MDA in plasma.

Internal standardization

To overcome unwanted variation caused by reactions with nucleophilic species during derivatization, sample preparation loss and matrix effects, D_2-MDA was prepared from D_2-TEP using a simple acidic hydrolysis step (Weber et al., 2004) and used as internal standard. We found that equivalent molar amounts of MDA salt and freshly produced D_2-MDA provided similar signals after derivatization using UHPLC-HRMS. The peak for D_2-MDA-DNPH eluted at 2.86 min and the mass spectrum was dominated by an intense $(M+H)^+$ ion at *m/z* 237.0594 matching with the molecular formula $C_9H_5D_2N_4O_4$ (error 0.6 mDa; calculated mass 237.0588; Figure 2.4). Because of the low mass difference (2 Da) between the unlabelled and labelled molecules, the M+2 isotope of MDA-DNPH could possibly interfere with the IS peak at high MDA concentrations. While this should not alter the method's precision and accuracy as both samples and calibration standards would be equally affected, it may, however, degrade the

linearity of the calibration curve. To ensure that the relative contribution of the M+2 isotope of MDA-DNPH to the D_2-MDA-DNPH peak would remain negligible (< 2% for the highest calibration standard at 64.27 µM), a high IS concentration of 30 µM was selected. At this concentration no peak was detected in the mass spectrum for *m/z* 235.0462 (i.e. the protonated ion of MDA-DNPH), demonstrating the high purity of D_2-TEP and, thereby, D_2-MDA-DNPH (Figure 2.4). D_2-MDA was therefore considered a suitable IS for MDA quantification.

Method validation

Validation was performed on 10 µl aliquots of human plasma for both free and total MDA, i.e. using acidic precipitation and alkaline hydrolysis followed by acidic precipitation, respectively. The method was found to be highly selective for MDA-DNPH as there were no interfering peaks for both free and total MDA in plasma samples (Figure 2.5 A,B). A blank sample prepared under identical conditions gave no detectable signal (Figure 2.5 C). Linearity was achieved from 1 ng/ml (4.3 nM) to 5000 ng/ml (21.4 µM) (Supplemental Table S2., Supplemental Figure S2.3). All points of the linear regressions obtained from a calibration curve built from the MDA salt subjected to the entire sample preparation process fell within +/- 15% of the nominal value (Supplemental Table S2.3; Supplemental Figure S2.4). The instrumental LLOD and LLOQ obtained from the injection of standard solutions were 2.1 nM and 6.5 nM, respectively. The method LLOD and LLOQ, which take into account the dilution factor and recovery of the sample preparation as well as the matrix effects in the MS source, were 32 nM and 100 nM, respectively. The results for precision, accuracy, recovery, matrix effects and process efficiency are presented in Table 2.. In brief, precision was excellent at high MDA concentrations (12.8 µM, within the range of expected total physiological levels) and good at low concentrations (240 nM, within the range of expected free physiological levels). At both levels of concentrations, accuracy fell within acceptable ranges (80-120%). Extraction

recovery was ca. 70%, due to losses during analyte extraction. Matrix effects were negligible and did not impact the MS response. The overall process efficiency was about 65-70%. Plasma samples stored for one month at -80 °C did not show any significant deviation from freshly collected samples (t-test: $n = 5$; $p = 0.97$).

Taken together, these results demonstrate that the developed method is sensitive, precise and accurate enough to measure both free and total MDA from minute volumes of plasma samples.

Analysis of plasma samples from different species

To illustrate the applicability of the developed method, free and total MDA levels were measured in plasma samples of humans as well as other vertebrates from various families and classes. In general, both free and total MDA levels were relatively similar among species at the exception of total MDA in house sparrow, which was much higher than in other species (Figure 2.6). Free MDA concentrations ranged from 89 to 195 nM and total MDA concentrations from 6.2 to 26.0 µM. Average values for free and total MDA in human plasma were 120 nM (SD 36.26) and 6.7 µM (SD 0.46), respectively. MDA bound to proteins through the formation of Schiff base adducts (calculated as total minus free MDA), represented about 98% of the total MDA in humans, and 97-99% in other species.

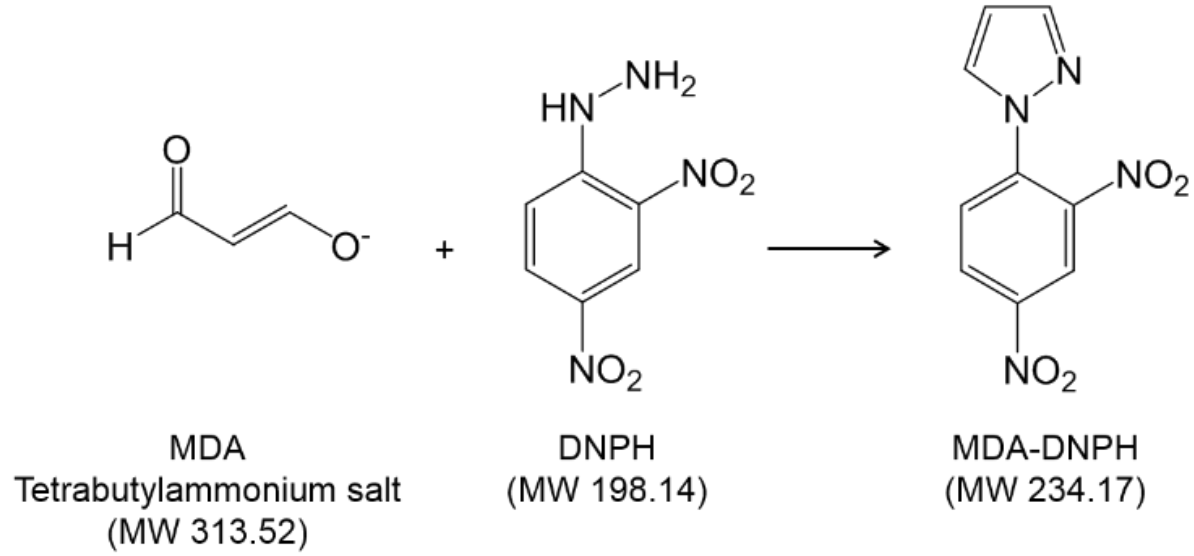


Figure 2.1 - Derivatization of MDA with DNPH yielding a stable MDA-DNPH derivative. The reaction was performed using the tetrabutylammonium salt of MDA. MDA: malondialdehyde; DNPH: 2,4-dinitrophenylhydrazine; MW: molecular weight.

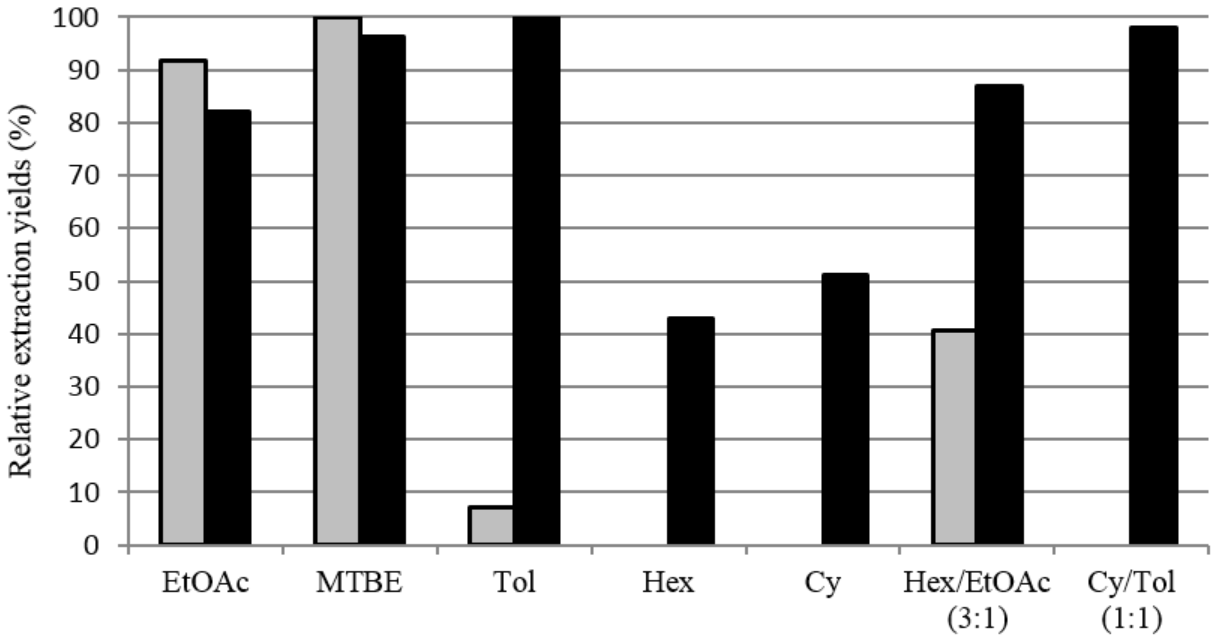


Figure 2.2 - Relative extraction yields for DNPH (grey bars) and MDA-DNPH (black bars) using various organic solvents and solvent mixtures. Extraction of the aqueous reaction mixture was performed twice with 250 µL of the mentioned solvents. Data are mean of 2-4 replicates. EtOAc: ethyl acetate; MTBE: methyltertbutylether; Tol: toluene; Hex: hexane; Cy: cyclohexane.

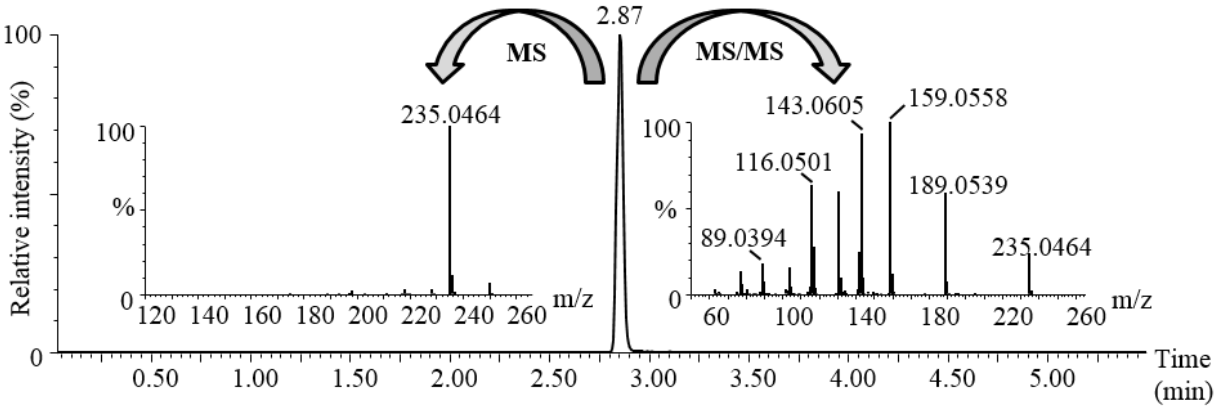


Figure 2.3 - Base peak intensity (BPI) chromatogram and high resolution mass spectra of MDA-DNPH. Data were obtained from an MDA-DNPH standard (500 ng/ml) on a QTOF mass spectrometer at a resolution of ca. 20000 (FWHM at m/z 500). MS spectrum acquired at low collision energy (4 eV) yielded the $(M+H)^+$ ion. MS/MS spectrum acquired at high collision energy (10-35 eV ramp) yielded typical fragments. For fragment annotation, refer to the text.

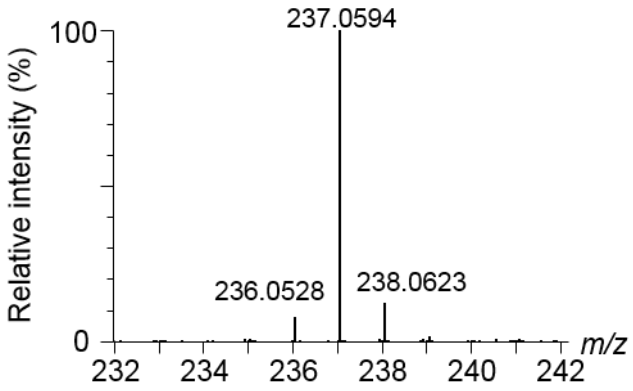


Figure 2.4 - High resolution mass spectrum obtained for D_2-MDA-DNPH at a final concentration of 3 μM. No detectable peak at *m/z* 235.0462 was observed.

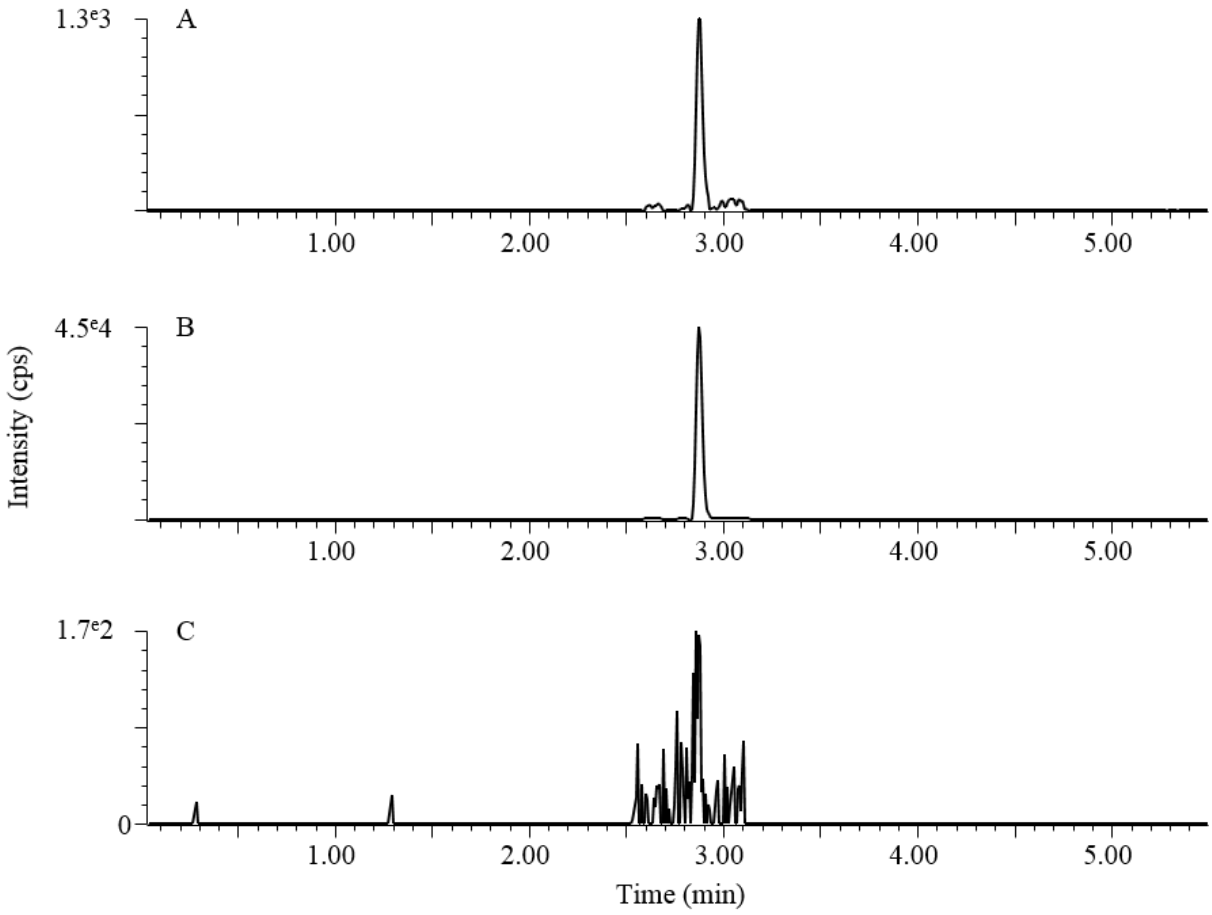


Figure 2.5 - Typical chromatograms obtained for plasma and blank samples. Extracted ion chromatograms centred on m/z 235.0462 using a mass extraction window of 0.012 Da are displayed. A: Representative human plasma sample, free MDA fraction; B: Representative human plasma sample, total MDA fraction; C: Blank sample.

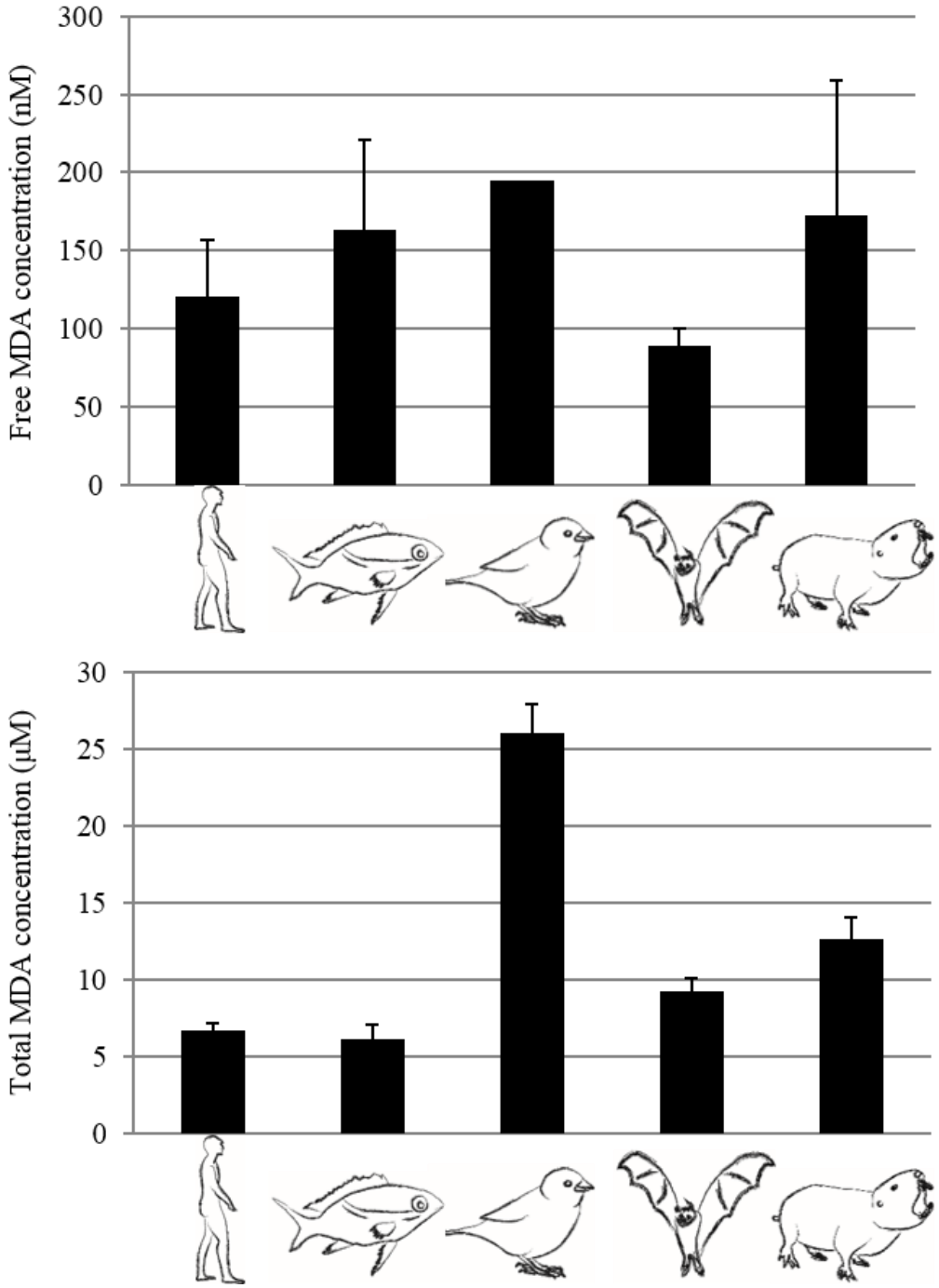


Figure 2.6 - Levels of free (top) and total (bottom) MDA in plasma of human and other vertebrates. Studied animal species were (from left to right) *Homo sapiens* (human), *Scolopsis bilineatus* (fish), *Passer domesticus* (bird), *Carollia perspicillata* (bat) and *Fukomys damarensis* (rodent). Data are from 3 replicates + SD, except for free MDA in house sparrow for which only one replicate was available.

Table 2.1 - Assay validation parameters of MDA quantification in human plasma. RSD_1: intraday precision (n = 5); RSD_2: interday precision over three consecutive days (n = 15).

Sample preparation	Concentration (μM)	Precision		Accuracy (%)	Recovery (%)	Matrix effects (%)	Process efficiency (%)
		RSD_1 (%)	RSD_2 (%)				
Free MDA	0.24	12.8	24.9	101	67	104	69
Total MDA	12.8	2.9	3.0	107	70	91	64

2.4. DISCUSSION

This study reports a novel method based on UHPLC-HRMS for the quantification of MDA after derivatization with DNPH. Both free and total MDA can be measured from minute amounts of plasma thanks to the high sensitivity of HRMS. To measure free levels at least 10 µl are needed, while for total MDA volumes as low as 0.5-1 µl would be sufficient to reliably quantify MDA. In comparison, Pilz and colleagues (2000) attempted to measure free MDA as DNPH derivative by HPLC-UV starting from 1 ml of plasma, but levels were below detection limits. An MS-based approach thus represents a real advantage over UV- or fluorescence-based methods, in particular when sample volumes are restricted (e.g. from new-borns, small animals, etc.). Another advantage of HRMS is its high selectivity due to the ability to set very narrow mass extraction windows (0.01-0.02 Da for QTOFMS) (Glauser et al., 2016): plasma samples provided particularly clean chromatograms with a single peak corresponding to MDA-DNPH and low background noise (Figure 2.5). The total chromatographic time was only 6 min, which increased throughput without compromising selectivity and sensitivity.

A key aspect of MDA analysis in general is the possible presence of artifactually produced MDA (Agarwal and Chase, 2002; Lykkesfeldt, 2001), which can be revealed by preparing blank (water) samples. For instance, in our laboratory, the TBA assay usually generates amounts of MDA up to 500 nM in blank matrices subjected to the extraction process, precluding the analysis of free MDA and complicating the determination of total MDA for small plasma volumes. Using the described method based on derivatization with DNPH, we were able to keep MDA levels in blank samples below detection limits (Figure 2.5). Nevertheless, it should be noted that very small amounts of MDA may still be occasionally present in blank samples and a series of measures should be taken to avoid interference, particularly during the analysis of free MDA in plasma samples. We determined that

contamination of blanks occurs only during LLE with the organic solvent mixture, therefore high quality solvents should be used and solutions regularly re-prepared. Moreover, the unwanted reaction of residual MDA with DNPH preferentially occurs at low pH, thus increasing the pH to 9-10 before solvent partitioning reduces the risk of contamination. It should be noted that the use of phase partitioning is not absolutely essential as plasma samples prepared without LLE did not generate more background noise or matrix effects during detection. Yet, excess DNPH over an extended period can be harmful to both HPLC and MS systems, as it may crystallize in the injector and the source, respectively (L. Lachat, personal observation). We found that a 1:1 (v/v) mixture of cyclohexane and toluene efficiently removes over 99% of unbound DNPH, while providing very good recovery of MDA-DNPH. Moreover, it enables the concomitant removal of TCA and other matrix compounds which might potentially affect the robustness of the method over time. We thus highly recommend the use of phase partitioning.

Previous methods for MDA analysis have either relied on external standardization (i.e. no internal standard) (Mateos et al., 2005; Pilz et al., 2000), or internal standardization using methyl-MDA (Claeson et al., 2001; Sim et al., 2003), or isotopically labelled MDA (Cighetti et al., 1999; Schmid-Siegert et al., 2016; Yeo et al., 1994). Isotopically labelled IS represent the gold standards in LC-MS quantification of complex matrices since they show identical or near identical behaviour during sample extraction, chromatography and MS ionisation but display different masses from their unlabelled isotopomers, which enables their separation in the MS analyser (Thevenet et al., 2017). In the present study, we used D_2-MDA as IS and showed its suitability for the quantification of MDA by UHPLC-HRMS. A high IS concentration (30 µM) was used to avoid the relative contribution of the M+2 isotope of MDA-DNPH to the IS peak becoming substantial and possibly affecting the linearity and accuracy of

the calibration curve. It should be noted that the M+2 isotope of MDA-DNPH is mostly caused by the presence of $^{13}C_2$ and that it could theoretically be separated from D_2-MDA-DNPH using high resolution mass spectrometry since both ions have different exact masses (*m/z* 237.0588 and 237.0529, respectively). However, complete resolution of both peaks would require a much higher resolution (> 80'000) than that afforded by our mass spectrometer (ca. 17000 at *m/z* 235). In this respect, Orbitrap-based systems may represent an attractive alternative to QTOF-based systems for MDA quantification in the future.

To our knowledge, this is the first study to assess MDA levels across different animal species and classes using the same method. The average levels found for free (120 nM) and total (6.7 µM) MDA in human plasma are within the range of previously found values (Carbonneau et al., 1991; Cighetti et al., 1999; Pilz et al., 2000; Sim et al., 2003; Steghens et al., 2001), although different methods were shown to yield quite different results (Del Rio et al., 2005; Kadiiska et al., 2005; Pilz et al., 2000; Yeo et al., 1994). These discrepancies were mainly observed for bound MDA and are likely due to different hydrolytic conditions during sample preparation (Sobsey et al., 2016). MDA levels in other species differed from those in humans, although they remained in a similar range. Yet, total MDA levels in the house sparrow constituted an exception, as they were about 4 times higher than in humans. These particularly high concentrations are possibly species but not class related as Pérez-Rodríguez and colleagues (2015) found large variations in MDA levels between adults of three different bird species.

Here we present a highly sensitive and selective method to measure free and total MDA with good precision and accuracy, allowing the use of sample volumes that can be as small as 0.5-1 µl. We show that the method is applicable not only to human samples, but also to a variety of other vertebrate species, potentiating its use for comparative studies. Finally, although our method has been validated for plasma samples, we believe that small adaptations can be done

to allow measurement of free and total MDA in cell homogenate samples, such as red blood cells. Currently, the physiological meaning of the two forms of MDA remains elusive. Future research should therefore clarify this difference to allow accurate interpretation of free and total/bound MDA measurements.

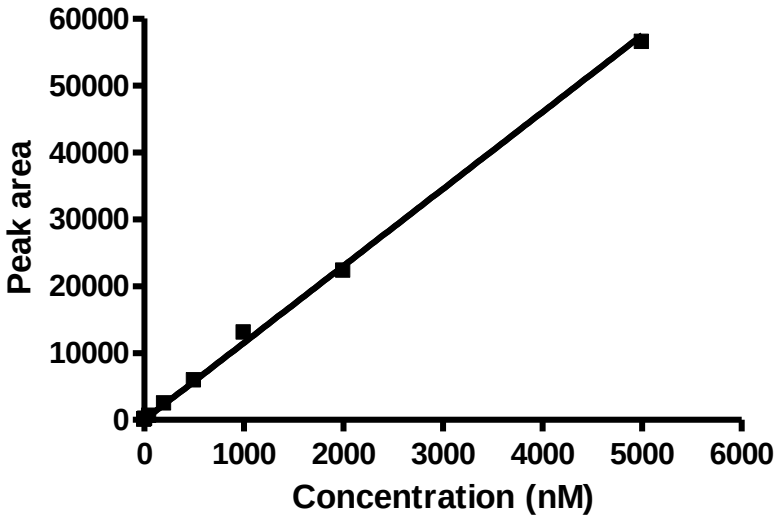
2.5. SUPPLEMENTAL MATERIAL

Supplemental Table S2.1 – Linearity assay using different concentrations of purified MDA-DNPH. Calibration equation was: y = 11.50x + 1.89, weighing 1/x, with r2 = 0.997.

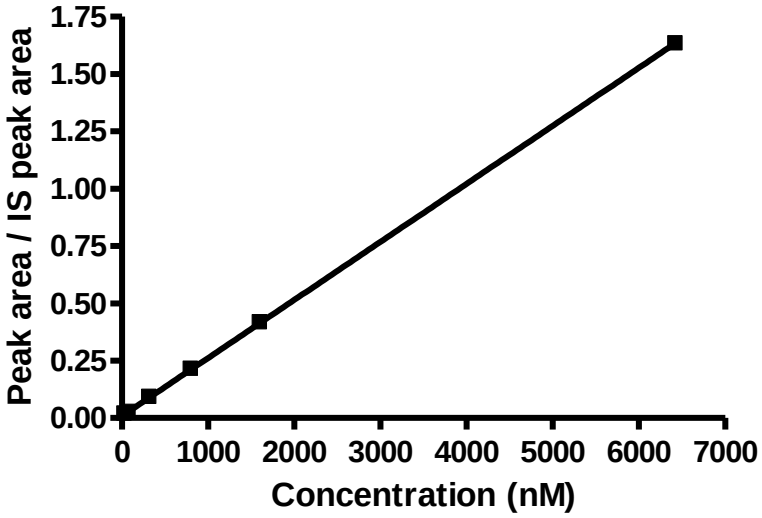
MDA-DNPH standards	Trace (m/z)	Predicted RT (min)	RT (min)	Back calculated concentration (ng/ml)	Standard concentration (ng/ml)	Accuracy (%)	Injection volume (µl)
Standard 1 ng/ml	235.0462	2.87	2.88	1.07	1	106.8	2.5
Standard 2.5 ng/ml	235.0462	2.87	2.88	2.55	2.5	102.1	2.5
Standard 5 ng/ml	235.0462	2.87	2.87	4.66	5	93.3	2.5
Standard 10 ng/ml	235.0462	2.87	2.87	10.23	10	102.3	2.5
Standard 50 ng/ml	235.0462	2.87	2.88	47.91	50	95.8	2.5
Standard 200 ng/ml	235.0462	2.87	2.87	211.86	200	105.9	2.5
Standard 500 ng/ml	235.0462	2.87	2.87	508.71	500	101.7	2.5
Standard 1000 ng/ml	235.0462	2.87	2.87	1134.25	1000	113.4	2.5
Standard 2000 ng/ml	235.0462	2.87	2.88	1938.33	2000	96.9	2.5
Standard 5000 ng/ml	235.0462	2.87	2.87	4908.94	5000	98.2	2.5

Supplemental Table S2.2 – Calibration assay using different concentrations of MDA salt with constant concentration (3000 nM) of D_2-MDA. Calibration equation was: y = 0.0253x + 0.0085, weighing: 1/x, with r^2 = 0.999.

MDA standards	Trace (*m/z*)	Predicted RT (min)	RT (min)	Back calculated concentration (nM)	Accuracy (%)	Injection volume (µl)
Standard 32 nM	235.0462	2.87	2.86	30.8	96.3	2.5
Standard 80 nM	235.0462	2.87	2.86	82.4	103.0	2.5
Standard 321 nM	235.0462	2.87	2.86	334.9	104.3	2.5
Standard 803 nM	235.0462	2.87	2.86	757.2	94.3	2.5
Standard 1606 nM	235.0462	2.87	2.86	1641.0	102.2	2.5
Standard 6427 nM	235.0462	2.87	2.86	6422.7	99.9	2.5



Supplemental Figure S2.3– Calibration curve obtained for the linearity assay using different concentrations of purified MDA-DNPH. Calibration equation was: y = 11.50x + 1.89, weighing 1/x, with r^2 = 0.997.



Supplemental Figure S2.4 – Calibration curve obtained using different concentrations of MDA salt. Calibration equation was: y = 0.0253x + 0.0085, weighing: 1/x, with r^2 = 0.999.

Chapter 3

Oxidative costs of cooperative behaviours in cooperatively breeding Damaraland mole-rats

ABSTRACT

Within cooperatively breeding societies, individuals adjust their cooperative contributions according to internal and external factors. The possibility that increased cooperative contributions are accompanied with physiological costs which could affect individuals' fitness has been largely overlooked. Oxidative stress, a state of imbalance between the formation of reactive oxygen species (ROS; inherent by-products of energy production) and antioxidant protection, may represent such a cost when cooperative behaviours are energetically demanding. Oxidative stress can lead to the accumulation of cellular damages, which may compromise survival and reproduction and thus mediate the trade-off between these competing life-history traits (i.e. between somatic and germline functions). Here, we provide the first experimental evidence that increases in cooperative contributions induce oxidative stress in somatic (erythrocytes) and germline (ejaculates) tissues, in a cooperatively breeding rodent, the Damaraland mole-rat (*Fukomys damarensis*). When cooperative contributions were experimentally doubled, erythrocytes showed increased cellular oxidation (oxidized/reduced glutathione), but no changes in the antioxidant superoxide dismutase (SOD) or in oxidative damage to lipids (malondialdehyde, MDA). The protective increase of SOD activity in the ejaculates of male helpers did not prevent the accumulation of MDA, suggesting that sperm quality may be negatively affected by increased cooperative contributions, and that male helpers may favour the maintenance of their somatic tissues, i.e. survival, over reproduction. Our results support the hypothesis that increases in energetically demanding cooperative contributions could ultimately affect individuals' fitness through oxidative stress.

3.1. INTRODUCTION

Individual variation in cooperative behaviours is frequent within animal societies where individuals behave to the benefit of others (Clutton-Brock, 2016; Koenig and Dickinson, 2016; West et al., 2007b). In cooperative breeders, where helpers postpone or forfeit reproduction and assist in raising the offspring of breeders and/or defending and maintaining their territory (Clutton-Brock, 2016; Koenig and Dickinson, 2016), individuals adjust their cooperative contributions according to environmental and individual cues (Cant and Field, 2001; Clutton-Brock et al., 2001a; Nichols et al., 2012a; Russell et al., 2003b; Sanderson et al., 2014). Although these adjustments seem to be adaptive, the possibility that increased cooperative contributions may generate physiological costs, which can ultimately affect an individual's fitness, has been largely overlooked (Heinsohn and Legge, 1999).

In cooperative breeders, several types of cooperative behaviours have been shown to be energetically demanding [e.g.: nest building (Rowley, 1978), incubation (Heinsohn and Cockburn, 1994); babysitting (Clutton-Brock et al., 1998), feeding young (Anava et al., 2001; Canestrari et al., 2007; Russell et al., 2003b), maintaining or expanding territories (Grantner and Taborsky, 1998; Lovegrove, 1989)]. Adjustments in cooperative contributions must thus be linked with appropriate tuning of metabolism and energy production (Lovegrove, 1989). Oxidative stress, which is an imbalance between the formation of reactive oxygen species (ROS; inevitable by-products of energy production) and antioxidant protection in favour of the former (Sies, 1985), may represent a physiological cost of cooperative behaviours, susceptible of affecting lifetime fitness. Under oxidative stress, important cellular components, such as DNA, proteins and lipids are more likely to be oxidized by ROS, and oxidative damage may accumulate, potentially disrupting cell and tissue functioning, thereby compromising survival and reproduction (Davies et al., 1982; Finkel and Holbrook, 2000; Harshman and Zera, 2007;

Monaghan et al., 2009). Intense physical activities, associated with increased energy production, have been shown to induce oxidative stress, as measured by increasing ROS formation, and greater accumulation of oxidative damage (Bejma and Ji, 1999; Davies et al., 1982; Leeuwenburgh and Heinecke, 2001; Radak et al., 2008). In cooperatively breeding birds, recent correlative work has suggested that individuals differing in their social status and cooperative contributions may be differentially affected by oxidative stress before (van de Crommenacker et al., 2011) and during (Cram et al., 2015b, 2014) the breeding season. Yet, it remains unclear whether this could be a direct consequence of differences in energetically demanding cooperative contributions. Experimental work is therefore needed to shed some light on the possibility that oxidative stress represents a physiological cost of cooperative behaviours.

Upon increased cooperative contributions, individuals may differ in the strategy used to circumvent the risk posed by a higher production of ROS. Many life-history traits are involved in trade-offs, and the most studied one is that between survival and reproduction, i.e. between somatic and germline functions (Metcalfe and Alonso-Alvarez, 2010; Stearns, 1976; Velando et al., 2008). Noticeably, the germline, and particularly sperm cells, are highly sensitive to ROS due to a high proportion of polyunsaturated fatty acids (especially vulnerable to ROS) in their membranes, and sensitive DNA (Agarwal et al., 2008; Tremellen, 2008). Hence, competing demands between somatic and germline functions in terms of protection against ROS may represent a physiological mechanism underlying this trade-off (Dowling and Simmons, 2009). Optimal investment in one or the other function is dependent on intrinsic and extrinsic conditions, and plastic adjustments at any given life-history stage are expected to maximise individual fitness. For instance, when extrinsic mortality (e.g. predation) is high, the benefits of investing in self-maintenance might never be enjoyed (Cichoń, 1997) and individuals may

benefit from investing in reproduction (Drenos and Kirkwood, 2005; Teriokhin, 1998). Conversely, when current reproductive opportunities are uncertain, individuals may benefit from increasing resource allocation towards survival, in order to preserve the possibility of future reproduction (Drenos and Kirkwood, 2005; Perrin and Sibly, 1993). Strikingly, whether differences in cooperative contributions affect the trade-off between somatic and germline functions remains untested.

Here, we experimentally tested whether increases in energetically demanding cooperative contributions affect (i) oxidative balance in both males and females and (ii) the trade-off between somatic and germline tissues in males of Damaraland mole-rats (*Fukomys damarensis*). Damaraland mole-rats are cooperative breeders with only one female and usually one male monopolizing reproduction (Bennett and Jarvis, 1988; Burland et al., 2004, 2002), and helpers are unlikely to breed (Bennett and Faulkes, 2000; Bennett and Jarvis, 1988; Burland et al., 2004; Jarvis and Bennett, 1993). Damaraland mole-rats are subterranean rodents, dwelling in niches where burrowing activities, to expand the tunnel system in search for food which is then made available to all colony members (Jarvis et al., 1998), are the most pronounced and energetically demanding form of cooperation (Lovegrove, 1989). All members of the colony contribute to burrowing activities, but large differences in cooperative contributions, within and between individuals, are evident (Bennett, 1990; Bennett and Jarvis, 1988; Zöttl et al., 2016).

In order to increase cooperative contributions, we increased the daily amount of sand provided to captive colonies. Oxidative balance was assessed in one somatic (erythrocytes) and one germline tissue (ejaculates). Oxidative balance is a complex system that integrates ROS formation, antioxidant defences and oxidative damage to various biomolecules (Monaghan et al., 2009). Therefore, and as encouraged by Hõrak and Cohen (2010), we described oxidative

balance through: (i) the activity of the antioxidant superoxide dismutase (SOD), a key enzymatic antioxidant which catalyses the dismutation of superoxide anions (the first ROS produced during energy production) into hydrogen peroxide (a less harmful ROS) and water (Halliwell and Gutteridge, 2007); (ii) the balance between oxidized and reduced glutathione. Reduced glutathione (GSH) is a tripeptide, which reduces hydrogen peroxide to form oxidized glutathione (GSSG) and water (Halliwell and Gutteridge, 2007). The molar ratio between its two forms (GSSG/GSH) provides a useful measure of cellular oxidation and ROS formation (Jones, 2006), and changes of this ratio are suggestive of disturbances of the oxidative balance (Flohé, 2013). And (iii) the concentration of malondialdehyde (MDA), a marker of oxidative damage to lipids (Halliwell and Gutteridge, 2007).

Owing to the energetically demanding nature of cooperative behaviours and based on the assumption that increasing energetic demands result in increased ROS formation, we predicted that antioxidant protection (SOD) and cellular oxidation (GSSG/GSH) would be increased to compensate the increases in ROS. We expected stable or increased levels of oxidative damage (MDA) to be related to a good or poor antioxidant protection, respectively. We also predicted that individuals with lower cellular oxidation levels, better antioxidant protection, and/or lower oxidative damage levels before the experimental manipulation could afford a greater increase in cooperative contributions (Meade and Hatchwell, 2010; Russell et al., 2003b; van de Crommenacker et al., 2011). Finally, we tested whether increased cooperative contributions affect the trade-off between somatic and germline tissues and predicted investment to be biased towards survival (somatic tissue) in subordinate males.

3.2. METHODS

Study animals and husbandry

We used six originally wild-caught colonies of Damaraland mole-rats (79 individuals: 36 females, 43 males; group size range = 9 – 20 individuals; mean = 13.2 individuals), that were maintained in captivity since early 2013 at the Kuruman River Reserve in the Northern Cape, South Africa. Each colony included one female and one male breeders. Female breeders were readily identified due to their elongated nipples, perforated vagina, and were the only ones giving birth since the colonies were captured. Male breeders were defined as those with the highest number of copulations with the breeding female of their colony, recorded through scan observations (see below) conducted for other purposes, in the 4 months preceding the end of the experiment.

Each colony was individually housed in standardized artificial tunnel systems with a total tunnel length of approximately 18 m. All individuals were marked with hair dye for visual recognition and the top of the tunnels was transparent, allowing behavioural observations. Each tunnel system contained a nesting box, food storing areas, and a large plastic waste box where animals could discard unwanted material. Three vertical pipes, hereafter referred as sand dispensers, were placed close to the extremities of the tunnel systems to provide sand to the colonies. Every morning, tunnels and waste boxes were cleaned and nest material (small pieces of paper towel) was provided to the animals, away from the nest. Animals were fed *ad libitum* twice per day with a diet of sweet potatoes and cucumbers. Food items were provided behind the sand dispensers to encourage burrowing activities and the sand dispensers were filled with clean sand on the same occasion. The temperature in the animal rooms was controlled with air-conditioning systems set to 23℃ during the whole experiment.

Experimental design

To increase the expression of burrowing behaviours, the sand provisioning regime was experimentally manipulated. Between October and December 2015, colonies were subjected to a control and a sand treatment, each lasting 8 days and separated by a resting period of 8 days (Figure 3.1). The sequence of the treatments was balanced between colonies, three starting with the control and the other three with the sand treatment. During the control treatment, sand dispensers were filled twice every day (7:00 and 19:00), following the non-experimental regime (see above). During the sand treatment, the sand dispensers were re-filled 12 times per day (i.e. once every hour from 7:00 to 19:00), even if they had only been partly emptied by the animals. This resulted in the daily provision of 16 kg of sand in the control treatment and of 92 kg (range: 70.4 – 112 kg) of sand in the sand treatment. To control for potential disturbances to the animals during the sand treatment, the sand dispensers were touched every hour during the control treatment. The amounts of food and nest material were standardized and kept constant across the two treatments. Small pieces of food, which the animals could transport to storage areas, were provided every morning.

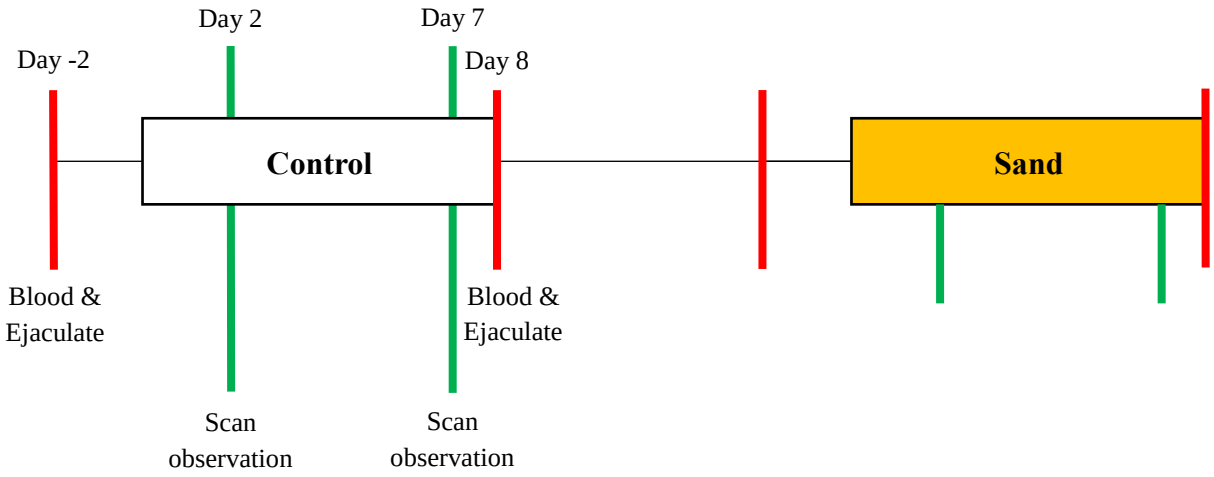


Figure 3.1 - Timeline of the experimental design. Three of the colonies used in this study started with the Control treatment, as shown in the figure, while the other three started with the Sand treatment (opposite sequence to the figure). Blood and ejaculate samples were collected two days before the start and on the last day of each treatment week (red bars). Scan observations were carried out for 12 hours on Day 2 and Day 7 of each treatment week (green bars). Refer to the text for more details.

Behavioural observations

Behavioural observations were carried out on Day 2 and Day 7 of each treatment (Figure 3.1). A scan protocol (Altmann, 1974) was used to record the behaviour of each colony member every 4 minutes, from a list of 17 pre-defined behaviours (Table 3.1). Scan observations lasted 12 hours, resulting in the collection of 180 behavioural data points for each group member. All observations were carried out by two observers, who equally alternated observation shifts every 2 to 4 hours.

Table 3.1 - Ethogram used for scan observations. The left side of the table shows how behaviours were grouped to form variables used in the statistical analyses.

Variables		Behaviour	Description
Activity	Food Carry	Food carry	Transporting food pieces
	Nest Build	Nest build	Preparing nest material for transport and transporting nest material
	Burrow	Dig	Excavating sand using incisors and front paws
		Sweep	Moving sand backwards using hind legs
		Kick	Compacting sand against tunnel using nose or hind legs
		Locomotion work	Moving between bouts of the above behaviours
	Non-Cooperation	Locomotion	Moving unrelated with cooperative behaviours
		Sniff	Investigating objects with the nose
		Eat	Ingesting food
		Self-Groom	Hygiene maintenance behaviours directed to the actor's body
		Social interaction	Any interaction with another individual
		Pump	Repetitive up and down movement of the body
		Other	Any behaviour that cannot be assigned to the described behaviours
		Gnaw	Chewing the plastic tunnels with incisors
Rest		Rest	Sleeping in the nest or tunnels
		Huddle	Resting in the tunnels in physical contact with at least one individual

Sampling procedures

Blood and ejaculates were collected from anaesthetised subjects 2 days before the start (Day -2) and on the last day of each treatment (Day 8) to assess oxidative balance (Figure 3.1). Subjects were removed from their tunnel systems and anaesthetized with 5% isoflurane (Isofor, Safe Line Pharmaceuticals, Johannesburg, South Africa) mixed with oxygen gas. This was delivered by a vaporizer at a rate of 1 l/min, through a cone gently fitted on the animal's snout. Once fully sedated, the concentration of isoflurane was reduced to 2% until the completion of the sampling procedures. Only animals over 6 months old were blood sampled, and ejaculates were collected from males older than 8 months.

For blood collection, a vein from a pre-warmed foot was pierced using a sterile needle. Blood was collected into lithium-heparinized tubes and centrifuged immediately at room temperature for 5 minutes at 2000 *g*. Plasma was separated from the cell pellet and both fractions were kept at -80℃ until laboratory analysis.

Ejaculates were collected by electro-ejaculation (EE), following minor adjustments to the procedure described by Fasel et al. (2015). EE was always performed after blood sampling, with the subjects still under anaesthesia. The anus and genitalia were cleaned and dried, a rectal probe (12 mm diameter; fitted with two electrodes of 16 mm length) was coated with an aqueous lubricant and inserted about 20 mm deep in the rectum. The electrodes were always facing the abdomen to ensure stimulation of the nerves surrounding the prostate and the probe was considered well placed when erection and contractions of the legs were observed (Fasel et al., 2015). An audio amplifier (Lepai LP.808 Super Bass) transmitted to the probe an electrical stimulation designed with an audio software (Audacity 2.1.1). Stimulation series were repeated until ejaculation was achieved, with a maximum of 3 series conducted and allowing a 60 s

resting period between them. Each series consisted in 10 stimuli of increasing intensity (0.3 – 3 mA, 50 Hz). Each stimulus spanned 4 s followed by a 4 s break, at the exception of the last stimulus of each series, which lasted 10 s at the maximum intensity. Electrical current was continuously monitored with a current meter (Top Tronic T3601 Digital Multimeter) and never exceeded 3 mA. Ejaculates were diluted 1:3 (v/v) in phosphate buffer solution (PBS) and stored at -80℃ until laboratory analysis.

After sampling, subjects were removed from anaesthesia and weighed before being placed back in their original tunnel system, once full mobility was regained. Additional animal procedures included collection of urine samples on Day -4 and Day 3 of both treatments, for other purposes.

Quantification of oxidative stress markers

We used cellular homogenates for all analysis. Erythrocytes and ejaculates were homogenized for one minute at 30 Hz in PBS using 4 glass beads, sonicated in ice-cold bath for 5 min and centrifuged for 10 min at 11180 *g* and 4℃. The supernatant was collected and aliquots were made for each marker.

SOD activity was determined in erythrocyte and ejaculate homogenates, using a commercial kit (Cayman Chemical) after adjustment of the final dilution (1:2250 for erythrocyte and 1:300 for ejaculate homogenates). Intra- and inter-assay coefficients of variation (CVs) were 10.7% and 7.5%, respectively.

Concentrations of GSH and GSSG were determined in erythrocyte homogenates following minor adjustments to the description of Mora et al. (2016). Briefly, 4 µl of homogenate was

mixed with 5 µl of TCA 5%, 5 µl of internal standard glutathione ethyl ester (GSHee 40 µg/ml; Sigma Aldrich) and 36 µl of water. The mixture was vortexed, kept on ice for 5min and centrifuged for 14 min at 21913 *g* and 4℃. Finally, 10 µl of the supernatant was diluted in 790 µl of water and 150 µl of the mixture was transferred into an HPLC vial fitted with a 250 µl conical insert. HPLC analysis followed that described by Mora et al. (2016). Intra- and inter-assay CVs were assessed by adding a control homogenate sample at the beginning and end of each assay. These CVs were 3.3% and 15.5% for GSH, and 2.7% and 16.9% for GSSG, respectively.

Total MDA was quantified following Mendonça et al. (2017), applying minor modifications to enable the analysis of a reduced volume of erythrocyte and ejaculate homogenates. Briefly, 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. Quantification method followed that described in the original method (Mendonça et al., 2017). The intra-assay CV was 8.2% and the inter-assay CV was 8.1%.

Data preparation and statistical analysis

Data exploration followed the recommendations of Zuur et al. (2013, 2010). Unless otherwise stated, a mixed modelling approach was used to account for the non-independency of the data due to repeated measures on the same individuals. All analyses and their graphical representations were done using the software R (version 3.4.0) (Team, 2017). Generalized Linear Mixed-Effects Models (GLMM) and Linear Mixed-Effects Models (LMM) were fitted using the package *lme4* (Bates et al., 2015). Model validation was based on normality of residuals and homogeneity of residual variation, following Zuur et al. (2009) recommendations. Additionally, when overdispersion was present, an observation level random effect (OLRE) was added for correction (Zuur et al., 2013). P-values for fixed effects were extracted from ANOVA tables using the package *car* (Fox and Weisberg, 2011). When interactions between categorical variables were significant, we compared group pairs using least-squares means and associated standard errors followed by Tuckey adjustment of p-values, with the package *lsmeans* (Lenth, 2016). Statistical significance was set at $p < 0.05$.

Some behaviours were grouped based on common functionality to form meaningful behavioural variables used for statistical analysis (Table 3.1). The variable Active included all the behaviours recorded excluding Rest and Huddle. The variable Burrow included the behaviours that we aimed to manipulate: Dig, Sweep, Kick, and Locomotion work. The variables Nest Build and Food Carry included the behaviours with the same name (Table 3.1). The variable Non-Cooperation was the sum of all non-cooperative behaviours (Table 3.1).

Effect of treatment on behaviour and body condition

To assess the effect of treatment on behavioural responses, and unless otherwise stated, we used binomial GLMMs. All behavioural analysis excluded the two pups (2 months old) present in one of the six colonies used, resulting in 77 individuals per scan observation (30 female helpers body mass range: 76 g – 175 g; 6 female breeders body mass range: 121 g – 174 g; 35 male helpers body mass range: 59 g – 216 g; 6 male breeders body mass range: 138 g – 187g). GLMMs included the fixed effects treatment (control, sand), sex (female, male), breeding status (breeder, helper) and all possible interactions, to test for different responses to the treatment dependent on individuals' sex and breeding position. Body mass (scaled by treatment), day of treatment (Day 2, Day 7) and their interaction with treatment were added as fixed effects to test if the effect of treatment depended on individuals' body mass and if animals performed equally throughout each treatment week, respectively. Individual identity, nested within colony identity, was always included as a random effect.

We started by testing the effect of treatment on individuals' activity levels, by specifying Active as a response variable in an OLRE binomial GLMM. We then investigated whether the treatment effect differed between non-cooperative and cooperative activities. For this, we used Non-Cooperation and Burrow as response variables in two OLRE binomial GLMMs, while Nest Building and Food Carry were analysed using Wilcoxon signed rank tests with continuity corrections. To test whether treatment affected feeding, Eat was used as a response variable in a binomial GLMM.

To assess the effect of treatment on changes in body condition, we used the difference in body mass between the last day (Day 8) and the beginning (Day -2) of each week as a response variable in a LMM. Treatment, sex, breeding status and all possible interactions were included

as fixed factors. To control for undesired regression to the mean effects (heavier animals have more body mass to lose than lighter ones), body mass at the beginning of the week was included as a covariate (Kelly and Price, 2005). Individual identity, nested within colony identity, was included as a random effect. Due to some missing weights, this model comprised 73 individuals from 6 colonies, and all breeders were included.

Effect of treatment on oxidative stress in erythrocytes

To evaluate the effect of treatment on erythrocyte oxidative balance, the ln-transformed levels of SOD, GSSG/GSH, and MDA measured at the end of each treatment (Day 8) were used as response variables in LMMs. The ln-transformed levels of GSSG and GSH were analysed in two additional LMMs to assess the contribution of oxidized and reduced glutathione, respectively, to the variation in their molar ratio. In all models, treatment, sex, breeding status and all possible interactions as well as body bass (scaled by treatment) and its interaction with treatment were specified as fixed effects. The interaction between treatment and the respective marker level at the start of the week (Day -2) was added to test if the treatment effect depended on oxidative balance at the start of the week. Individual identity, nested within colony identity, was included as a random effect.

Effect of treatment on oxidative stress in ejaculates

To evaluate the effect of treatment on ejaculate oxidative balance, the ln-transformed levels of SOD and MDA measured at the end of each week were specified as response variables in two distinct LMMs. Due to the small sample size, only treatment, body mass on Day 8 (scaled by treatment), and their interaction were included as fixed effects. Individual identity, nested within colony identity, was specified as random effect. A small number of samples from male

breeders were included in the models since they exhibited levels of oxidative stress markers within the range of that of the helpers (SOD activity: range for helpers = 11 – 186 U/ml; range for breeders: 13 – 70 U/ml; MDA: range for helpers = 705 – 17843 ng/ml; range for breeders = 1292 – 4728 ng/ml). These included SOD activity levels from 4 male breeders in both treatments, and MDA levels from 3 male breeders during the control and two during the sand treatment.

Effect of treatment on the trade-off between somatic and germline tissues

To evaluate the effect of treatment on the trade-off between the two tissues, the ln-transformed ratio between ejaculate and erythrocyte (germline/soma) levels of SOD and MDA on Day 8 were fitted as response variables in two distinct LMMs. Model structure followed the one described for the effect of treatment on oxidative stress in ejaculates.

Relationship between cooperative contributions and oxidative stress

To assess whether cooperative contributions can predict levels of oxidative stress markers in erythrocytes and ejaculates, models similar to those specified above were used, except that the percentage of Burrow displayed on Day 7 replaced treatment as a fixed effect and the models contained no interactions between continuous variables.

Relationship between initial oxidative stress and changes in cooperative contributions

Finally, we tested whether individuals better equipped to cope with a potential increase in ROS formation could raise their cooperative contributions to a greater extent. We used the difference in the percentage of Burrow between the two treatments (sand – control) as a response variable

in five LMMs (one for each marker). The oxidative stress marker level measured before the start of the sand treatment, sex, breeding status and all possible interactions were specified as fixed effects. Additionally, body mass and its interaction with the marker level were also included as fixed effects. Finally, to control for undesired regression to the mean effects, percentage of Burrow during the control treatment was included as a fixed effect (Kelly and Price, 2005). Colony identity was specified as a random effect.

3.3. RESULTS

Effect of treatment on behaviour and changes in body condition

Animals were more active during the sand treatment, compared to the control treatment (Treatment: estimate ± SE = 0.411 ± 0.165, χ^2 = 38.4, p < 0.001; Day: estimate ± SE = -0.088 ± 0.063, χ^2 = 12.2, p < 0.001). This effect was specifically driven by a significant increase in the expression of energetically demanding burrowing behaviours (Table 3.2a, Figure 3.a). Indeed, the other cooperative and non-cooperative activities were either not increased (Nest Build: females n = 36, V = 145, p = 0.642; males n = 41, V = 142.5, p = 0.595; Food Carry: females n = 36, V = 117, p = 0.972; Figure 3.b, c), or even decreased (Food Carry males: n = 41, V = 382.5, p-value = 0.008; Non-Cooperation: Table 3.2b; Figure 3.,d) during the sand treatment. Furthermore, the expression of the analysed behaviours throughout the week was not affected by treatment (Treatment * Day: all models p > 0.138).

Despite the increased activity levels and significantly decreased amount of time spent eating during the sand treatment (Table 3.3), weekly changes in body mass did not significantly differ between treatments (all p > 0.143).

Effect of treatment on oxidative stress in erythrocytes

Treatment did not significantly affect the antioxidant SOD activity. Only SOD activity on Day -2 significantly predicted SOD activity on Day 8 (Initial SOD: estimate ± SE = 0.085 ± 0.026, χ^2 = 13.817, p < 0.001; all other fixed effects: p > 0.234; data from 70 individuals belonging to six colonies, in each treatment).

Cellular oxidation (GSSG/GSH) was significantly higher at the end of the sand treatment, compared to the control treatment (Table 3.4a, Figure 3.3a). This effect was driven by a greater increase in oxidized glutathione (GSSG; 23.3%) than in reduced glutathione (GSH; 9.6%) in the sand, compared to the control treatment (Table 3.4b,c; Figure 3.3b,c). While controlling for the effects of treatment, helpers had significantly higher levels of GSH than breeders. However, removing the female breeder with the lowest GSH concentration from the analysis rendered this effect non-significant (Breeding status: estimate ± SE = -0.010 ± 0.052, χ^2 = 3.189, p = 0.074), without qualitatively affecting the other terms in the model.

Treatment alone did not significantly affect oxidative damage to lipids (MDA). Yet, the effect of treatment on MDA levels appeared to depend on sex (Table 3.5; Figure 3.4), with females having lower levels of MDA at the end of the sand treatment compared to the control, while males maintained similar MDA levels across the treatments (post-hoc comparisons; females: estimate ± SE = 0.104 ± 0.038, t = 2.762, p = 0.037; males: estimate ± SE = 0.011 ± 0.039, t = -0.275, p = 0.993). Regardless of treatment, there was a significant effect of body mass on MDA, with heavier individuals showing lower levels of MDA.

Effect of treatment on oxidative stress in ejaculates

Antioxidant (SOD) activity in ejaculates was significantly higher at the end of the sand treatment, compared to the control treatment (Table 3.6a; Figure 3.5a). Concerning oxidative damage to lipids (MDA), an initial model revealed a significant effect of the interaction between treatment and body mass (Treatment * Body Mass: estimate ± SE = 0.567 ± 0.258, χ^2 = 4.831, p = 0.028). However, visual inspection of this interaction suggested that one single data point was driving this effect. Refitting the model excluding this point (n = 41) rendered the interaction non-significant and revealed that MDA levels in ejaculates were significantly

higher at the end of the sand treatment, compared to the control (Table 3.6b; Figure 3.5b). These effects most likely best reflect the pattern experienced by male helpers, who represented 84% and 88% of the sample size on the models for SOD and MDA, respectively.

Effect of treatment on the trade-off between somatic and germline tissues

SOD activity and MDA levels were both increased in ejaculates to a greater extent than in erythrocytes during the sand treatment (Table 3.7, Figure 3.6).

Relationship between cooperative contributions and oxidative stress

Cooperative contributions explained the significant effects of treatment on erythrocytes' but not on ejaculates' oxidative stress markers on Day 8. Cellular oxidation (GSSG/GSH) in erythrocytes was positively correlated with the expression of burrowing contributions on the previous day (Burrow: estimate ± SE = 0.015 ± 0.031, χ^2 = 10.345, p = 0.001; Initial marker level: estimate ± SE = 0.503 ± 0.078, χ^2 = 41.451, p < 0.001; all other fixed effects: p > 0.281) (Figure 3.7a). This was due to a greater effect of burrowing contributions on GSSG (Burrow: estimate ± SE = 0.015 ± 0.032, χ^2 = 9.827, p = 0.002; Initial marker level: estimate ± SE = 0.062 ± 0.048, χ^2 = 54.197, p < 0.001; all other fixed effects: p > 0.117) than on GSH (Burrow: estimate ± SE = 0.004 ± 0.007, χ^2 = 4.025, p = 0.045; Initial marker level: estimate ± SE = 0.894 ± 0.034, χ^2 = 674.253, p < 0.001; Status: estimate ± SE = 0.049 ± 0.065, χ^2 = 4.866, p = 0.027 all other fixed effects: p > 0.281) (Figure 3.7b,c). Burrowing contributions did not significantly correlate with SOD or MDA in erythrocytes (p > 0.157) or ejaculates (p > 0.415).

Relationship between initial oxidative stress and changes in cooperative contributions

The initial levels of oxidative stress markers did not significantly predict the changes in percentage of time spent burrowing between the two treatments (Table 3.8).

Table 3.2 – OLRE binomial GLMMs examining the effect of treatment on (a) percentage of burrowing activities, and (b) percentage of non-cooperative activities, during 12h scan observations. Dataset included 308 observations, from 77 individuals belonging to 6 different colonies. Reference levels of categorical fixed effects are: [1]control; [2]breeder; [3]female; [4]Day2. Significant terms are shown in bold.

	Estimate	SE	χ^2	p-value	Estimate	SE	χ^2	p-value
Fixed effects	*(a) Burrow*				*(b) Non-Cooperation*			
Intercept	-3.076	0.311			-1.400	0.121		
Treatment [1]	**0.962**	**0.281**	**207.149**	**< 0.001**	**0.074**	**0.137**	**23.669**	**< 0.001**
Body mass	0.046	0.097	0.014	0.905	**-0.052**	**0.037**	**7.958**	**0.005**
Breeding Status [2]	0.111	0.338	0.001	0.981	**0.153**	**0.125**	**4.778**	**0.029**
Sex [3]	0.479	0.428	1.469	0.225	0.064	0.161	0.047	0.828
Day [4]	**-0.155**	**0.110**	**4.205**	**0.040**	**-0.058**	**0.052**	**9.162**	**0.002**
Treatment * Body mass	-0.060	0.085	0.507	0.477	-0.059	0.041	2.022	0.155
Treatment * Status	0.116	0.299	0.483	0.487	-0.175	0.146	0.040	0.842
Treatment * Sex	-0.024	0.376	0.024	0.877	-0.436	0.191	1.593	0.207
Status * Sex	-0.365	0.464	0.673	0.412	-0.005	0.174	1.790	0.181
Treatment * Day	0.005	0.148	0.001	0.975	-0.108	0.073	2.151	0.142
Treatment * Status * Sex	0.057	0.408	0.019	0.890	0.401	0.206	3.802	0.051
Random effects								
OLRE	0.328	0.573			0.068	0.261		
Animal/Colony	0.315	0.561			0.024	0.156		
Colony	0.000	0.000			0.007	0.081		

Table 3.3 – Binomial GLMM examining the effect of treatment on percentage of time eating, during 12h scan observations. Dataset included 308 observations, from 77 individuals belonging to 6 different colonies. Reference levels of categorical fixed effects are: [1]control; [2]breeder; [3]female; [4]Day2. Significant terms are shown in bold.

Fixed effects	Estimate	SE	χ^2	p-value
Intercept	-2.857	0.121		
Treatment [1]	**-0.148**	**0.145**	**17.554**	**< 0.001**
Body mass	**-0.086**	**0.037**	**8.753**	**0.003**
Breeding Status [2]	0.051	0.125	2.290	0.130
Sex [3]	0.038	0.162	1.581	0.209
Day [4]	-0.030	0.052	0.003	0.957
Treatment * Body mass	-0.004	0.041	0.012	0.914
Treatment * Status	-0.041	0.154	0.661	0.416
Treatment * Sex	-0.265	0.206	0.078	0.780
Status * Sex	0.044	0.175	1.384	0.239
Treatment * Day	0.069	0.075	0.831	0.362
Treatment * Status * Sex	0.282	0.221	1.629	0.202
Random effects				
Animal/Colony	0.022	0.148		
Colony	0.006	0.077		

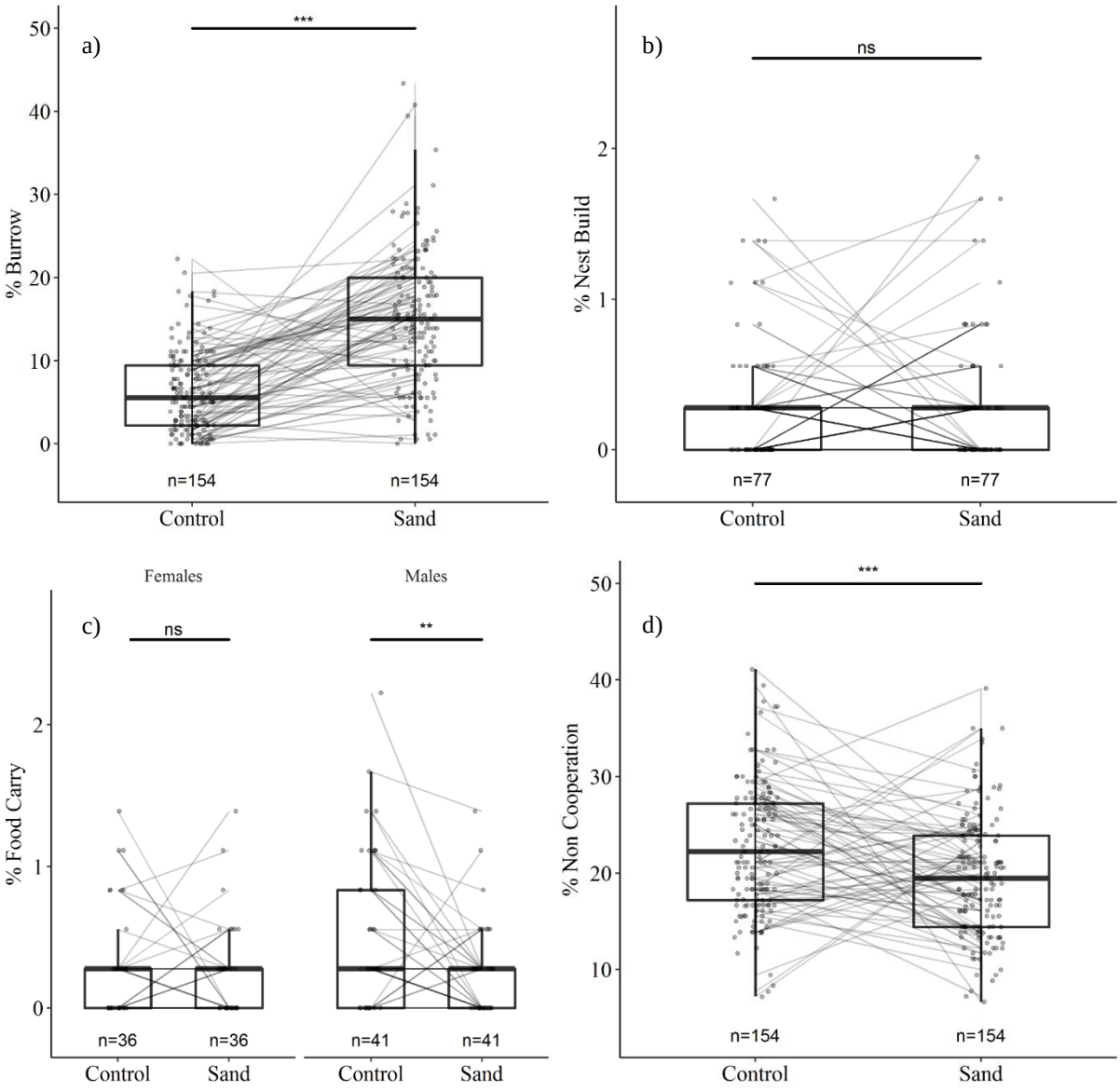


Figure 3.2 – Effect of treatment on the percentage of time spent (a) burrowing, (b) nest building, (c) carrying food and (d) expressing non-cooperative behaviours, in two sessions of 12h scan observations. Each individual is represented twice in each treatment, once from scan on day 2 and once from scan on day 7. Lines between points illustrate the repeated measurements of same individuals. ***, $p < 0.001$; ns, non-significant.

Table 3.4 – LMMs examining the effect of treatment on levels of (a) GSSG/GSH, (b) GSSG and (c) GSH in erythrocytes. Dataset included 138 observations, from 74 individuals belonging to 6 different colonies. Reference level of categorical fixed effects are: [1]control; [2]breeder; [3]female. Significant terms are shown in bold.

	Estimate	SE	χ^2	p-value	Estimate	SE	χ^2	p-value	Estimate	SE	χ^2	p-value
Fixed effects	(a) GSSG/GSH				(b) GSSG				(c) GSH			
Intercept	-3.976	0.238			2.888	0.232			6.796	0.045		
Treatment [1]	**0.174**	**0.256**	**5.414**	**0.020**	**0.129**	**0.288**	**9.434**	**0.002**	**0.089**	**0.071**	**24.970**	**< 0.001**
Breeding Status [2]	0.010	0.200	0.151	0.698	-0.037	0.223	0.004	0.949	**0.047**	**0.051**	**5.194**	**0.023**
Sex [3]	0.056	0.249	0.252	0.616	-0.014	0.267	0.692	0.405	-0.023	0.064	0.320	0.571
Body Mass	-0.039	0.063	0.062	0.804	-0.053	0.069	0.619	0.431	0.010	0.015	0.976	0.323
Initial marker level	**0.442**	**0.064**	**50.283**	**< 0.001**	**0.525**	**0.071**	**66.385**	**< 0.001**	**0.249**	**0.014**	**634.51**	**< 0.001**
Treatment * Status	0.009	0.277	0.011	0.918	0.220	0.321	0.080	0.778	0.015	0.078	0.008	0.931
Treatment * Sex	-0.007	0.351	0.126	0.722	0.099	0.399	0.763	0.382	0.000	0.099	0.166	0.684
Status * Sex	-0.083	0.273	0.225	0.635	0.032	0.298	0.221	0.639	0.023	0.071	0.077	0.781
Treatment * Body Mass	0.093	0.077	1.465	0.226	0.158	0.089	3.148	0.076	0.001	0.021	0.002	0.965
Treatment * Initial marker level	-0.120	0.071	2.878	0.090	-0.145	0.084	2.999	0.083	0.002	0.020	0.007	0.931
Treatment * Status * Sex	-0.053	0.372	0.020	0.888	-0.295	0.431	0.467	0.494	-0.020	0.107	0.035	0.852
Random effects												
Animal/ Colony	0.034	0.184			< 0.001	< 0.001			< 0.001	< 0.001		
Colony	0.151	0.390			< 0.001	< 0.001			< 0.001	< 0.001		
Residual	0.144	0.379			< 0.001	< 0.001			< 0.001	< 0.001		

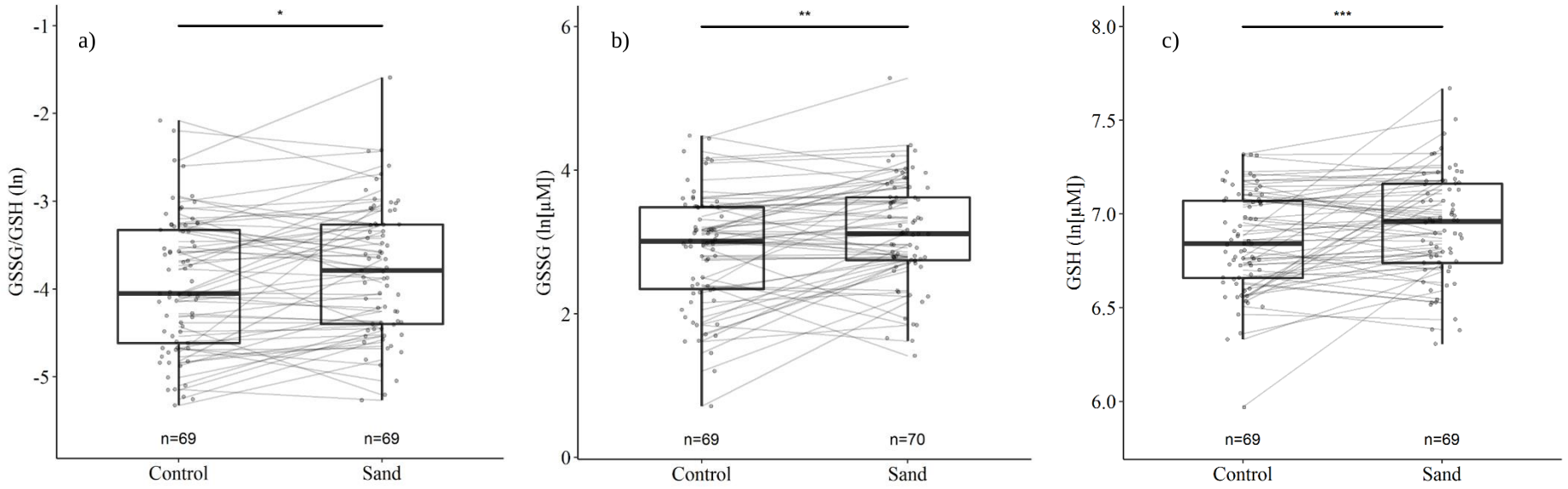


Figure 3.3 – Effect of treatment on (a) cellular oxidation (GSSG/GSH), (b) oxidized glutathione (GSSG) and (c) reduced glutathione (GSH). Lines between points illustrate the repeated measurements of same individuals. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Table 3.5 - LMM examining the effect of treatment on concentrations of MDA (oxidative damage to lipids), in erythrocytes. Dataset included 138 observations, from 73 individuals belonging to 6 different colonies. Reference level of categorical fixed effects are: [1]control; [2]breeder; [3]female. Significant terms are shown in bold.

Fixed effects	Estimate	SE	χ^2	p-value
Intercept	8.573	0.054		
Treatment [1]	-0.169	0.070	1.240	0.265
Breeding Status [2]	-0.131	0.054	1.106	0.293
Sex [3]	-0.130	0.068	0.694	0.405
Body Mass	**-0.012**	**0.019**	**6.631**	**0.010**
Initial marker level	**0.184**	**0.026**	**93.140**	**< 0.001**
Treatment * Status	0.129	0.078	1.902	0.168
Treatment * Sex	0.166	0.098	3.834	0.050
Status * Sex	0.128	0.076	1.972	0.160
Treatment * Body Mass	-0.035	0.023	2.265	0.132
Treatment * Initial marker level	-0.001	0.030	0.001	0.978
Treatment * Status * Sex	-0.102	0.106	0.912	0.340
Random effects				
Animal/Colony	0.001	0.031		
Colony	0.003	0.059		
Residual	0.012	0.110		

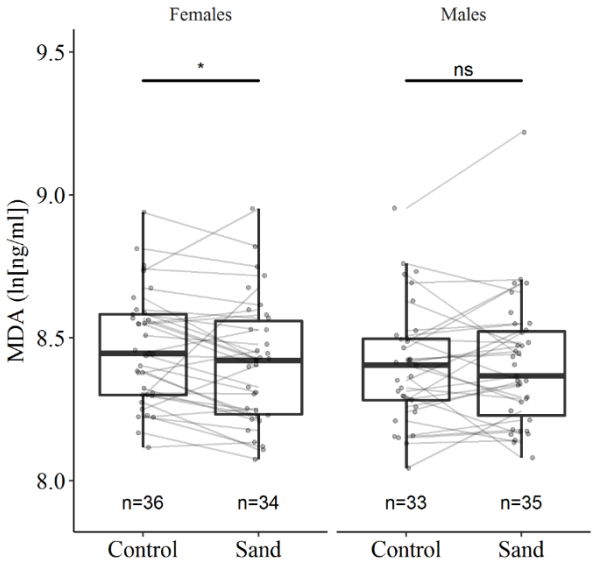


Figure 3.4 – Effect of treatment dependent on sex on oxidative damage to lipids (MDA). Lines between points illustrate the repeated measurements of same individuals. Significance levels represented refer to least square means comparisons: *, $p < 0.05$; ns, non-significant.

Table 3.6 - LMMs examining the effect of treatment on levels of (a) SOD activity and (b) MDA (oxidative damage to lipids), in ejaculates. Dataset for SOD included 51 observations, from 30 males belonging to 6 different colonies. Dataset for MDA included 41 observations from 27 males belonging to 6 colonies. Reference level of categorical fixed effect is: [1]control. Significant terms are shown in bold.

	Estimate	SE	χ^2	p-value	Estimate	SE	χ^2	p-value
Fixed effects	(a) SOD				(b) MDA			
Intercept	3.325	0.154			8.200	0.173		
Treatment [1]	**0.499**	**0.190**	**6.921**	**0.009**	**0.482**	**0.243**	**4.804**	**0.028**
Body Mass	-0.052	0.153	0.163	0.687	-0.261	0.170	1.540	0.215
Treatment * Body Mass	0.015	0.198	0.006	0.940	0.259	0.284	0.837	0.360
Random effects								
Animal/Colony	0.062	0.248			0.060	0.245		
Colony	0.011	0.104			0.000	0.000		
Residual	0.441	0.664			0.531	0.729		

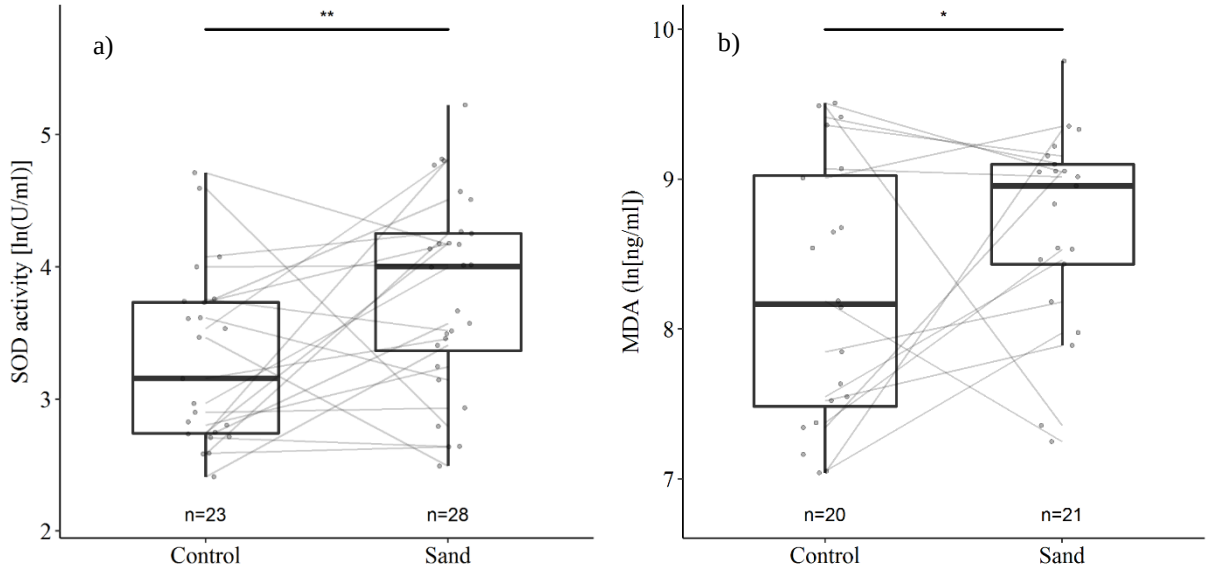


Figure 3.5 - Effect of treatment on (a) SOD activity and (b) MDA concentration (oxidative damage to lipids), in ejaculates. Lines between points illustrate the repeated measurements of same individuals. *, $p < 0.05$; **, $p < 0.01$.

Table 3.7 - LMMs examining the effect of treatment on the ratio germline/soma of (a) SOD activity and (b) MDA levels (oxidative damage to lipids). Dataset for SOD included 51 observations, from 30 males belonging to 6 different colonies. Dataset for MDA included 41 observations from 27 males belonging to 6 colonies. Reference level of categorical fixed effect is: [1]control. Significant terms are shown in bold.

	Estimate	SE	χ^2	p-value	Estimate	SE	χ^2	p-value
Fixed effects	(a) SOD				(b) MDA			
Intercept	-3.161	0.150			-0.196	0.170		
Treatment [1]	**0.421**	**0.176**	**5.746**	**0.016**	**0.516**	**0.246**	**5.044**	**0.025**
Body Mass	0.091	0.153	0.264	0.607	-0.126	0.167	0.246	0.620
Treatment * Body Mass	-0.058	0.185	0.099	0.753	0.173	0.282	0.374	0.541
Random effects								
Animal/Colony	< 0.001	< 0.001			0.013	0.113		
Colony	< 0.001	< 0.001			0.000	0.000		
Residual	< 0.001	< 0.001			0.556	0.745		

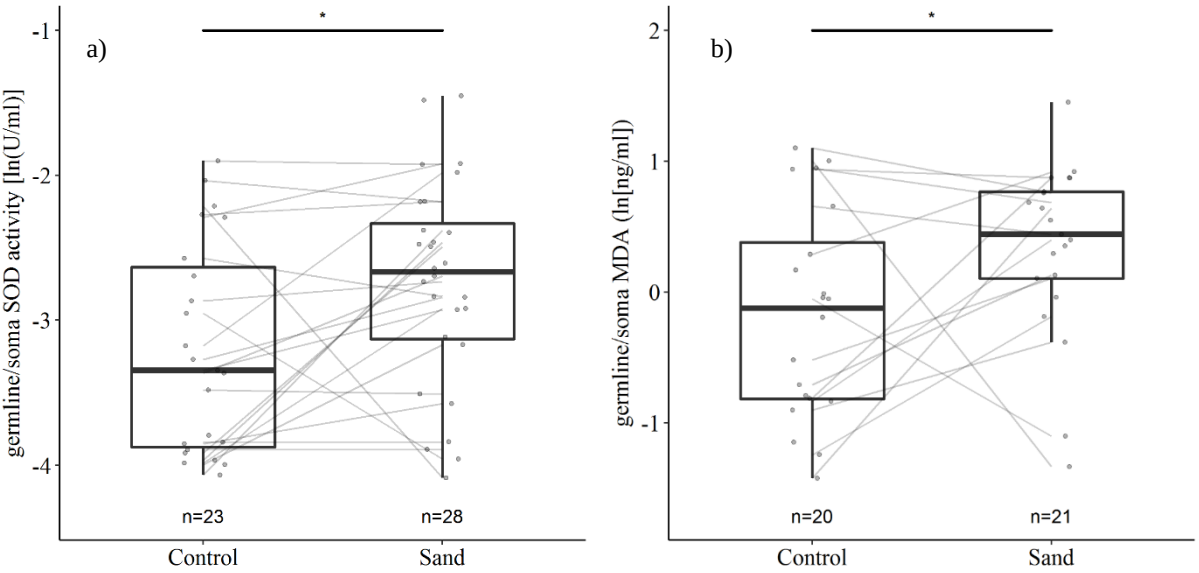


Figure 3.6 – Effect of treatment on the ratio germline/soma of (a) SOD activity and (b) MDA levels (oxidative damage to lipids). Y-axis is a logarithmic scale, where negative values indicate greater levels in the soma, and positive values indicate greater levels in the germline.

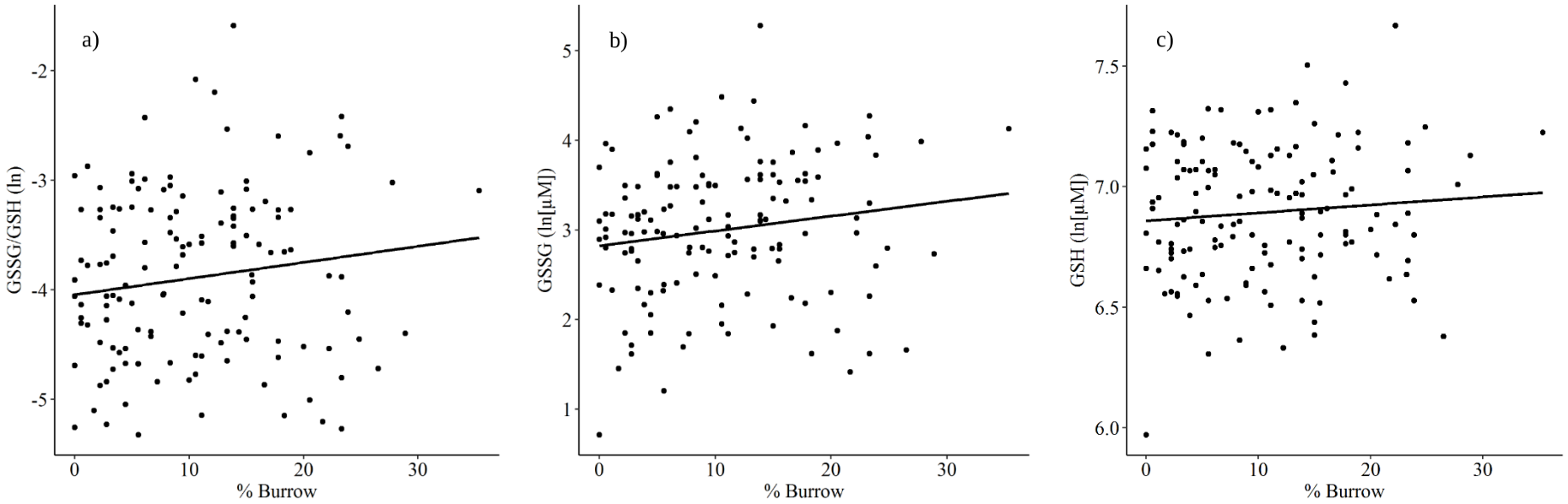


Figure 3.7 – Relationship between the percentages of burrowing behaviour on Day 7 and levels of (a) cellular oxidation (GSSG/GSH), (b) oxidized glutathione (GSSG) and (c) reduced glutathione (GSH), in erythrocytes on Day 8.

Table 3.8 - LMMs examining whether initial levels of (a) GSSG/GSH, (b) SOD, (c) and MDA in erythrocytes predicted changes in the percentage of burrowing (from the control to the sand treatment). Datasets included 70 observations, from 70 individuals belonging to 6 different colonies. Reference levels of categorical fixed effects are: [1]female; [2]breeder. Significant terms are shown in bold.

	Estimate	SE	χ^2	p-value	Estimate	SE	χ^2	p-value	Estimate	SE	χ^2	p-value
Fixed effects	(a) GSSG/GSH				(b) SOD				(c) MDA			
Intercept	23.619	29.521			30.851	140.674			-83.153	181.664		
Initial concentration	3.696	7.751	0.184	0.668	-2.480	21.641	0.113	0.737	10.111	19.661	0.008	0.927
Sex [1]	-7.014	32.577	1.788	0.181	31.274	126.515	1.407	0.236	-9.085	278.429	2.694	0.101
Breeding Status [2]	-10.828	30.337	0.934	0.334	-1.476	118.915	0.919	0.338	11.158	193.126	1.638	0.201
Body Mass	-2.774	4.375	0.691	0.406	-0.302	0.520	0.775	0.379	-47.275	28.169	0.597	0.440
Control Burrow	**-0.842**	**0.189**	**19.934**	**< 0.001**	**-0.841**	**0.184**	**20.769**	**< 0.001**	**-0.846**	**0.181**	**21.809**	**< 0.001**
Initial concentration * Sex	-2.536	8.881	0.651	0.420	-4.332	19.557	1.651	0.199	1.451	30.390	2.799	0.094
Initial concentration * Status	-3.675	8.077	0.596	0.440	0.645	18.467	0.028	0.868	-0.817	20.940	0.269	0.604
Sex * Status	2.723	33.681	0.062	0.804	15.326	130.226	0.003	0.955	147.661	285.844	0.113	0.736
Initial concentration * Body Mass	-0.631	1.369	0.212	0.645	0.041	0.078	0.279	0.597	5.061	3.062	2.733	0.098
Initial concentration * Sex * Status	0.366	9.286	0.002	0.969	-2.415	20.131	0.014	0.904	-16.310	31.201	0.273	0.601
Random effects												
Colony	4.310	2.076			2.795	1.672			6.444	2.538		
Residual	52.070	7.216			52.559	7.250			48.431	6.959		

3.4. DISCUSSION

The results presented here offer the first experimental demonstration that oxidative stress represents a physiological cost of energetically demanding cooperative behaviours in a cooperatively breeding rodent species. Experimental manipulation of sand provisioning led to increased activity levels due to a specific increase in cooperative contributions and generated oxidative stress in somatic (erythrocytes) and germline (ejaculates) tissues. Additionally, we show that upon increased cooperative contributions, male helpers privileged somatic over germline functions, as would be expected by a trade-off between survival and reproduction. It could be argued that the effects of our experimental treatment on oxidative balance were instead driven by its significant negative effect on feeding behaviour. However, changes in body mass did not differ between treatments, suggesting that animals compensated by eating more during the night, outside of the observation time, when no sand was being provided.

Increased cooperative contributions, and its associated energetic demands, represent an oxidative threat to somatic tissues. By experimentally increasing the amount of sand provided to the colonies, individuals doubled the expression of energetically demanding cooperative behaviours (burrowing), regardless of sex and breeding status. Increased cooperative contributions induced oxidative stress, as witnessed by the increased levels of cellular oxidation (GSSG/GSH) in erythrocytes, generated by a greater increase in oxidized glutathione (GSSG) than in its reduced form (GSH) (Flohé, 2013; Urso and Clarkson, 2003). Furthermore, the degree of cellular oxidation was significantly and positively correlated with the expression of cooperative behaviours, suggesting that greater cooperative contributions are paralleled by greater oxidative threats (Flohé, 2013; Urso and Clarkson, 2003). These results are in line with the effects of physical exercise in rats, which leads to increased ROS formation (Davies et al., 1982), accumulation of GSSG and increased levels of GSH in several somatic tissues (Lew et

al., 1985). ROS are neutralized by GSH, via the catalytic action of glutathione peroxidase (GPx), producing GSSG which is accumulated in the cells and converted back to GSH by glutathione reductase (GR) when the source of the oxidative threat is removed (Morgan et al., 2013). Despite the extensive oxidation of GSH, its concentration was also increased during the sand treatment, suggesting that GPx, GR and the enzymes involved in *de novo* synthesis of GSH played a major antioxidant role in minimizing oxidative damage (Halliwell and Gutteridge, 2007). Finally, regardless of treatment and sex, helpers had higher levels of GSH than breeders, but care should be taken since this effect was sensitive to the removal of one female breeder which showed unusually low GSH concentration. Although this result deserves attention in further studies, it may indicate that helpers are better prepared to deal with a surge in ROS formation and avoid accumulation of somatic oxidative damage, compared to breeders. Yet, this possibility contrasts with a lack of effect of breeding status on lipid peroxidation in our study (see below), and with the results of a previous correlative study in Damaraland mole-rats where, compared to female breeders, female helpers exhibited higher (not lower) levels of oxidative damage to lipids in heart, kidney and liver (Schmidt et al., 2014). It should however be noted that in the study of Schmidt et al. (2014), these differences in oxidative damage were not evident in blood plasma and skeletal muscle.

Interestingly, increased cooperative contributions did not affect the activity of the antioxidant SOD in erythrocytes, suggesting that this was within sufficient levels to neutralize the exceeding superoxide anions formed upon the increased energetic requirements of the sand treatment (Selman et al., 2002). In agreement with our result, mice selected to double the expression of energetically demanding activities (voluntary wheel-running), compared to randomly bred controls, did not show differences in SOD activity in several tissues (Vaanholt

et al., 2008). Also, in short-tailed field voles (*Microtus agrestis*), voluntary exercise during a 7-day period did not significantly affect SOD activity in the muscle (Selman et al., 2002).

Despite the oxidative threat posed by the increased cooperative contributions, animals were able to avoid increased somatic oxidative damage. Indeed, regardless of sex and breeding status, oxidative damage to the lipids (MDA) was not increased during the sand treatment. One possible explanation is that individuals adjusted cooperative contributions according to their oxidative status, in order to minimize oxidative damage. Yet, baseline oxidative status did not predict the extent to which individuals increased cooperative contributions during the sand treatment. In other species, it has been shown that individual condition and oxidative status affect the decision, and the extent, to engage in energetically demanding activities, such as cooperative ones (Clutton-Brock et al., 1998; Cram et al., 2014; Eden, 1987; van de Crommenacker et al., 2011). In our study, such an effect may have been shadowed by the captivity conditions and *ad libitum* food provision, keeping animals in good condition. Direct and biologically relevant experimental manipulations of individual oxidative balance (Koch and Hill, 2016) are now needed to test whether oxidative status influences individual cooperative contributions.

Female Damaraland mole-rats may be better equipped than males to prevent and even reduce somatic oxidative damage upon increased cooperative contributions. Females tended to have lower MDA levels at the end of the sand treatment, compared to the control, whereas males' levels did not differ between treatments. Three possibilities could explain the reduction in MDA levels in females. Firstly, oxidative damage may be reduced through increased antioxidant defences (Costantini and Verhulst, 2009). SOD and reduced glutathione levels did not differ between sexes, yet the possibility that females upregulated other antioxidants not measured here cannot be discarded. Secondly, sex differences in the efficiency of mechanisms

repairing oxidative damage to lipids could be responsible for the observed result (Monaghan et al., 2009). Finally, owing to their negative effect on ROS formation, mitochondrial uncoupling proteins could be pointed as a possible mechanism explaining the reduction of oxidative damage. This is, however, unlikely since it would limit the efficiency of energy production (Brand, 2000). Further studies are necessary to ascertain whether and how males and females differently deal with increased energetic requirements, as current work often focus on either sex (Davies et al. 1982; Lew, Pyke & Quintanilha 1985; Ji 1993; Bejma & Ji 1999; Vaanholt et al. 2008; but see Bize et al. 2008; Kamper et al. 2009; Costantini et al. 2010).

Energetically demanding cooperative behaviours may incur additional costs to males through negative effects on sperm quality. Although the higher SOD activity levels in ejaculates at the end of the sand treatment suggest an adaptive protection response against ROS, increased cooperative contributions resulted in increased oxidative damage in the ejaculates, highlighting the high sensitivity of sperm cells to ROS (Agarwal et al., 2008; Tremellen, 2008). Since lipid peroxidation of sperm cells has been shown to result in reduced sperm motility and compromised function, such as the ability to fuse with oocytes (Aitken et al., 1989; Suleiman et al., 1996), it is possible that increased cooperative contributions compromise the fitness of male Damaraland mole-rats. Determination of sperm quality markers would be necessary to support this possibility, as shown previously in great tits (*Parus major*), where increased chick provisioning led to greater oxidative damage to lipids in ejaculates and a concomitant decreased sperm quality (Helfenstein et al., 2010). Although Damaraland mole-rat helpers do not reproduce within their natal colony, reproductive opportunities in the wild may arise when the underground tunnel systems of two colonies become connected, for example due to animals extending their tunnels searching for new food sources. In such situations, compromised sperm quality can negatively affect the direct fitness of male helpers. Yet, whether the effects reported

here represent only short-term or can translate into long-term costs is currently unknown and may depend on whether undifferentiated germline cells are equally affected by increased energetic demands. Also, whether male breeders are subjected to the same costs deserves further attention.

Our study shows that somatic and germline tissues are differently affected during the sand treatment, supporting the hypothesis that oxidative stress can, at least partly, mediate the trade-off between survival and reproduction. Recently, it has been proposed that resources involved in the antioxidant machinery may be traded-off between somatic and germline functions (Rojas Mora et al., 2017). Our results indicate that SOD does not represent a limited resource involved in such trade-off since the increased SOD activity in the germline was not accompanied by a concomitant decrease in the soma. However, we found that males accumulated oxidative damage in the germline to a greater extent than in the soma, suggesting that other antioxidant resources have been traded-off. Indeed, oxidative damage integrates the negative effects of ROS and the protective effects of antioxidants and repair mechanisms (Halliwell and Gutteridge, 2007) and thus provides reliable information about the outcome of a potential trade-off between somatic and germline tissues. In this respect, our results suggest that, overall, males prioritized survival over reproduction by avoiding increased oxidative damage to the soma, at the expense of damage to the germline. For male helpers, who represented over 84% of our sample size, such strategy may be adaptive because reproduction is unlikely while staying in their natal colony (Bennett and Faulkes, 2000; Bennett and Jarvis, 1988; Burland et al., 2004; Jarvis and Bennett, 1993) and investment in survival may result in increased indirect fitness and increased prospects of future reproduction. Furthermore, this result is in agreement with theoretical work suggesting that under uncertain reproductive opportunities, individuals should prioritize survival, at the cost of current reproduction (Drenos and Kirkwood, 2005; Perrin and

Sibly, 1993). Finally, the exciting possibility that male breeders and helpers have different solutions for the trade-off between somatic and germline functions deserves further examination.

In summary, our findings suggest that oxidative stress may represent a key mechanism underlying the physiological costs of energetically demanding cooperative behaviours, susceptible to ultimately compromise individuals' fitness. Furthermore, we highlight that assessing such costs in somatic and germline tissues may provide insight into the physiological mechanisms underpinning one of the best studied life-history trade-offs, that between survival and reproduction.

Chapter 4

Glucocorticoids, cooperative contributions and oxidative stress in cooperatively breeding Damaraland mole-rats

ABSTRACT

Within cooperatively breeding societies, individuals fine-tune their cooperative contributions according to internal and external factors. Glucocorticoid hormones (GCs) represent a physiological (internal) mechanism that may facilitate the expression of energetically demanding cooperative behaviours. Additionally, GCs may induce oxidative stress, a state in which the formation of reactive oxygen species (ROS; inherent by-products of energy production) exceeds the antioxidant protection capacity, leading to accumulation of oxidative damage which may negatively impact fitness. Building upon our previous finding, that increasing the expression of energetically demanding cooperative behaviours generates oxidative stress, we investigate a possible physiological link between GC levels, cooperative contributions and oxidative stress in female helpers of a cooperatively breeding rodent, the Damaraland mole-rat (*Fukomys damarensis*). We show that experimental increases of GC levels over a period of 7 days, significantly raised plasma oxidative damage to lipids (MDA) but did not affect oxidative stress markers in erythrocytes [superoxide dismutase (SOD), cellular oxidation, oxidized or reduced glutathione (GSSG/GSH, GSSG, GSH), or lipid peroxidation (MDA)]. Although our experimental treatment increased GC levels, energetically demanding cooperative contributions and oxidative stress, the different relationships between these variables suggest that the oxidative costs induced by elevated GCs and cooperative contributions may independently affect an individual's fitness. Also, we provide the first indication that the oxidative costs of elevated GC levels can be induced by GC levels falling within a range that is not necessarily associated with stressful events.

4.1. INTRODUCTION

Within cooperatively breeding societies, where helpers forego their own reproduction and assist breeders in raising their offspring (Clutton-Brock, 2016; Koenig and Dickinson, 2016), individuals fine-tune the expression of cooperative behaviours according to internal and external factors (Cant and Field, 2001; Clutton-Brock et al., 2001a; Nichols et al., 2012b; Russell et al., 2003b; Sanderson et al., 2014). These adjustments explain the observable within and between individual variations in cooperative contributions (e.g. Clutton-Brock et al., 2001a; Field et al., 2006; Hodge, 2007; Zöttl et al., 2016). The ultimate causes of such variation as well as the endocrine mechanisms regulating them are increasingly well understood. Yet, the possibility that increased cooperative contributions could incur physiological costs that may ultimately affect individuals' fitness has remained largely unexplored (Heinsohn and Legge, 1999).

Many cooperative behaviours are likely to incur physiological costs due to their energetic requirements. In fact, cooperative behaviours such as incubation of eggs, baby-sitting and feeding youngsters, as well as maintaining and expanding the territory of the breeders have been shown to be energetically demanding, inducing loss of body condition and increasing metabolism (Clutton-Brock et al., 1998; Grantner and Taborsky, 1998; Heinsohn and Legge, 1999; Lovegrove, 1989; Russell et al., 2003b), and may culminate in reduced fecundity and survival (Clutton-Brock, 1991; Reyer, 1984; Russell et al., 2003b; Stacey and Koenig, 1990). A physiological cost that has been associated with increased energetic needs and metabolism is oxidative stress. Oxidative stress is characterized by an imbalance between the formation of reactive oxygen species (ROS; inherent by-products of cellular energy production) and the protective antioxidant system, in favour of the former (Sies, 1985). Under oxidative stress, ROS are more likely to oxidise important cellular components such as DNA, proteins, and

lipids, leading to an increasing rate of oxidative damage accumulation (Davies et al., 1982), which can compromise individuals' survival and reproduction (Costantini, 2014; Finkel and Holbrook, 2000; Harshman and Zera, 2007; Monaghan et al., 2009). Energetically demanding activities have been shown to increase ROS formation, induce oxidative stress, and ultimately lead to a higher accumulation of oxidative damage (Bejma and Ji, 1999; Davies et al., 1982; Leeuwenburgh and Heinecke, 2001; Radak et al., 2008). In cooperative breeders, correlative studies have suggested that elevated energetically demanding cooperative contributions may induce oxidative stress (Cram et al., 2015b, 2014; van de Crommenacker et al., 2011), and findings from our own experimental work support this hypothesis (Chapter 3). However, whether such oxidative costs of cooperation are directly caused by increased energy demands, or if other mechanisms are involved, is currently unknown.

Glucocorticoid hormones (GCs) may mediate a link between energetically demanding cooperative contributions and their oxidative stress costs. On one side, GCs facilitate the mobilization of stored energy and stimulate metabolism (Sapolsky et al., 2000), characteristics that make these hormones important regulators of energetically demanding behaviours. In cooperative breeders, correlational and experimental work has indeed suggested that higher GC levels are positively associated with helpers' cooperative contributions (Carlson et al., 2006b, 2006a, Dantzer et al., 2017b, 2017a; Sanderson et al., 2014; Young et al., 2010). However, this relationship may be dependent on factors such as the type of cooperative activity, the species, the sex and the nutritional state of the animals (Bender et al., 2008; Dantzer et al., 2017a; Mota et al., 2006; Raynaud and Schradin, 2015; Sanderson et al., 2014). On the other side, GCs are frequently shown to generate oxidative stress (Costantini et al., 2011), increasing ROS formation (Wilson et al., 2013), decreasing antioxidant protection and resistance to oxidative stress (McIntosh et al., 1998; Orzechowski et al., 2002; Pereira et al., 1994; Rajashree

and Puvanakrishnan, 1998; Stier et al., 2009), and ultimately increasing oxidative damage (Caro et al., 2007; Costantini et al., 2008; Cote et al., 2010; Eid et al., 2003; Lin et al., 2004a; Zafir and Banu, 2009). To date, the possibility for a link between GCs, oxidative stress and cooperative contributions has not been addressed. Here, we aim to shed some light on this possibility.

Using captive Damaraland mole-rats (*Fukomys damarensis*), we experimentally tested whether increased GC levels induce oxidative stress. Damaraland mole-rats are subterranean cooperatively breeding rodents, living in colonies composed of one breeding pair and their non-reproductive offspring, the helpers (Bennett and Jarvis, 1988; Burland et al., 2004, 2002). In this species, the most common cooperative activities relate to burrow maintenance and extension used to search for food, which is then made available for all colony members (Bennett, 1990; Bennett and Faulkes, 2000; Jarvis et al., 1998). These cooperative activities have been shown to be energetically demanding (Lovegrove, 1989), stimulated by increased GC levels (Vullioud et al., in preparation) and to generate oxidative stress (Chapter 3).

We experimentally elevated cortisol (CORT) levels [the predominant GC produced in this species (Clarke et al., 2001)] of female helpers, for a period of 7 days, and assessed their oxidative balance in erythrocytes and plasma. Oxidative balance is a complex system that integrates ROS formation, antioxidant defences and oxidative damage (Cohen and McGraw, 2009; Hõrak and Cohen, 2010). We thus described oxidative balance through: (i) the activity of the antioxidant superoxide dismutase (SOD), the first line of defence in the chain of antioxidant reactions, catalysing the dismutation of superoxide anion (the first ROS produced during energy production) into hydrogen peroxide (a less harmful ROS) and oxygen (Halliwell and Gutteridge, 2007); (ii) the balance between oxidized and reduced molecules (oxidized/reduced glutathione). Reduced glutathione (GSH) is a tripeptide, which reduces

hydrogen peroxide into oxidized glutathione (GSSG) and water (Halliwell and Gutteridge, 2007). The molar ratio between its two forms (GSSG/GSH) provides a useful measure of cellular oxidation and ROS formation (Jones, 2006), and changes of this ratio are suggestive of disturbances of the oxidative balance (Flohé, 2013). And (iii) the concentration of malondialdehyde (MDA), a measure of oxidative damage to lipids (Halliwell and Gutteridge, 2007). Owing to the described effects of GCs on oxidative balance (see above), we expected our treatment to result in the downregulation of the antioxidant SOD, increased cellular oxidation (GSSG/GSH), possibly driven be decreased levels of reduced glutathione (GSH), and increased oxidative damage (MDA). We further predicted that if CORT mediates a physiological link between energetically demanding cooperative behaviours and their oxidative costs, correlations between oxidative stress markers and CORT levels or cooperative contributions would both follow similar patterns.

4.2. METHODS

Study animals and husbandry

We used seven originally wild caught colonies (group-size range: 6 to 21 individuals, mean = 10.71, SD = 5.12), maintained in captivity at the Kuruman River Reserve in the Northern Cape, South Africa. Each colony was individually housed in a standardized artificial tunnel system with a total tunnel length of about 18 m. All individuals were marked with hair dye for visual recognition and the upper part of the tunnels was transparent, allowing behavioural observations. Each tunnel system contained a nesting box, food storing areas, and a large plastic box where animals could discard unwanted material. Three vertical pipes, hereafter referred to as sand dispensers, were placed close to the extremities of the tunnel systems.

Every morning the tunnel systems were cleaned and nest material (small pieces of paper towelling) was provided. Animals were fed *ad libitum* twice per day with a diet of sweet potatoes and cucumbers. Food items were provided behind the sand dispensers to encourage burrowing activities and the sand dispensers were filled with clean sand on the same occasion. The temperature in the animal rooms was controlled with air-conditioning systems set to 23℃ during the whole experiment.

Experimental design

In order to manipulate glucocorticoid levels, two originally wild-caught adult female helpers of similar body mass, from each of the seven experimental colonies, alternately received a control (0.001 mg CORT; Control treatment) and a cortisol (5 mg CORT; Cortisol treatment) 7-day release implant. Two weeks after the end of the first treatment, each female received the opposite implant treatment (Figure 4.1). Energetically demanding cooperative opportunities (i.e. sand dispensers filled with sand) were provided approximately every 2h, for a period of 12h on each treatment day.

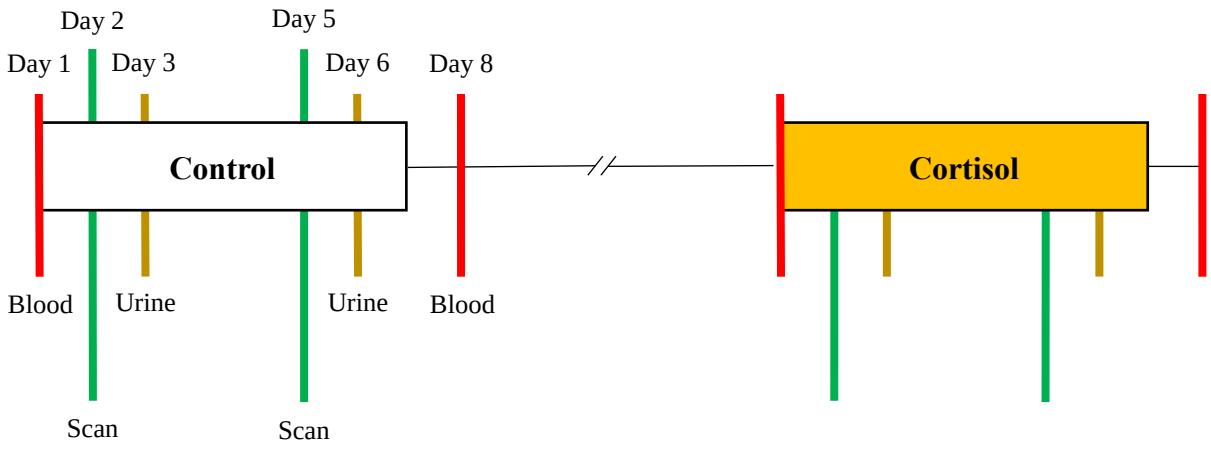


Figure 4.1 - Timeline of the experimental design. Within each of the six pairs of female helpers used in this study, one female started with the Control treatment, as shown in the figure, while the other female started with the Cortisol treatment (opposite sequence to the figure). Blood was collected right before implantation and on Day 8 of each treatment (red bars). Scan observations were carried out during 12 hours on Day 2 and Day 5 of each treatment week (green bars), while urine samples were collected on the days after each scan (yellow bars). Fourteen days were allowed between the two treatments (black line). Refer to the text for more details.

Sampling procedures

Implants and blood collections were done under anaesthesia. Subjects were removed from their tunnel systems and anaesthetized with 5% isoflurane (Isofor, Safe Line Pharmaceuticals, Johannesburg, South Africa) mixed with oxygen gas. This was delivered by a vaporizer at a rate of 1 l/min, through a cone gently fitted on the animal's snout. Once fully sedated, the concentration of isoflurane was reduced to 2% until the completion of the procedures.

On Day1 and Day 8 of each treatment week, a blood sample was collected (Figure 4.1) by piercing a vein from a pre-warmed foot with a sterile needle. Blood was collected into lithium heparinized tubes and centrifuged immediately at room temperature for 5 minutes at 2000 *g*. Plasma was separated from the cell pellet and both fractions were kept at -80℃ until laboratory analysis.

On Day 1 of each treatment week, and after blood sampling, implants (Innovative Research of America) were inserted subcutaneously in the neck area of anaesthetised subjects (Figure 4.1), using a 10-gauge precision trochar (Innovative Research of America). Procedures did not exceed 15 minutes and subjects were removed from anaesthesia and weighed before being placed back in their original tunnel system once full mobility was regained.

Urine samples were collected on the morning of Days 3 and 6 (Figure 4.1) to determine the effect of treatment on hormonal profile and for treatment validation (Vullioud et al., in preparation). Animals were individually kept in urine chambers, which consisted of cylindrical containers with a metal mesh on the bottom. Urine was collected from a Plexiglas surface fitted underneath the mesh. Urination usually occurred shortly after transfer to the chamber, and

animals were never kept in the chambers for longer than 180 min. A piece of food was always provided to each animal.

Measurement of cooperative contributions

Cooperative contributions were calculated through behavioural observations that were carried out on Days 2 and 5 of each treatment week (Figure 4.1). A scan protocol was used to record the behaviour of each colony member every 4 minutes, from a list of 17 pre-defined behaviours (Table 3.1). Scan observations lasted 12 hours, resulting in the collection of 180 behavioural data points for each group member. Sand was provided to the colonies every 2 hours, through the sand dispensers, during scan observations. All observations were carried out by two observers, who equally alternated observation shifts every 2 to 4 hours.

Quantification of oxidative stress

Superoxide dismutase (SOD) activity, glutathione (GSSG and GSH), and malondialdehyde (MDA) concentrations were assessed in erythrocyte homogenates. The latter was also assessed in plasma samples.

Erythrocytes were homogenized using 4 glass beads in phosphate buffer solution (PBS) for one minute at 30 Hz, sonicated in ice-cold bath for 5 min and centrifuged for 10 min at 11180 *g* at 4℃. The supernatant was collected, and aliquots were made for each of the three markers.

SOD activity was determined using a commercial kit (Cayman Chemical), after adjustment of the final dilution (1:2250 v/v). Samples were run in triplicates, yielding an intra-assay CV of

15.6%. The inter-assay CV was 7.6% and was calculated with control samples analysed 3 to 7 days later. These samples showed a lower intra-assay CV (7.8%).

Concentrations of GSSG and GSH were determined in erythrocyte homogenates following the protocol described by Mora et al. (2016) with minor modifications. Briefly, 4 µl of homogenate was mixed with 5 µl of trichloroacetic acid (TCA 5%), 5 µl of internal standard glutathione ethyl ester (GSHee 40 µg/ml; Sigma Aldrich), and 36 µl of water. The mixture was vortexed, kept on ice for 5min and centrifuged for 14 min at 21913 *g* and 4℃. Finally, 10 µl of the supernatant was diluted in 790 µl of water and 150 µl of the mixture was transferred into an HPLC vial. A standard curve containing GSH (Sigma Aldrich) and GSSG (Sigma Aldrich) at 2, 20, 100, 500 and 2000 ng/ml and GSHee at a constant concentration of 50 ng/ml was made. HPLC-MS analysis followed the description in Mora et al. (2016). All samples were analysed in one batch only, which yielded intra-assay CVs of 2.4% for GSSG and 10.0% for GSH.

Oxidative damage to lipids was assessed by quantifying the concentrations of MDA through derivatization with thiobarbituric acid (TBA), followed by separation by ultra-high pressure liquid chromatography (UHPLC) with fluorescence detection, following Mora et al. (2016). Although the MDA-TBA assay is increasingly criticized due to the potential artefactual MDA produced during the heating step, this was the method available and validated in our laboratory at the time of analysis. We nonetheless strongly advise the use of more accurate and sensitive methods, such as that described by Mendonça et al. (2017). To 15 µl of homogenate, or plasma, or standard (1,1,3,3-tetraethoxypropane, TEP; Sigma), 80 µl of TCA 5%, and 300 µl water, and 40 µl of TBA (Sigma) were added. Samples were vortex-mixed for 5 s and centrifuged for 14 minutes at 21913 *g* and 4℃. From the epiphase, 400 µl were transferred to a screw-top microcentrifuge, heated for 60 minutes at 100℃ in a dry bath incubator, and then cooled on ice for 5 minutes. The resulting TBA-adducts were extracted twice with 300 µl butanol. Organic

phases were combined and evaporated in a centrifugal evaporator (Labconco) at 35 °C for about 90 min. The dry residue was reconstituted in 90 µl of MeOH 30% and the resulting solution was sonicated, centrifuged and transferred into an HPLC vial. Quantification followed the method described by Mora et al. (2016). All samples were analysed in one batch only, which yielded intra-assay CVs of 2.3% for erythrocytes and 0.2% for plasma.

Quantification of urinary cortisol levels

Urinary CORT was quantified by radio-immunoassay (RIA), using a commercial kit (Coat a Count, Diagnostic Products Corporation), previously validated for Damaraland mole-rats (Clarke et al., 2001). The intra-assay CV was 5.5% and the inter-assay CV was 7.6%. CORT concentrations were corrected for urine dilution by determining the specific gravity of each sample using a digital hand-held pen refractometer (Atago, Ltd), following the description by Miller et al. (2004).

Data preparation and statistical analysis

Data exploration followed the recommendations of Zuur et al. (2013, 2010). A mixed modelling approach was used to account for the non-independency of the data, which was due to repeated measures on the same individuals. All analyses and their graphical representations were done using the software R (version 3.4.0) (Team, 2017). Linear mixed-effects models (LMMs) were fitted using the package *lme4* (Bates et al., 2015). Model validation was based on normality of residuals and homogeneity of variance, following Zuur et al. (2009) recommendations. Models' p-values reported were extracted from ANOVA tables using the package *car* (Fox and Weisberg, 2011). Statistical significance was set at $p < 0.05$.

The experiment of the present Chapter was the result of a collaboration, thus some relevant statistical analyses have been produced and presented elsewhere but are yet not published (Vullioud et al., in preparation). These include:

1) Effect of treatment on urinary CORT levels;
2) Treatment validation: comparison of urinary CORT levels during the Cortisol treatment with those measured in non-experimental female helpers under (i) a stable social environment (baseline) and (ii) an unstable social environment (stressful event; characterized by unusually high levels of conflict and aggression directed towards female helpers);
3) Effect of treatment on cooperative contributions;
4) Effect of treatment on changes in body mass.

Effect of treatment on oxidative stress

To evaluate the effect of treatment on oxidative balance, the levels (ln-transformed) of SOD, GSSG/GSH, GSSG, GSH, and MDA measured on Day 8 of each treatment were specified as response variables in different LMMs. Treatment (Control, Cortisol) was included as the explanatory variable of interest. Additionally, the respective marker level before implant (Day 1) was included as a covariate to control for individual initial oxidative status. Individual identity, nested within colony identity, was specified as a random effect in all models to account for the repeated-measures inherent to our design.

Relationship between CORT levels and oxidative stress

To assess whether CORT levels can predict levels of oxidative stress markers, models similar to those specified above were used, with the difference that urinary CORT level measured on Day 6 (ln-transformed) replaced the variable Treatment. We decided to use CORT levels of Day 6 due to their closer proximity in time to the oxidative stress measurements.

Relationship between cooperative contributions and oxidative stress

To assess whether cooperative contributions can predict levels of oxidative stress markers, models similar to those specified above were used, with the difference that the percentage of Burrow, the cooperative activity specifically increased by the Cortisol treatment (see Results), displayed on Day 5 replaced the variable Treatment. Response variables were ln-transformed whenever the normality of residuals assumption was not met. The variable Burrow was obtained by grouping all behaviours associated with tunnel maintenance and extension (Dig, Sweep, Kick, and Locomotion work; Table 3.1).

4.3. RESULTS

As mentioned above, some relevant results of the present experimental manipulation are part of a separate manuscript not yet published (Vullioud et al., in preparation). These include:

1) The Cortisol treatment significantly increased urinary CORT levels, causing a 3-fold increase in CORT, in comparison to the Control treatment (Appendix Figure A4.1);
2) Urinary CORT levels induced by the Cortisol treatment were significantly higher than those measured in non-experimental female helpers experiencing a stable social environment (baseline) and were not significantly different from the CORT levels measured in female helpers experiencing an unstable social environment (stressful event) (Appendix Figure A4.2). Nonetheless, the range of the CORT levels induced by the Cortisol treatment overlapped with that of the Control treatment and of female helpers experiencing a stable social environment (Appendix Figure A4.2). Altogether, these results show that the Cortisol treatment increased CORT levels within the physiological range secreted by Damaraland mole-rat female helpers;
3) Compared to the Control, the Cortisol treatment significantly increased cooperative contributions due to a specific effect on the expression of energetically demanding burrowing behaviours (Appendix Figure A4.3);
4) Treatment did not significantly affect changes in body mass (LMM; Treatment: estimate ± SE = 1.214 ± 0.928, $\chi^2 = 1.727$, p = 0.189).

Effect of treatment on oxidative stress

The effect of treatment partially met our initial predictions. Although the Cortisol treatment did not significantly affect antioxidant protection (SOD; Table 4.1, Model 1), cellular oxidation (GSSG/GSH; Table 4.1, Model 2), oxidized or reduced glutathione (GSSG or GSH; Table 4.1, Models 3 and 4, respectively) or oxidative damage to lipids (MDA; Table 4.1, Model 5) in erythrocytes (n = 24), it significantly increased the concentration of MDA in the plasma (n = 23; Table 4.1, Model 6; Figure 4.2), in comparison to the Control treatment.

Because the higher CORT levels induced by the Cortisol treatment were similar to those measured during a stressful event (see point 2 above), we tested if the effect of treatment was retained when only the CORT levels closer to baseline were considered (i.e. stable social environment). For this, we repeated the models above after removing observations associated with CORT levels higher than the third quartile of the Cortisol treatment (i.e. those similar to a stressful event). This did not qualitatively change the results (SOD: $p = 0.704$; GSSG/GSH: $p = 0.460$; GSSG: $p = 0.912$; GSH: $p = 0.159$; erythrocyte MDA: $p = 0.159$; plasma MDA: $p = 0.005$).

Relationship between CORT levels and oxidative stress

In line with our predictions, the relationship between CORT and oxidative stress markers followed the pattern described for the effect of treatment. Urinary CORT levels measured on Day 6 did not show any significant relationship with antioxidant protection (SOD; Table 4.2, Model 1), cellular oxidation (GSSG/GSH; Table 4.2, Model 2), oxidized or reduced glutathione (GSSG or GSH; Table 4.2, Models 3 and 4, respectively) or oxidative damage to lipids (MDA; Table 4.2, Model 5)) in erythrocytes (n = 24), but tended to positively correlated with the

concentration of MDA in the plasma (n = 23; Table 4.2, Model 6; Figure 4.3). These effects did not qualitatively change if the average weekly urinary CORT concentration (Day 3 and 6) was used as fixed effects instead (all $p > 0.06$).

Relationship between cooperative contributions and oxidative stress

The relationship between cooperative contributions and oxidative stress markers followed a different pattern to our prediction. The percentage of burrowing on Day 5 was negatively correlated with erythrocyte oxidation (GSSG/GSH) on Day 8, due to a significant positive effect of burrowing on reduced glutathione (GSH), but no effect on its oxidized form (GSSG) (Table 4.3; Models 2-4; Figure 4.4). The percentage of burrowing did not significantly correlate with the levels of the other markers in erythrocytes or plasma (Table 4.3, Models 1, 5 and 6).

Table 4.1 – LMMs examining the effect of treatment on levels of oxidative stress markers in erythrocytes and plasma on Day 8. Dataset used included 24 observations for models 1 to 5 and 23 observations for model 6, from 14 individuals belonging to 5 different colonies. Reference level of categorical fixed effect is: [1]control. Significant terms are shown in bold.

	Fixed effects	Estimate	SE	χ^2	p-value
Model 1. SOD					
Fixed effects	Intercept	2.017	0.867		
	Treatment [1]	-0.012	0.091	0.017	0.895
	Initial marker level	**0.697**	**0.135**	**26.608**	**< 0.001**
Random effects	Animal/Colony	0.000	0.000		
	Colony	0.000	0.000		
	Residual	0.046	0.214		
Model 2. GSSG/GSH					
Fixed effects	Intercept	-0.956	0.387		
	Treatment [1]	-0.089	0.114	0.608	0.435
	Initial marker level	0.329	0.220	2.235	0.135
Random effects	Animal/Colony	0.079	0.282		
	Colony	0.000	0.000		
	Residual	0.064	0.253		
Model 3. GSSG					
Fixed effects	Intercept	4.892	1.177		
	Treatment [1]	0.044	0.135	0.108	0.743
	Initial marker level	0.112	0.219	0.262	0.609
Random effects	Animal/Colony	0.000	0.000		
	Colony	0.026	0.161		
	Residual	0.100	0.317		
Model 4. GSH					
Fixed effects	Intercept	6.753	1.266		
	Treatment [1]	0.076	0.057	1.727	0.189
	Initial marker level	0.038	0.179	0.045	0.833
Random effects	Animal/Colony	< 0.001	< 0.001		
	Colony	< 0.001	< 0.001		
	Residual	< 0.001	< 0.001		
Model 5. MDA erythrocytes					
Fixed effects	Intercept	0.574	0.134		
	Treatment [1]	0.149	0.118	1.584	0.208
	Initial marker level	0.043	0.146	0.089	0.766
Random effects	Animal/Colony	0.000	0.000		
	Colony	0.022	0.148		
	Residual	0.081	0.284		
Model 6. MDA plasma					
Fixed effects	Intercept	-0.091	0.068		
	Treatment [1]	**0.182**	**0.076**	**5.789**	**0.016**
	Initial marker level	**0.542**	**0.101**	**28.834**	**< 0.001**
Random effects	Animal/Colony	0.000	0.000		
	Colony	0.011	0.104		
	Residual	0.030	0.173		

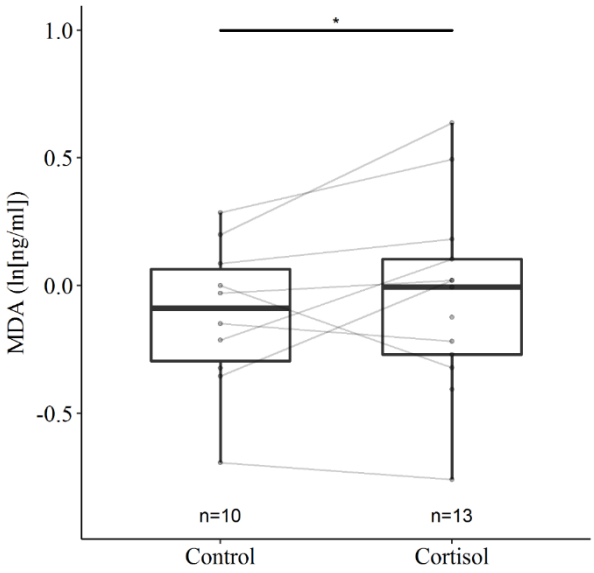


Figure 4.2 – Effect of treatment on plasma MDA concentrations. Lines between points illustrate the repeated measurements of same individuals. *, $p < 0.05$.

Table 4.2 – LMMs examining the effect of CORT concentration on levels of oxidative stress markers in erythrocytes and plasma on Day 8. Dataset used included 24 observations for models 1 to 5 and 23 observations for model 6, from 14 individuals belonging to 5 different colonies. Significant terms are shown in bold.

	Fixed effects	Estimate	SE	χ^2	p-value
Model 1. SOD					
Fixed effects	Intercept	2.064	0.861		
	CORT level	-0.023	0.044	0.267	0.605
	Initial marker level	**0.693**	**0.133**	**27.145**	**< 0.001**
Random effects	Animal/Colony	0.001	0.038		
	Colony	< 0.001	0.024		
	Residual	0.044	0.209		
Model 2. GSSG/GSH					
Fixed effects	Intercept	-1.099	0.366		
	CORT level	0.004	0.065	0.004	0.947
	Initial marker level	0.274	0.214	1.646	0.199
Random effects	Animal/Colony	< 0.001	< 0.001		
	Colony	< 0.001	< 0.001		
	Residual	< 0.001	< 0.001		
Model 3. GSSG					
Fixed effects	Intercept	4.738	1.126		
	CORT level	0.077	0.066	1.332	0.248
	Initial marker level	0.130	0.207	0.394	0.530
Random effects	Animal/Colony	0.000	0.000		
	Colony	0.025	0.157		
	Residual	0.095	0.308		
Model 4. GSH					
Fixed effects	Intercept	7.073	1.205		
	CORT level	0.052	0.031	2.826	0.093
	Initial marker level	-0.009	0.171	0.003	0.957
Random effects	Animal/Colony	0.031	0.175		
	Colony	0.000	0.000		
	Residual	0.014	0.118		
Model 5. MDA erythrocytes					
Fixed effects	Intercept	0.661	0.145		
	CORT level	-0.018	0.064	0.079	0.778
	Initial marker level	0.066	0.152	0.189	0.664
Random effects	Animal/Colony	< 0.001	< 0.001		
	Colony	< 0.001	< 0.001		
	Residual	< 0.001	< 0.001		
Model 6. MDA plasma					
Fixed effects	Intercept	-0.073	0.072		
	CORT level	0.074	0.040	3.422	0.064
	Initial marker level	**0.514**	**0.105**	**24.146**	**< 0.001**
Random effects	Animal/Colony	0.000	0.000		
	Colony	0.012	0.111		
	Residual	0.033	0.181		

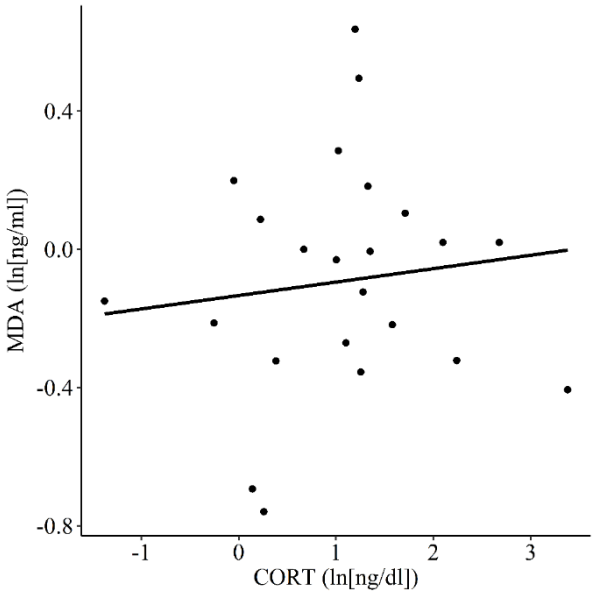


Figure 4.3 – Relationship between urinary CORT concentrations on Day 6 and plasma MDA concentrations on Day 8.

Table 4.3 – LMMs examining the effect of cooperative contributions (% Burrow) on levels of oxidative stress markers in erythrocytes and plasma on Day 8. Dataset used included 24 observations for models 1 to 5 and 23 observations for model 6, from 14 individuals belonging to 5 different colonies. Significant terms are shown in bold.

	Fixed effects	Estimate	SE	χ^2	p-value
Model 1. SOD					
Fixed effects	Intercept	2.120	0.864		
	Burrow	-0.003	0.005	0.234	0.628
	Initial marker level	**0.685**	**0.131**	**27.163**	**< 0.001**
Random effects	Animal/Colony	0.000	0.000		
	Colony	< 0.001	0.007		
	Residual	< 0.001	0.213		
Model 2. GSSG/GSH					
Fixed effects	Intercept	0.225	0.048		
	Burrow	**-0.005**	**0.002**	**8.716**	**0.003**
	Initial marker level	0.328	0.188	3.054	0.081
Random effects	Animal/Colony	0.003	0.059		
	Colony	0.002	0.040		
	Residual	0.002	0.050		
Model 3. GSSG					
Fixed effects	Intercept	218.851	59.540		
	Burrow	-0.651	2.171	0.090	0.764
	Initial marker level	0.225	0.233	0.934	0.334
Random effects	Animal/Colony	0.000	0.000		
	Colony	2102	45.85		
	Residual	5627	75.01		
Model 4. GSH					
Fixed effects	Intercept	6.594	1.144		
	Burrow	**0.011**	**0.004**	**7.625**	**0.006**
	Initial marker level	0.047	0.162	0.084	0.772
Random effects	Animal/Colony	0.013	0.115		
	Colony	0.000	0.000		
	Residual	0.018	0.132		
Model 5. MDA erythrocytes					
Fixed effects	Intercept	0.645	0.128		
	Burrow	0.038	0.068	0.315	0.575
	Initial marker level	0.055	0.148	0.137	0.711
Random effects	Animal/Colony	0.000	0.000		
	Colony	0.028	0.168		
	Residual	0.083	0.289		
Model 6. MDA plasma					
Fixed effects	Intercept	-0.012	0.089		
	Burrow	0.002	0.006	0.074	0.785
	Initial marker level	**0.490**	**0.106**	**21.388**	**< 0.001**
Random effects	Animal/Colony	0.000	0.000		
	Colony	0.009	0.094		
	Residual	0.040	0.201		

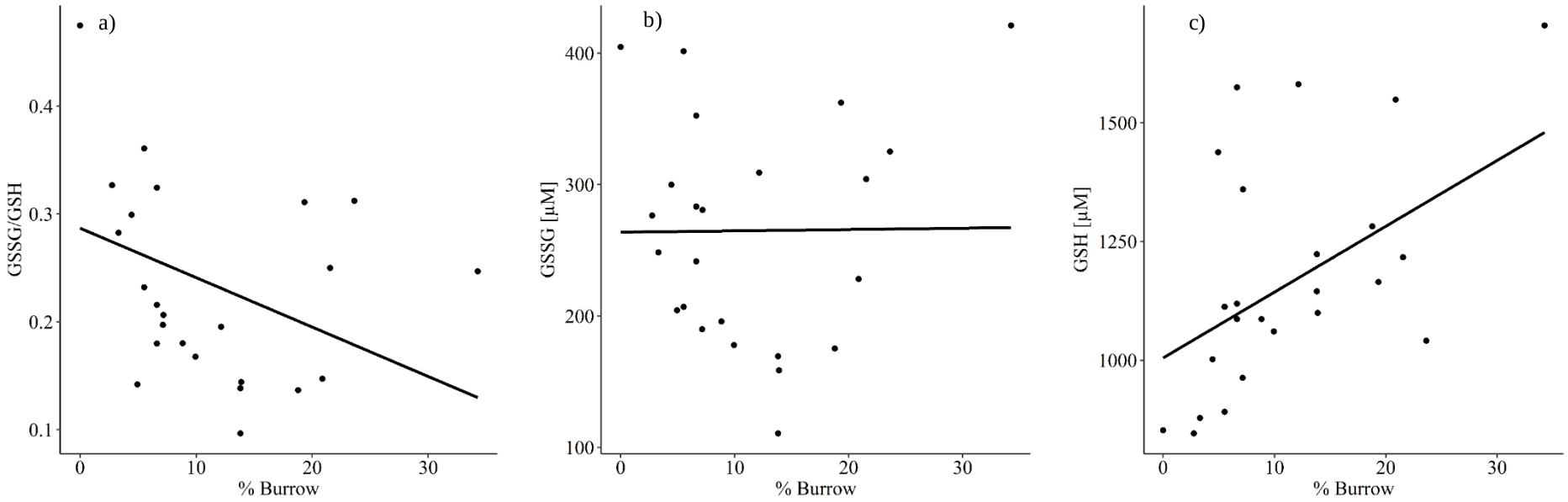


Figure 4.4 – Relationship between the percentages of burrowing behaviour on Day 5 and levels of (a) cellular oxidation (GSSG/GSH), (b) oxidized glutathione (GSSG) and (c) reduced glutathione (GSH), in erythrocytes on Day 8.

4.4. DISCUSSION

In this study, we present the first experimental evidence that increasing GC levels induces oxidative stress and leads to the accumulation of oxidative damage in the plasma, but not in erythrocytes, of Damaraland mole-rat female helpers. Our results also provide the first indication that GC-induced oxidative stress can arise within a range of GC levels which is not associated with stressful events. The different correlations between GC levels or cooperative contributions and oxidative stress markers do not support the hypothesis that GCs represent a link between energetically demanding cooperative contributions and their oxidative stress costs. Nonetheless, this is the first time that a causal link between elevated GC levels and oxidative stress is reported in a cooperative breeder, a pattern that corroborates the findings in non-cooperative species (Costantini et al., 2011).

Experimentally increased CORT levels over a period of seven days, resulted in significantly higher levels of oxidative damage to lipids (MDA) in the plasma, suggesting that, at the organismal level, the Cortisol treatment induced oxidative stress. Blood plasma interacts with all tissues and is responsible for transporting their excretion products, including MDA, thus oxidative damage measured in the plasma provides an integrated overview of the whole organism. The negative impact of higher GC levels on overall oxidative balance and associated damage is in line with findings for reptiles (Cote et al., 2010), birds (Costantini et al., 2008; Eid et al., 2003; Lin et al., 2004b), and mammals (Orzechowski et al., 2000; Rajashree and Puvanakrishnan, 1998). In rats, for instance, experimentally increased GC levels over a period of at least eight days induces oxidative stress and results in higher oxidative damage to lipids in blood plasma (Orzechowski et al., 2002, 2000), heart and kidneys (Rajashree and Puvanakrishnan, 1998).

Our results suggest that increases in GC levels both within baseline and stress-induced ranges, are accompanied by oxidative costs. Baseline GC levels are associated with the predictable daily and seasonal variations in environmental and life-history demands, whereas stress-induced levels refer to unpredictable threatening events, which demands surpass the ones of the current season/life-history stage (Landys et al., 2006; Wingfield et al., 1998). Our Cortisol treatment induced CORT levels that were similar to the levels measured around events of high social instability, characterized by a threatening setting where female helpers are aggressively marginalized from their natal group by the dominant breeder. Interestingly, the effect of treatment on plasma MDA was still significant when only CORT levels similar to those within the baseline range were considered, suggesting that increases within baseline GC levels can also incur physiological costs in the form of oxidative damage. This is ecologically relevant since (i) increases in baseline GC levels can upregulate energetically demanding cooperative contributions in Damaraland mole-rats (Young et al., 2010; Vullioud et al., in preparation) and other cooperative breeders (Carlson et al., 2006a; Dantzer et al., 2017b; Sanderson et al., 2014) and (ii) such activities are commonly expressed in the absence of a stressor in cooperatively breeding societies (Dantzer et al., 2017b). This is in line with the finding that Damaraland mole-rat female helpers have higher CORT levels during the wet season (Young et al., 2010), probably to support increased energetic demands related to higher expression of burrowing behaviours (Jarvis and Bennett, 1993), but may also reflect increased social instability during the wet season (Young et al., 2010). Furthermore, the almost significant and positive correlation between CORT and MDA levels of our study casts doubt on the idea that GC-induced oxidative stress is independent of experimental GC dosage (Costantini et al., 2011). Instead, our results suggest that the higher CORT levels may induce the greater levels of oxidative stress. It should, however, be noted that the suggestion by Costantini et al. (2011), was based exclusively on studies where GC levels were raised towards physiological

maximums (stress-induced) levels. We advocate that the effects of baseline GC fluctuations on oxidative stress should not be neglected, and that research in other species must also investigate this issue.

As opposed to our predictions, the oxidative balance of erythrocytes was not affected by the Cortisol treatment, suggesting that these cells may have low susceptibility to GC-induced oxidative stress. It has recently been proposed that tissues/organs differ in their vulnerability to GC-induced oxidative stress and that blood is among the less vulnerable ones (Costantini et al., 2011). Because in that meta-analysis results of studies assessing effects in whole-blood, plasma, serum and erythrocytes were grouped together, it is impossible to infer the susceptibility of each blood fraction separately. In addition, few studies on the specific effect of GCs on erythrocytes are currently available and the resilience of erythrocytes towards GC-induced oxidative stress remains elusive (Orzechowski et al., 2002; Stier et al., 2009). An alternative explanation to our result is that increased CORT levels might have stimulated erythropoiesis (production of new erythrocytes), which could have diluted any effect of treatment on erythrocytes' oxidative stress markers (Bauer et al., 1999; Golde et al., 1976).

The significant and negative relationship between cooperative contributions and cellular oxidation (GSSG/GSH) identified here, appears to be driven by an increase in reduced glutathione (GSH) and a lack of effect on its oxidized form (GSSG). This result contrasts with the pattern detected in our previous research, where experimentally increasing burrowing activities through increased sand provisioning resulted in higher (not lower) levels of cellular oxidation, due to a greater increase in oxidized glutathione (GSSG) than its reduced form (GSH) (Chapter 3). The opposing results of the two studies possibly reflect differences in the delay between stimulation of burrowing activities (i.e. sand provisioning) and sampling. Whereas in our previous experiment blood samples were collected while cooperative

contributions were being stimulated (high sand provisioning during sampling day), in the present study, blood samples were collected 12h after the last stimulation of cooperative activities (high sand provisioning was interrupted at the end of day 7, and blood samples collected in the morning of day 8). The pattern presented here may thus represent a recovery from increased oxidation levels following greater energetically demanding cooperative contributions. Upon an oxidative threat, GSH is oxidized into GSSG to neutralize ROS, but is converted back to GSH when the threat is removed (Morgan et al., 2013). If cells increased GSH production as a protective measure during the oxidative threat, this may lead to an increase in GSH upon recovery of the oxidative balance, and consequently a decrease in cellular oxidation, as observed in our results. These apparently contradictory results highlight the importance of appropriate sampling timing and consistent experimental designs if results are to be compared.

Finally, our experimental results raise the question as to whether GCs may represent a physiological mechanism linking energetically demanding cooperative contributions to their oxidative stress costs. The simultaneous increases of cooperative contributions (Vullioud et al., in preparation) and plasma oxidative damage caused by the Cortisol treatment, and the significant and positive relationship between CORT levels and plasma oxidative damage, support this notion. These results also suggest that higher CORT levels are associated with both greater energetic demands and oxidative costs. However, the different relationship between oxidative stress markers and cooperative contributions do not support our hypothesis: burrowing activities were significantly and negatively correlated with cellular oxidation (GSSG/GSH) in erythrocytes. Together, these results suggest that the impact of GCs on oxidative balance may be independent from the impact of energetically demanding cooperative contributions. Further experimental work is thus necessary to fully disentangle the effects of

GC levels and those of cooperative contributions on oxidative balance, and to test how these costs interact (i.e. additive, synergistic, or antagonistic interactions).

In conclusion, our findings show that increases in CORT levels over one week induce oxidative stress and result in higher levels of plasma oxidative damage to lipids, in female helpers of the cooperatively breeding Damaraland mole-rat. It has been suggested that chronic stress-related GC levels can affect an individual's fitness through its oxidative costs (Costantini et al., 2011). Our results further support the possibility that fluctuations within baseline GC levels may have similar effects. In Damaraland mole-rats, baseline CORT levels are higher during the wet season of the Kalahari desert, a period that lasts about seven months (approximately from October to May) and where breeding opportunities for helpers may be more frequent (Young et al., 2010). We therefore hypothesise that during the wet season oxidative damage is increased, possibly to levels even higher than those reported here, which may negatively impact an individual's fitness.

4.5. APPENDIX

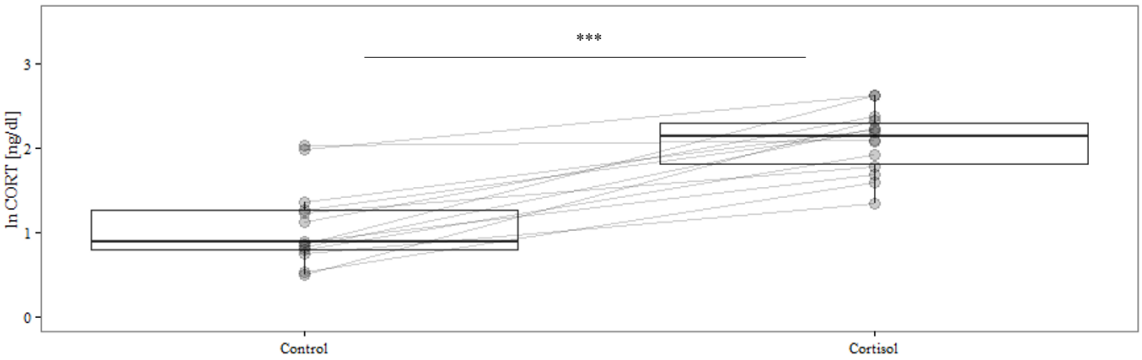


Figure A4.1 - Effect of treatment on CORT levels measured during the control (left) and cortisol (right) treatments. Lines between points illustrate the repeated measurements of same individuals. LMM: ***, $p < 0.001$. (Source: Vullioud et al., in preparation).

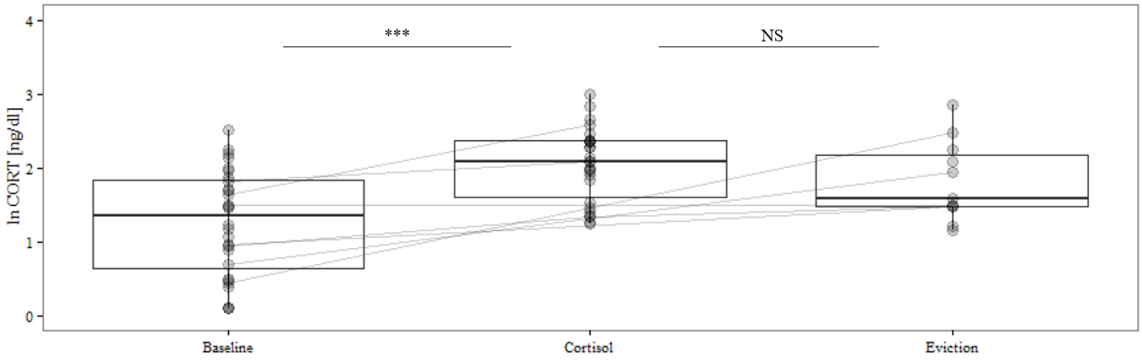


Figure A4.2 - Comparison of the CORT levels measured during the Cortisol treatment (middle) with those measured in female helpers experiencing a stable social environment (left) and an unstable social environment (right). LMM: NS, $p > 0.05$; ***, $p < 0.001$. (Source: Vullioud et al., in preparation).

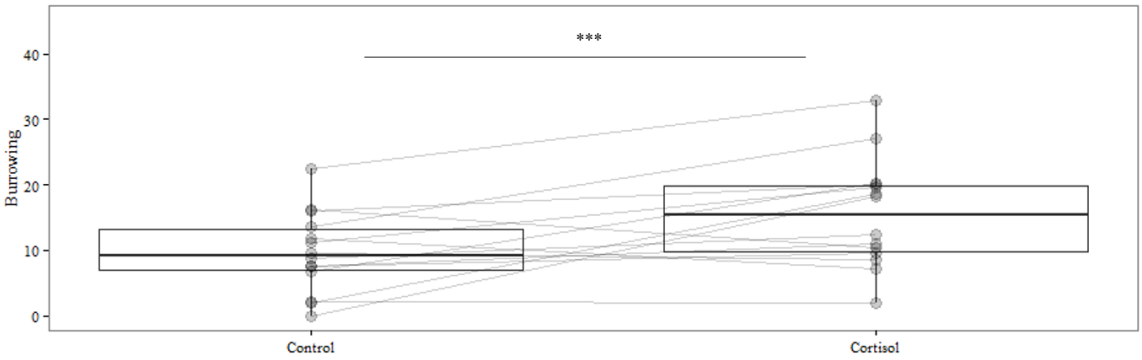


Figure A4.3 - Effect of treatment on individual percentages of Burrowing activities, displayed during the 12 hours scan sessions (averaged over day 2 and day 5). Lines between points illustrate the repeated measurements of same individuals. LMM: ***, $p < 0.001$). (Source: Vullioud et al., in preparation)

Chapter 5

General Discussion

With this thesis, I investigated the physiological costs of cooperative behaviours using a cooperatively breeding mammal, the Damaraland mole-rat, as a model species. I hypothesized that one such cost could be oxidative stress, particularly when cooperative behaviours are energetically demanding.

In Chapter 2, I developed a method to quantify the concentration of malondialdehyde (MDA), a marker of lipid peroxidation, i.e. oxidation of phospholipids caused by reactive oxygen species (ROS). This is a more precise, accurate, and sensitive method than the one previously available, which was based on the derivatization of MDA with thiobarbituric acid (TBA). The greatest weakness of the MDA-TBA assay is the consistent presence of MDA peaks in blank samples (where no MDA should be detected), produced as an artefact of the high temperatures (100 ºC) used during the derivatization step. MDA peaks in blank samples lead to high limits of quantification (LOQ), thereby precluding its quantification in samples with low MDA content. This issue applied particularly to ejaculate samples of Damaraland mole-rats whose volumes are small and MDA concentrations are low, only quantifiable with the method developed here. This method relies on the use of 2,4-dinitrophenylhydrazine (DNPH) as an alternative derivatization agent to TBA and allows this step to occur at a lower temperature (60 ºC), resulting in the absence of artificial MDA peaks in blank samples and in a lower LOQ compared to the MDA-TBA assay. The MDA-DNPH method has now been established as the standard methodology to quantify MDA in samples from vertebrates, invertebrates, and plants, by several research groups at the University of Neuchâtel.

In Chapter 3, I conducted the first experimental investigation of the oxidative costs of energetically demanding cooperative behaviours in a cooperatively breeding species. I experimentally increased the voluntary expression of burrowing behaviours by increasing the daily total amount of sand provided to captive colonies of Damaraland mole-rats. I tested

whether increases in cooperative contributions could generate oxidative stress and lead to accumulation of oxidative damages (MDA), and whether in males, protection against such potential damages may be traded-off between the soma and the germline. I showed that independent of sex, breeding status, or body mass, individuals increased their cooperative contributions and a week-long treatment generated oxidative stress, as evidenced by the higher cellular oxidation in erythrocytes, measured as the ratio between oxidized and reduced glutathione (GSSG/GSH). Such increases in cellular oxidation did not translate into higher levels of oxidative damage to lipids (MDA) in erythrocytes, yet it did so in ejaculates of male helpers, suggesting that, overall, helpers may favour somatic over germline protection against oxidative stress.

In Chapter 4, I investigated whether the oxidative costs of energetically demanding cooperative behaviours are the direct consequence of an increase in their expression, or the indirect consequence of an increase in cortisol (CORT), a glucocorticoid (GC) hormone that upregulates the expression of burrowing behaviours in Damaraland mole-rats. A week-long experimental increase in CORT levels of female helpers did not affect cellular oxidation but resulted in increased plasma MDA levels. The different results obtained in Chapter 3 and 4 indicate that the oxidative costs imposed by elevated CORT levels and those imposed by energetically demanding cooperative behaviours may be, at least partially, independent.

Overall, the results presented in my thesis suggest that the energetically demanding burrowing behaviours may induce oxidative costs. Yet, whether other forms of energetically demanding cooperative behaviours pose similar oxidative costs in Damaraland mole-rats and other species is currently unknown. Furthermore, my work highlights the short-term oxidative costs of cooperative behaviours and further studies are necessary to understand whether these can translate into long-term costs susceptible of affecting individuals' fitness.

5.1. Short-term oxidative costs of cooperative behaviours

The results presented in this thesis suggest that the expression of cooperative behaviours can alter the oxidative balance, with greater cooperative contributions inducing higher levels of oxidative stress (Chapter 3). This is likely a widespread phenomenon, specifically when a cooperative behaviour entails an energetic demand. Indeed, production of cellular energy under the form of ATP is a requirement for the expression of such cooperative behaviours, and may lead to the by-product formation of ROS, with the potential to disrupt oxidative balance (Davies et al., 1982; Halliwell and Gutteridge, 2007).

Several forms of cooperative behaviours have been directly shown to be energetically demanding through their positive effects on metabolism and may, therefore, be hypothesized to induce oxidative costs similar to those presented in Chapter 3. In cooperatively breeding cichlids, digging behaviours used for territory maintenance, lead to a 5-fold increase of the resting metabolic rate (Grantner and Taborsky, 1998), similarly to that found in Damaraland mole-rats (Lovegrove, 1989). Also, in cooperatively breeding birds, provisioning food to dependent nestlings has been shown to increase metabolic rate (Anava et al., 2001) and to disrupt the oxidative balance (Cram et al., 2015b).

Other forms of cooperative behaviours have been indirectly shown to pose an energetic demand through their negative effects on body mass. In species where the expression of energetically demanding cooperative behaviours also negatively impacts food intake, the oxidative costs may be more pronounced than the ones shown in Damaraland mole-rats. In many cooperatively breeding species, offspring provisioning entails the energetic demand associated with searching for food items and the forgoing of that meal, which is provisioned to the dependent pups (Clutton-Brock, 2016; Koenig and Dickinson, 2016). Although the effects of short-term

partial food restriction on oxidative balance are still unclear, long periods of food restriction can stimulate the formation of ROS, affecting antioxidant protection and leading to the accumulation of oxidative damage (Costantini, 2014).

When GC secretion positively influences the expression of energetically demanding cooperative behaviours, it may impose additional oxidative costs (Chapter 4). The influence of GCs on cooperative behaviours is a largely unexplored field with most evidence available from cooperatively breeding mongooses (Carlson et al., 2006a, 2006b; Dantzer et al., 2017b; Sanderson et al., 2014; Santema et al., 2013). It is, however, generally recognized that high GC levels negatively impact oxidative balance by means of increased ROS formation (e.g. Wilson et al., 2013) and/or decreased antioxidant protection (e.g. Pereira et al., 1994). It could thus be hypothesized that in the cases where high GC levels positively influence the expression of cooperative behaviours (or vice-versa), oxidative costs may be expected.

The energetic and oxidative costs of cooperative behaviours may be mitigated by external variables such as group size. In many cooperatively breeding species individual cooperative contributions are lower in larger groups (Brown et al., 1978; Carlson et al., 2006a, 2006b, Clutton-Brock et al., 2001a, 2000; Heinsohn and Cockburn, 1994; Mumme and de Queiroz, 1985; Ridley, 2016; Wright, 1997). Group size may thus alleviate the energetic (Anava et al., 2001) and oxidative costs of cooperation due to its effects on diminishing the required contribution per individual (Cram et al., 2015b; Lardy et al., 2016). In my thesis, I could not explore the effect of group size on burrowing contributions and oxidative balance since the experimental work was carried out in groups with minor differences in group size (Chapter 3). It is possible that in wild Damaraland mole-rats, the costs of burrowing behaviours are mitigated in larger groups. Indeed, large groups usually inhabit areas of high food density

(Jarvis et al., 1998), which may result in smaller distances that need to be burrowed by a larger workforce to obtain food.

In Damaraland mole-rats, cooperative contributions other than burrowing activities could alter oxidative balance and add up to the oxidative costs shown for burrowing activities. Transport of food items, which serves to the building of food stores (Jarvis et al., 1998), is likely to be energetically demanding and thus to induce changes in oxidative balance. In wild conditions, food items weighing up to 10% of an adult's average body mass (Jarvis et al., 1998) can be carried for distances equivalent to 2000 times the average adult body length (Thomas et al., 2016), from the extremities of the tunnel system to storage areas usually located close to the nest (Jarvis et al., 1998). The results of Chapter 3 did not comprise the oxidative costs of food transport since this activity was not changed by the experimental manipulation of sand provisioning of the colonies. Manipulations of the amount and size of transportable food and of the distance between food source and storage area, while keeping burrowing activities constant, would be necessary to test the possibility that food transport generates oxidative costs.

In contrast to food transport, allo-parental care and nest building activities are less likely to alter individual oxidative balance in Damaraland mole-rats because their energetic demands and frequency of expression are expected to be low (Zöttl et al., 2016). Allo-parental care is most evidently expressed when helpers retrieve young wandering pups back to the safety of the nest, a behaviour only sporadically expressed in captive conditions during a few weeks following the birth of the pups, (Bennett and Jarvis, 1988; Zöttl et al., 2016). Nest building, consisting of collection, transport and accumulation of light material (dried vegetable material in the wild and paper towelling in the laboratory) to the nesting area is unlikely to pose great energetic cost. Nonetheless, the energetic and oxidative costs of these behaviours have not been explored in my thesis and further experimental manipulation would be necessary to support

this claim. Standardized tests aimed at manipulating these behaviours, coupled with measurements of metabolic rates and oxidative stress markers would be crucial to shed some light on their physiological costs.

Further studies are now necessary to evaluate the oxidative costs of cooperative behaviours in other species, and whether and how external (e.g. social, environmental) and internal (e.g. hormonal) variables influence such costs. Additionally, whether the short-term oxidative costs of cooperation can translate into long-term costs is currently unknown and deserves further attention as they may affect individuals' fitness.

5.2. Short-term oxidative costs vs long-term fitness costs of cooperative behaviours

The short-term oxidative costs of energetically demanding cooperative behaviours may accumulate and, in the long-term, negatively affect individuals' fitness. This possibility stems from research showing that oxidative stress is often negatively correlated with health, fecundity and survival (Bize et al., 2008; Cram et al., 2015a; Saino et al., 2011), explained by the detrimental effects of accumulating oxidative damage to lipids, proteins, and DNA on cell and tissue functioning (Costantini, 2014; Finkel and Holbrook, 2000; Harshman and Zera, 2007; Monaghan et al., 2009). Empirical evidence for this hypothesis comes from earlier studies suggesting that in the stripe-backed wren (Campylorhychus nuchalis; Stacey and Koenig, 1990) and in the pied kingfisher (Ceryle rudis; Reyer, 1984), more generous helpers during a breeding season have lower chances of survival to the next year. More recent work using meerkat female helpers has suggested that cooperative contributions may decrease fecundity, as cooperative contributions were negatively correlated with the probability of conceiving in

their natal group (Russell et al., 2003b). In Damaraland mole-rats, increased cooperative contributions lead to higher levels of oxidative damage in the germline (Chapter 3), prone to impact fertility (Tremellen, 2008). Altogether, these studies support the possibility that short-term oxidative costs of cooperative behaviours may accumulate and negatively impact the survival and fecundity of helpers.

High GC levels may represent an additional physiological mechanism promoting the long-term fitness costs of energetically demanding cooperative behaviours. GCs have been shown both to promote and to be raised after the expression of cooperative behaviours in cooperatively breeding mammals (Carlson et al., 2006a, 2006b; Dantzer et al., 2017b; Sanderson et al., 2014), including the Damaraland mole-rat, where experimental manipulations showed that GC levels raise cooperative contributions and vice-versa (Vullioud et al., in preparation). Additionally, high GC levels have been shown to induce oxidative stress (e.g. Costantini et al., 2011), including in the Damaraland mole-rat where experimentally increased GC levels led to higher levels of oxidative damage (Chapter 4), and to negatively affect survival (Romero and Wikelski, 2001). Thus, elevated GC levels maintained over long periods of time (as a consequence or as a means to promote cooperative behaviours) could lead to a constant generation and accumulation of oxidative damage, with the potential to affect the lifetime fitness of helpers (Costantini, 2014; Finkel and Holbrook, 2000; Harshman and Zera, 2007; Monaghan et al., 2009).

Individuals may, however, employ strategies to avoid the building-up of the short-term oxidative costs of cooperative behaviours into long-term fitness costs. Behavioural adjustments could represent one of such strategies. Individual adjustments of cooperative contributions according to their past contributions and current conditions have been shown in a variety of species (Meade and Hatchwell, 2010; Mumme, 1992; Russell et al., 2003b; Sanderson et al.,

2014; Wright, 1997) and proposed as an explanation for the general lack of a negative relationship between cooperative contributions and fitness costs (Clutton-Brock, 2016). Supporting this possibility, in the Seychelles warbler, females that refrained from provisioning the breeding pair's brood had higher levels of oxidative damage prior to the breeding season, compared to those that helped (van de Crommenacker et al., 2011), suggesting that only individuals with low levels of oxidative damage could afford the additional oxidative cost of energetically demanding cooperative behaviours. Yet, in Damaraland mole-rats, investment in cooperative activities was not conditional on initial oxidative status (Chapter 3). Although the captive setting may have promoted good physiological conditions that precluded the detection of such effect, a lack of behavioural adjustment according to oxidative balance was reported in wild white-browed sparrow weavers (Cram et al., 2014). In the absence of such behavioural adjustments, the short-term oxidative costs of cooperative contributions are more likely to accumulate over time and lead to fitness costs.

Physiological adaptations could represent another strategy employed to avoid long-term costs of energetically demanding cooperative behaviours. These may include upregulation of antioxidant production, upregulation of oxidative damage repairing mechanisms, and changes of the lipid composition of cellular membranes (Costantini, 2008; Hulbert et al., 2007; Radak et al., 2008, 2001). For instance, increasing the saturation level of phospholipids in cellular membranes may be a beneficial structural defence against lipid peroxidation, but can drastically change the fluidity of the cell and alter its functioning (Clandinin et al., 1991; Spector and Yorek, 1985). Also, upregulation of antioxidant defences and repairing mechanisms may face limitations and generate trade-offs between competing demands/tissues (Chapter 3).

The hypothesized long-term costs of energetically demanding cooperative contributions remain merely speculative. Experimental manipulations of cooperative contributions coupled

with measures of fitness are now much needed since evidence of long-term fitness costs (or the lack of it) is, so far, exclusively correlational (Heinsohn and Legge, 1999).

5.3. Oxidative stress and life-history trade-offs

Plastic adjustments of life-history trade-offs, according to external (ecological) and internal (physiological) cues, are expected to maximise an individual's fitness (Drenos and Kirkwood, 2005; Perrin and Sibly, 1993; Stearns, 2000, 1992, 1989). In recent years, oxidative stress has been proposed to mediate life-history trade-offs (Dowling and Simmons, 2009; Metcalfe and Alonso-Alvarez, 2010; Monaghan et al., 2009; Speakman and Garratt, 2013), such as that between survival and reproduction, yet empirical evidence is still scarce. In this thesis, I attempted to shed some light as to whether and how individuals trade-off resources for oxidative protection between somatic and germline tissues under an oxidative challenge. Although life-history trade-offs are expected to be dependent on variables such as reproductive status, I was unable to specifically test this hypothesis due to the small sample size of ejaculates obtained from breeding males.

Cooperatively breeding species represent interesting model systems to understand how reproductive opportunities shape the trade-off between survival and reproduction. Particularly in species with high reproductive skew, breeding status greatly predicts reproductive prospect since breeders monopolize reproduction and helpers do not reproduce while in their natal colony (Clutton-Brock, 2016). Furthermore, reproductive prospects are expected to influence the trade-off between survival and reproduction (Perrin and Sibly, 1993). Individuals with certain reproductive opportunities are expected to favour reproduction over survival, while those facing uncertain reproductive opportunities are predicted to favour survival over

reproduction, a strategy that would increase the chances of living until reproductive opportunities become more promising. Accordingly, my results suggest that Damaraland mole-rat male helpers, who lack reproductive opportunities, prioritize somatic over germline protection against the accumulation of oxidative damage during an oxidative challenge, as evidenced by a greater increase in oxidative damage in ejaculates than in erythrocytes (Chapter 3). Such strategy may be highly advantageous for non-reproducing individuals of cooperatively breeding species and reflect their investment in current indirect fitness benefits.

Whether breeders and helpers differentially trade-off resources between somatic and germline protection against oxidative stress remains, at the moment, purely speculative. From the results of Chapter 3, it can be gathered that, similar to helpers, male breeders facing an oxidative threat did not accumulate somatic oxidative damage. Three different possible scenarios can thus be formulated regarding breeders' trade-off between somatic and germline protection (Figure 5.1). The first two possible scenarios (Figure 5.1a,b) imply that the protection resources against accumulation of oxidative damage of breeders are not subjected to limitations. Male breeders would thus be able to equally protect their soma and germline, avoiding accumulation of oxidative damage in both tissues (Figure 5.1a), or even prioritize germline protection and damage repair, thus decreasing oxidative damage to this tissue, as long as somatic tissues are not affected (Figure 5.1b). These two possibilities rely on the assumption that breeders have privileged access to resources (Clutton-Brock, 2016), which allows them to enjoy a superior oxidative protection compared to helpers, similarly to what has been found in honeybees (*Apis mellifera*; Aamodt, 2009; Haddad et al., 2007). Moreover, the high predictability of reproductive opportunities may favour breeders' higher investment in germline protection, compared to helpers (Perrin and Sibly, 1993).

The third possible scenario suggests that breeders are subjected to similar limitations and follow the same trade-off strategy as helpers, prioritizing somatic over germline protection during an oxidative challenge (Figure 5.1c). This scenario would imply that the germline of breeders would accumulate damage during an oxidative threat. In stable colonies of Damaraland mole-rats, the lack of reproductive competition may have favoured male breeders who do not invest heavily in germline quality, as long as their spermatozoa are capable of fertilization. In favour of this possibility, a correlative study examining the number and motility of spermatozoa in Damaraland mole-rats did not find any differences between breeders and helpers (Faulkes et al., 1994), suggesting that, in this species, breeding status may not play a significant role in germline investment.

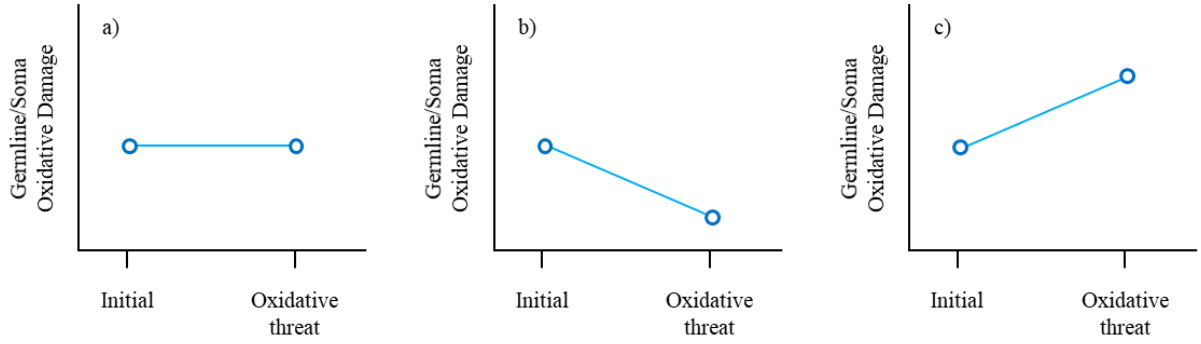


Figure 5.1 – Possible scenarios for the outcome of a trade-off between germline and soma protection against accumulation of oxidative damage upon an oxidative threat, in Damaraland mole-rat male breeders. These scenarios are built upon the result from Chapter 3: oxidative damage is not accumulated in a somatic tissue. Increasing values on the y-axis thus must result from an accumulation of damage in the germline. In scenario a), oxidative damage is not accumulated neither in the germline nor in the soma, suggesting that male breeders are not limited in antioxidant protection. In scenario b), oxidative damage is decreased in the germline of breeders suggesting a biased protection of this tissue. In scenario c), oxidative damage is increased in the germline, suggesting that breeders face comparable limitations and follow a similar trade-off strategy in antioxidant protection to that displayed by helpers.

Population density may represent a relevant cue for adjustments of the trade-off between somatic and germline protection against oxidative stress, through effects on reproductive opportunities. Indeed, individuals living in highly populated areas may face reproductive opportunities and competition more often than in low population density areas. In Damaraland mole-rats, this could be due to a greater chance that the tunnels of two neighbouring colonies overlap and individuals encounter potential mates (i.e. unfamiliar conspecifics). It could then be hypothesized that individuals living in high population density areas invest in their germline to a greater degree than those in low density areas. Specifically, it would be expected that helpers, who are limited in some antioxidant machinery (Chapter 3), would have lower levels of ejaculate oxidative damage and higher levels of somatic damage in high population density areas. Male breeders would also be expected to have lower damage in their ejaculates in high populated areas, but if this would negatively impact their soma greatly depends on whether they face the same limitations as helpers. Such a strategy would result in increased direct fitness benefits for both breeders and helpers.

The possibility that GCs play a role in the modulation of life-history trade-offs, and particularly that between the soma and the germline, remains largely unexplored. Recently it has been proposed that insulin signalling, which secretion is modulated by GC levels (Sapolsky et al., 2000), may be involved in the regulation of resource allocation between tissues (Harshman and Zera, 2007). In the context of cooperative breeding, where GC secretion promotes and is responsive to cooperative behaviours, it will be highly relevant to assess whether individual differences in GC secretion reflect different trade-offs between survival and reproduction.

5.4. Future perspectives

During the elaboration of this dissertation, it became increasingly evident that further work will be greatly valuable to the expansion of the knowledge developed here. Three main working lines should, in my opinion, be prioritized for further understanding the short-term oxidative costs of cooperative behaviours and the trade-offs faced by cooperatively breeding species.

Firstly, and as frequently expressed during this discussion, understanding whether and how breeders and helpers differentially solve the trade-off between survival and reproduction remains to be specifically assessed. Because in captivity such differences may not be evident under stable conditions, an option would be to inflict an oxidative challenge (e.g. increase cooperative contributions, or direct manipulation of the oxidative balance) and evaluate its effects on the accumulation of oxidative damage in somatic and germline tissues of breeders and helpers. It also remains to be assessed whether females similarly trade-off resources between somatic and germline tissues. Although such work in mammals is usually restricted by the difficulty in accessing female germline, studies in cooperatively breeding birds would provide invaluable insight. Furthermore, it remains to be identified which limiting resources may drive the trade-off between somatic and germline protection against accumulation of oxidative damage. Progress in this area would require a broader evaluation of antioxidants and repair mechanisms and would allow more precise predictions on the factors affecting life-history trade-offs.

Secondly, a future challenge would be to disentangle between the effects of increased cooperative contributions and increased GC levels on oxidative stress. The work presented in this thesis has highlighted the effects of increased cooperative contributions (Chapter 3) and GC levels (Chapter 4), yet these manipulations led to increased GC levels and cooperative

contributions, respectively (Vullioud et al., in preparation). Further experimental work could involve manipulations of cooperative contributions while blocking GC receptors and manipulations of GC levels while cooperative opportunities are not available.

Finally, it remains to be assessed whether and how the oxidative balance of captive animals differs from those living in wild conditions. In wild conditions, animals may face different challenges which may exacerbate (e.g. harsh environmental conditions, large territory areas) or lighten (e.g. low oxygen concentrations, diverse diet composition) the effects demonstrated in the laboratory. While experimental work in laboratory conditions may provide invaluable insights allowing the control of external variables, information from natural settings will allow us to draw parallels and firm conclusions about laboratory studies.

5.5. Concluding remarks

Understanding the pronounced variation in cooperative contributions, so frequently observable in cooperative societies, remains a key research ambition in evolutionary ecology. The progress achieved so far in explaining this variation is mostly bound to the study of the relatedness between individuals and the indirect fitness benefits accrued from cooperative behaviours. Yet, the study of the direct fitness costs of cooperative behaviours, which has long been hypothesised to explain the expression of cooperative behaviours, remains a largely unexplored field of research. While some correlative studies have shown that higher investment in some forms of cooperation leads to decreased body condition and increased metabolic rate, it remains largely unknown whether such immediate costs could cause direct fitness costs. Oxidative stress represents an exciting physiological mechanism underlying the costs of energetically demanding cooperative behaviours and life-history trade-offs. Such short-term costs may

accumulate and lead to long-term fitness costs linked to lower fecundity and survival. In this thesis, I explored the short-term oxidative costs of increased cooperative contributions and increased GC levels in Damaraland mole-rats. Further studies are now necessary to understand whether cooperative behaviours in other cooperatively breeding species induce similar short-term costs to those reported here. Also, it would be interesting to recognise whether and how helpers and breeders differentially trade-off protection against accumulating oxidative damage in other species and how such trade-offs may be affected by levels of reproductive skew, population densities, food availability, or other relevant ecological variables. Finally, I advocate the need for ecologically relevant experimental manipulations in this field, to concretely demonstrate a causal link between the expression of cooperative behaviours and their oxidative costs.

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