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Reproduction Is Associated with a Tissue-Dependent Reduction of Oxidative Stress in Eusocial Female Damaraland Mole-Rats (*Fukomys damarensis*)

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Abstract

Oxidative stress has been implicated as both a physiological cost of reproduction and a driving force on an animal's lifespan. Since increased reproductive effort is generally linked with a reduction in survival, it has been proposed that oxidative stress may influence this relationship. Support for this hypothesis is inconsistent, but this may, in part, be due to the type of tissues that have been analyzed. In Damaraland mole-rats the sole reproducing female in the colony is also the longest lived. Therefore, if oxidative stress does impact the trade-off between reproduction and survival in general, this species may possess some form of enhanced defense. We assessed this relationship by comparing markers of oxidative damage (malondialdehyde, MDA; protein carbonyls, PC) and antioxidants (total antioxidant capacity, TAC; superoxide dismutase, SOD) in various tissues including plasma, erythrocytes, heart, liver, kidney and skeletal muscle between wild-caught reproductive and non-reproductive female Damaraland mole-rats. Reproductive females exhibited significantly lower levels of PC across all tissues, and lower levels of MDA in heart, kidney and liver relative to non-reproductive females. Levels of TAC and SOD did not differ significantly according to reproductive state. The reduction in oxidative damage in breeding females may be attributable to the unusual social structure of this species, as similar relationships have been observed between reproductive and non-reproductive eusocial insects.

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Introduction

Investment in the production of offspring is customarily linked to compromised survival, as prolific reproduction tends to be coupled with a relatively shorter life span [1,2,3]. This relationship has been classically characterized as being driven by the diversion of resources from self-maintenance towards reproduction, however, attention has been more recently turned to the investigation of physiological costs of reproduction that impair functionality of one or more physiological processes[4]. Such costs may result from the reduction of resources available for self-maintenance, but they may also be direct effects of the process of reproduction, itself [4,5].The production of offspring is associated with a myriad of physiological adjustments, and it has been frequently proposed that oxidative stress resulting from such changes is one such cost of reproduction.

Oxidative stress arises when the production of reactive oxygen species (ROS), which damage proteins, lipids and DNA, exceeds the capacity of antioxidants and repair mechanisms to prevent or mitigate ROS damage [6,7,8]. It has been proposed that elevated reproductive effort should increase an animal's vulnerability to oxidative stress [9,10,11], and this has been demonstrated in some bird and reptile species [9,12,13,14,15,16]. In eutherian mammals, ROS production is elevated, in part, by mitochondrial activity of the placenta[17,18], and during gestation sows, humans and sheep exhibit an increase in oxidative damage along with a reduction in antioxidant capacity[19,20,21,22,23](but see[24]).Oxidative damage also increases with number or mass of offspring produced in sheep, mice and Eastern chipmunks (*Tamias striatus*) [25,26,27,28].

Oxidative stress is also associated with numerous pathologies (see [29]), and the accumulation of oxidative damage has long been considered a mechanism by which animals age [30,31]. Across several animal taxa there is a negative correlation between maximum lifespan and endogenous levels of tissue antioxidants [32] and long-lived species exhibit low rates of ROS production relative to oxygen consumption near DNA paired with high rates of DNA repair [33]. Thus, oxidative stress that accompanies the production of offspring has frequently been suggested to be the physiological link between reproduction and lifespan.

It is important to note that many investigations into the relationship between reproduction and oxidative stress have measured biomarkers of oxidative stress only in serum or plasma samples (see [34]). While this type of sampling is more practical for field and longitudinal studies, individual tissue types may vary in the level of oxidative damage exhibited at a given time within an animal (see [35]). Reproductive female Sprague-Dawley rats have higher levels of lipid oxidation in lung, uterus, brain, kidney and thymus, but not in the liver or spleen, relative to non-reproductive

females [36] and reproductive female mice have less protein and lipid oxidation, higher concentration of antioxidants in the liver, and less lipid oxidation in skeletal muscle than do non-reproductive females (this difference was not detected in the serum; [26]). In bank voles (*Myodes gareolus*) lipid oxidation was lower in skeletal muscle and kidney and protein oxidation tended to be lower in the heart of reproductive females relative to non-reproductive females, whereas there was no difference in levels of oxidative damage in the liver between reproductive and non-reproductive females [37]. Thus, serum and plasma measurements by themselves may not accurately portray the balance between ROS production and antioxidant activity.

Upon investigating oxidative stress in various tissues, the aforementioned studies also reveal that in some species reproducing females are experiencing less oxidative damage than those that do not reproduce. This relationship has been observed in social insect species (see [38]) and, interestingly, these reproductive females, or queens, live much longer than non-reproductive members of the colony [39,40,41]. In mammals, social species of African mole-rats (Bathyergidae) exhibit both a reproductive hierarchy and extended lifespan of reproducing females akin to that of social insects [42,43,44,45,46,47,48], and Damaraland mole-rats (*Fukomys damarensis*), which, along with naked mole-rats (*Heterocephalus glaber*), are argued to be the only truly eusocial mammals[43]. Like social bees, ants and termites, there is only one female in a Damaraland mole-rat colony that reproduces, all other female colony members are reproductively suppressed [44] and the reproductive female lives longer than her non-reproductive counterparts [45]. Upon separation from the reproductive female, a reproductively-suppressed female commences ovulation and thus can feasibly begin to reproduce [49], suggesting that reproductive status is not genetically regulated. It has been suggested that reproductive female Ansell's mole-rats possess stronger defenses against oxidative damage relative to non-reproductive females [50], however, to date little is known about how reproduction affects susceptibility to oxidative stress in this uniquely social group of mammals.

If reproductive mole-rats experience less oxidative stress than their non-reproductive cohorts, this may serve as a mechanism that underlies their extended lifespan. To determine this, we measured oxidative stress in several different tissues of reproductive and non-reproductive female Damaraland mole-rats, predicting that reproductive females have less oxidative damage and better antioxidant defense relative to their non-reproductive cohorts, and that there will be variation between tissue types as to how this difference in relation to reproduction is manifested. Although it has been suggested that a comparison between reproductive and non-reproductive females can be confounded by a female's ability to adjust reproductive investment [51], any degree of reproductive investment will result in an elevation of metabolic rate relative to non-reproductive females [34]. Additionally, many potential problems may accompany suggested means for controlling and manipulating reproductive investment, such as the impact of physiological constraints that are unaffected by experimental manipulation (see [34]). Thus, our approach is argued to be not only valid, but ideal for assessing oxidative stress as a potential cost of reproduction [34].

Materials and Methods

Ethics Statement

Research protocols were approved by the animal ethics committee at the University of Pretoria and complied with their guidelines for animal research (protocol number EC008-12).

Animals

We selected 9 reproductive and 14 non-reproductive females from colonies at the University of Pretoria that had been recently collected (following [52]) from the area surrounding the towns of Hotazel and Blackrock, Northern Cape Province, South Africa. Reproductive females were initially differentiated from non-reproductive females by their swollen teats or perforate vaginas [44] and later confirmed by the presence of placental scars. The number of previous reproductive bouts could not be determined. As these were wild-caught animals, exact age was not known, but they were all adult based on body mass measurements. Reproductive and non-reproductive females were kept together with other members of their colony to maintain their reproductive status, and were housed in large plastic boxes lined with wood shavings and paper nesting materials. We provided all animals with *ad libitum* access to a combination of sweet potatoes, carrots, apples and gemsbok squash.

Sample Collection

On 3–4 July 2012, we euthanized animals via decapitation and immediately collected about 1 mL of blood in heparinized tubes. We then centrifuged the sample for 10 min at $1,000\times$g, drew off the plasma, transferred it to plastic tubes, and stored plasma and erythrocytes in a -80 freezer until time of analysis, which was within a period of 40 days. We removed the heart, left kidney, a section of the left median lobe of the liver, and the vastus lateralis of the left leg (herein, skeletal muscle) immediately following decapitation and snap froze them in liquid nitrogen. We homogenized liver, heart, skeletal muscle and kidney on ice in 10% weight per volume 100 mM HEPES (N-2 hydroxyethylpiperazine-N′-2-ethanesulfonic acid) buffer solution for 1 (liver and kidney) or 2 (heart and skeletal muscle) minutes on an Ultra Turrax T18 Basic Homogenizer (IKA, Staufen, Germany), and stored all homogenates in a -80 freezer until time of analysis.

Analyses of Oxidative Stress

Oxidative stress represents an imbalance between ROS production, resulting in oxidative damage, and antioxidant defenses or repair mechanisms, and is thus more accurately characterized by including a range of assays of these damage and protection mechanisms [34,53]. We quantified oxidative damage by concentrations of malondialdehyde (MDA), a marker of lipid peroxidation [54], and protein carbonyls (PC), which indicates protein oxidation [55]. We assessed antioxidant levels as superoxide dismutase (SOD) activity as well as total antioxidant capacity (TAC). The specific tissues used for each of these assays are described below.

MDA

We measured concentrations of MDA in all tissue homogenates (i.e. liver, kidney, skeletal muscle, heart) and in plasma using high performance liquid chromatography (HPLC) as described by Nussey et al. [24]. We prepared samples following Nussey et al.[24] and injected 20 µL of sample into a Dionex HPLC system (Dionex Corporation, California, USA) fitted with a 5 µm ODS guard column and a Hewlett-Packard Hypersil 5 µ ODS 100×4.6 mm column maintained at 37°C. The mobile phase was methanol-buffer (40:60, v/v; 50 mM anhydrous solution of potassium monobasic phosphate at pH 6.8), running isocratically over 3.5 min at a flow rate of 1 mL min^{-1}. We collected data using a fluorescence detector (RF2000; Dionex) set at 515 nm (excitation) and 553 nm (emission). For calibration, we prepared a standard curve using a TEP stock solution (5 µM in 40% ethanol)

serially diluted using 40% ethanol. Results are expressed as nmol MDA per g tissue or ml plasma.

PC

We measured concentrations of PC in all tissue homogenates (i.e. liver, kidney, skeletal muscle, heart) and in plasma. Oxidation or oxidative cleavage of proteins results in the production of carbonyl groups [55], which covalently reacts with 2,4-dinitrophenylhydrazine (DNPH) to form 2,4-dinitrophenyl (DNP) hydrazone. DNP can then be detected via spectrophotometry at a wavelength of 370nm [56]. We measured PC concentration using a commercially available kit (Protein Carbonyl Assay Kit, Cayman Chemical Co., Ann Arbor, MI, USA), reading absorbances using a Spectramax M2 plate reader (Molecular Devices Corp., Sunnyvale, CA, USA). We then quantified the protein content of each sample from a bovine serum albumin (BSA) standard curve. Results are expressed as nmol per mg protein.

SOD

We measured SOD activity in all tissue homogenates (i.e. liver, kidney, skeletal muscle, heart) and in erythrocytes. SOD is an enzymatic antioxidant that catalyses the dismutation of superoxide anions to oxygen and hydrogen peroxide [57]. We measured SOD content with a commercially available kit (Superoxide Dismutase Assay Kit, Cayman Chemical Co., Ann Arbor, MI, USA) that measures the percentage of superoxide radicals that undergo dismutation in a given sample. Absorbances were read at 440 nm using a Spectramax M2 plate reader (Molecular Devices Corp., Sunnyvale,CA, USA). Erythrocyte lysate was used instead of plasma for this assay only. Results are expressed as units of SOD activity per mg tissue.

TAC

We measured TAC in homogenates of liver, kidney and heart, and in plasma. We did not measure TAC in skeletal muscle because intracellular antioxidant defenses (e.g. SOD) are likely to be more important in this tissue type. TAC was quantified using a commercially available kit (Antioxidant Assay Kit, Cayman Chemical Co., Ann Arbor, MI, USA) which measures the oxidation of ABTS (2,2′-Azino-di-[3-ethybenzthiazoline sulphonate]) by metmyoglobin, which is inhibited by non-enzymatic antioxidants contained in the sample. Oxidized ABTS can then be detected via spectrophotometry at a wavelength of 740 nm. The capacity of antioxidants in the sample to inhibit oxidation of ABTS is compared with the capacity of known concentrations of Trolox, and results are expressed as nmol of Trolox equivalents per g tissue or ml plasma.

Statistical Analyses

Data were examined for normality, homoscedasticity, and outliers. For MDA and SOD, data were $\log_{10}$-transformed to improve the approximation of normality. Individual markers of oxidative damage and antioxidant defense may vary in their association with reproductive state, and such relationships are likely to differ amongst tissues [58]. Therefore, we assessed whether each marker of oxidative damage or antioxidant defense in turn differed between reproductive and non-reproductive females, using General Linear Mixed Models (GLMM) with reproductive state and tissue (and their interaction) as fixed factors, and with individual identity and colony membership included as random factors. Degrees of freedom were calculated using Satterthwaite's correction. Models were developed by backward elimination of non-significant terms (where $P>0.05$) starting with the reproductive state x tissue interaction term. Significant reproductive state x tissue interactions were followed by post-hoc GLMMs for each tissue in turn, including reproductive state as a fixed factor and colony membership as a random factor. In the absence of any association with reproductive state, we were not interested in differences in oxidative damage or antioxidant defenses amongst tissues *per se*. Such differences are inevitable given the wide variety of tissues included in this study. Therefore, any significant main effect of tissue was not followed by post-hoc tests. Not all tissue samples were available for each individual and each assay, resulting in a variation in sample size between tissue types (Table S1). All analyses were performed using Genstat (16th edition) (VSN International Ltd., Hemel Hempstead, UK). Results are reported as means ± s.e.

Results

Levels of MDA were significantly decreased in reproductive compared to non-reproductive females, although this differed amongst tissue types. Post-hoc analyses revealed that MDA was significantly lower in the heart, kidney and liver in reproductive compared to non-reproductive females, but did not differ significantly according to reproductive state in skeletal muscle or plasma (Fig. 1a and Table 1). Levels of PC were significantly decreased in reproductive compared to non-reproductive females, and this was apparent across all tissues (Fig. 1b and Table 1). SOD activity did not differ significantly according to reproductive state, but varied markedly amongst tissues (Fig. 1c and Table 1). Levels of TAC showed a similar pattern, not differing significantly between reproductive and non-reproductive females, but varying markedly amongst tissues (Fig. 1d and Table 1).

Discussion

Lower concentrations of both markers of oxidative damage observed in reproductive females suggest that either these females produced less ROS than non-reproductive females, or that antioxidant defenses were more active in reproductive females. Mitochondrial uncoupling can result in reduced ROS production, although studies of mice have indicated that expression of the genes that code for uncoupling proteins is reduced or unchanged in breeders compared with non-breeders [5]. Therefore, mitochondrial uncoupling seems an unlikely general explanation for reduced levels of oxidative damage in various tissues of female Damaraland mole-rats. However, since we did not explicitly quantify ROS production and scavenging, we cannot rule out the possibility that reproductive females may have produced less ROS than non-reproductive females. Elevated ROS production has been observed in other mammal species during reproduction, and a variety of taxa, including sheep, mice, Pacific oysters (*Crassostrea gigas*) and painted dragon lizards (*Ctenophorus pictus*), all exhibited increased expression of antioxidants during reproduction [16,59,60,61,62]. Thus, the difference in oxidative damage observed in Damaraland mole-rats seems more likely to be attributable to variation in antioxidant activity. The absence of variation in TAC and SOD concentrations between reproductive and non-reproductive female Damaraland mole-rats intimates that either antioxidant defenses matched or exceeded ROS production during reproduction, or that other antioxidant systems play a more substantial role in this process.

Several physiological adjustments accompany a non-reproductive Damaraland mole-rat's transition to reproductive status, including increased body length, brain volume, reproductive hormone concentrations and pituitary sensitivity [52,63,64,65,66]. It is possible that any combination of these adjustments may reflect

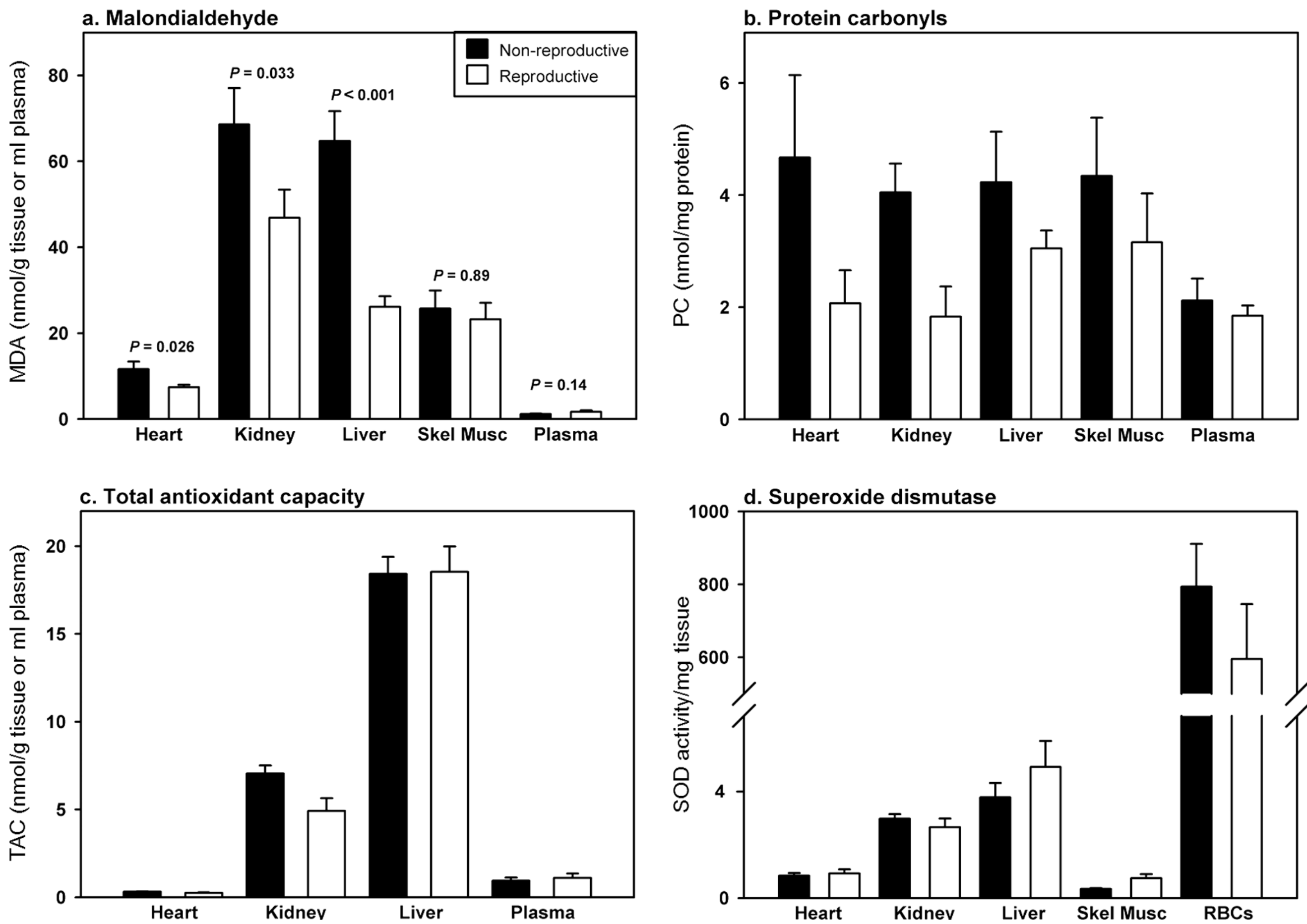

Figure 1. Oxidative stress markers of different tissue types for reproductive and non-reproductive females. Concentrations of markers of oxidative damage (a. malondialdehyde and b. protein carbonyls) and antioxidant activity (c. superoxide dismutase and d. total antioxidant capacity) in the heart, kidney, liver, skeletal muscle (skel musc) and plasma or erythrocytes (RBCs) of non-reproductive (black boxes) and reproductive (white boxes) adult female Damaraland mole-rats.
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Table 1. Variation in markers of oxidative damage (MDA and PC) and antioxidant defence (TAC and SOD) in relation to reproductive state and tissue.

Response	Explanatory	*F*	d.f.	*P*
MDA	Reproductive state	17.07	1,17.9	**<0.001**
	Tissue	298.31	4,81.2	**<0.001**
	Reproductive state x tissue	5.54	4,81.5	**<0.001**
PC	Reproductive state	10.40	1,16.6	**0.005**
	Tissue	2.04	4,65.8	0.10
	Reproductive state x tissue	0.76	4,60.8	0.55
SOD	Reproductive state	0.23	1,21.5	0.64
	Tissue	212.19	4,78.9	**<0.001**
	Reproductive state x tissue	0.48	4,74.2	0.75
TAC	Reproductive state	1.14	1,16.0	0.30
	Tissue	349.14	3,62.3	**<0.001**
	Reproductive state x tissue	1.54	3,58.9	0.21

Results are from General Linear Mixed Models including individual identity and colony membership as random factors. See Methods for details. Significant *P* values are shown in bold.
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or drive a decreased susceptibility to oxidative stress, and this effect could be enhanced by physiological adjustments associated with gestation and lactation, themselves. An example of this is found in honey bees (*Apis mellifera*), in which reproduction is associated with an increased production of the protein vitellogenin, which defends against oxidative stress [67,68]. Some vitellogenin sequences are conserved amongst vertebrates, insects and nematodes [69], and vitellogenin is thought to be related to mammalian low-density lipoproteins [70,71]. However, to date this potential relationship in mammals has yet to be explored.

Additionally, the reduction of oxidative stress found in reproductive Damaraland mole-rats and other species may be indicative of hormetic response, in which exposure to ROS associated with reproduction or becoming reproductive primes the female's system to defend against long-term oxidative challenges [72,73,74,75,76]. Exercise also factors into the hormetic framework; whereas vigorous and intermittent physical activity promotes oxidative stress, regular and moderate exercise reduces oxidative stress [77,78]. This is noteworthy, as non-reproductive Damaraland mole-rats, along with other social mole-rat species (naked mole-rats and common mole-rats (*Cryptomys hottentotus*)),-spend much more time sleeping than the reproductive female [44,79,80].Thus, the difference in physical activity between reproductive and non-reproductive females may play a role in differentiating oxidative stress characteristics of these two groups.

Levels of PC were reduced in all tissues of reproductive females, however, levels of MDA showed different patterns amongst tissues in relation to reproduction; MDA was reduced in heart, kidney and liver, but did not differ in skeletal muscle or plasma. This observation underscores the importance of assessing oxidative stress in more than one tissue in order to obtain a more thorough depiction of oxidative stress dynamics within an animal [34]. Our findings support observed differences in biomarkers of oxidative stress between tissues in other species [35,81], with some tissues being more susceptible, such as the liver relative to skeletal muscle [77], which is likely attributed to variation in metabolic rate between tissues [82,83]. For example, about 60% of resting energetic expenditure is attributed to metabolic activity of the brain, liver and kidneys in humans [83]. The composition of the different tissues may also drive variation in susceptibility. Cells that comprise the parenchyma of kidney and liver are constantly dividing, whereas those of the heart and skeletal muscle are post-mitotic, and it is generally thought that age-associated changes are stronger and more widespread in the latter cell type [84].

While several exceptions to the oxidative stress theory of aging have emerged (see [74]), our results do not negate the idea that accumulated oxidative damage is related to aging. Instead, our results show that less oxidative damage is present in reproductive female Damaraland mole-rats, which live longer than non-reproductive females. This may be, in part, attributable to our analysis of multiple tissues. Given our results, the question arises as to whether oxidative stress can be considered to be a cost of reproduction in Damaraland mole-rats, and, indeed, whether this species experiences any physiological costs of reproduction. Our results correlate reproduction, oxidative stress and lifespan, but it is important to quantify oxidative stress relative to survival rate, and to control for, or experimentally manipulate, reproductive effort before strong conclusions can be made regarding this relationship [85].

There is evidence that lifespan is not traded off against reproduction in some social insect species [41,68,86,87,88] and in Ansell's mole-rats it has been proposed that, given no difference in activity or intrinsic quality between reproductive and non-reproductive individuals, reproduction may drive increased longevity in breeding females [46]. Since the reproductive success of a eusocial colony is almost solely dependent on the condition of the queen, Damaraland mole-rats and other eusocial species may have acquired adaptations to ensure or enhance survival that are expressed when a female obtains dominance and commences breeding. Ascension to dominant status is largely driven by environmental stimuli [49,66], however, it is possible that an individual's capacity to defend against oxidative damage may also influence likelihood of successfully attaining reproductive status.

Thus, in this and other eusocial species, oxidative stress may serve as a potential link between reproduction and lifespan which could function as a basis for prospective avenues for investigating the evolution of sociality as well as life-history traits and reproductive strategies.

Supporting Information

Table S1 Data used for the analyses of markers of oxidative damage and antioxidant defence in various tissues in relation to reproductive state.
(DOCX)

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Author Contributions

Conceived and designed the experiments: CMS JDB NCB. Performed the experiments: CMS JDB. Analyzed the data: CMS JDB. Contributed reagents/materials/analysis tools: JDB NCB. Wrote the paper: CMS.

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