

Defining the link between oxidative stress, behavioural reproductive suppression and heterothermy in the Natal mole-rat (*Cryptomys hottentotus natalensis*)

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Highlights

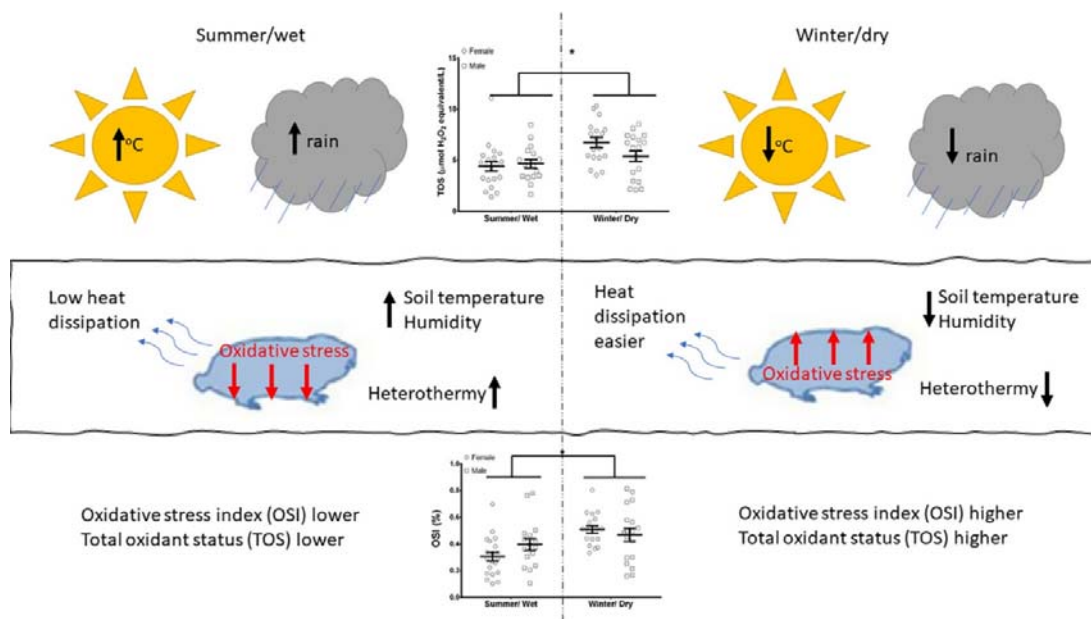
- Seasonal factors significantly affected oxidative stress.
- Male body size is positively correlated to oxidative damage.
- Significant oxidative damage in males linked to exercised-induced oxidative stress.
- Female reproductive strategies mediated by seasonal factors and reproductive state.
- First mole-rat to demonstrate sex differences in exercise-induced oxidative stress.

Abstract

Sub-lethal effects, such as oxidative stress, can be linked to various breeding and thermophysiological strategies, which themselves can be linked to seasonal variability in abiotic factors. In this study, we investigated the subterranean, social living Natal mole-rat (*Cryptomys hottentotus natalensis*), which, unlike other social mole-rat species, implements heterothermy seasonally in an attempt to avoid exercise-induced hyperthermia and relies solely on behavioural reproductive suppression to maintain reproductive skew in colonies. Subsequently, we investigated how oxidative stress varied between season, sex and breeding status in Natal mole-rats. Oxidative markers included total oxidant status (TOS measure of total peroxides present), total

antioxidant capacity (TAC), OSI (oxidative stress index) and malondialdehyde (MDA) to measure oxidative stress. Breeding and non-breeding mole-rats of both sexes were captured during the summer (wet season) and winter (dry season). Seasonal environmental variables (air temperature, soil temperature and soil moisture) had a significant effect on TOS, OSI and MDA, where season affected each sex differently. Unlike other social mole-rat species that use both physiological and behavioural means of reproductive suppression, no oxidative costs to reproduction were present in the Natal mole-rats. Males had significantly higher MDA than females, which was most apparent in summer (wet season). We conclude that the significant oxidative damage in males is a consequence of exercise-induced oxidative stress, exacerbated by increased burrow humidities and poorer heat dissipation abilities as a function of body mass. This study highlights the importance of both breeding and thermophysiological strategies in affecting oxidative stress.

Graphical abstract



Keywords: Oxidative stress, heterothermy, hormesis, mole-rat, exercise-induced oxidative damage, reproductive suppression

1. Introduction

All mammals possess both behavioural and physiological adaptations that not only allow for survival, but also reproduction in their respective habitat (Brommer, 2000; Chai et al., 2016; Dantas et al., 2021; Varpe, 2017; Withers et al., 2016). Abiotic factors that mammals must adapt to, such as variation in ambient temperature or resource availability can vary across seasons resulting in seasonal physiological and behavioural changes. These physiological changes can occur in reproduction (Alagaili et al., 2017; Hart et al., 2021a; Hart et al., 2020a), body condition (Scantlebury et al., 2006; Yom-Tov and Geffen, 2011), body temperature (Riek et al., 2017), energetics (Zelová et al., 2011) and thermoregulation (Moshkin et al., 2001), whereas behavioural changes can include variation in activity profiles (Finn et al., 2022; Riek et al., 2017). Consequently, mammals resort to physiological (Hart et al., 2019; Mitchell et al., 2002; Schmidt-Nielsen et al., 1956; van Jaarsveld et al., 2021) and behavioural (Farsi et al., 2020; Jacobs et al., 2020a) adaptations to mitigate these environmental stressors.

One of the most well-known physiological adaptations to an abiotic stressor is the implementation of adaptive heterothermy in the Dromedary camel (*Camelus dromedaries*) in response to heat and dehydration stress (Schmidt-Nielsen et al., 1956). Heterothermy is one mechanism by which animals deal with variations in their environment to avoid heat and water stress and for energy conservation (Giroud et al., 2021; Giroud et al., 2020; Hetem et al., 2009; Morales et al., 2021; Oosthuizen et al., 2021). A heterothermic response includes the facultative, reversible decrease in metabolic rate (metabolic depression) and body temperature in response to the environment (ambient temperature and/or reduced availability of energy resources) (Lovegrove, 2000; McKechnie and Mzilikazi, 2011). It is generally accepted that animals use long and deep bouts of heterothermy (hibernation) during winter/dry period (i.e., low resource availability and increased cold stress) for energy conservation and use short and shallow bouts of heterothermy in summer/wet period (accompanied by high humidity) to avoid heat stress (hyperthermia) (Hetem et al., 2009; Oosthuizen et al., 2021; Ozgul et al., 2010; Williams et al., 2012). Fitness consequences of heterothermy may directly increase short-term survival and possible future reproductive success (Desforges et al., 2021; Ozgul et al., 2010).

Heterothermy involves the process of metabolic depression (hypometabolism) (Drew et al., 2004; Landes et al., 2020) and rewarming through either increased

metabolism or ambient rewarming, both of which vary in reactive oxygen species production (Giroud et al., 2020). A lowered metabolic rate can reduce oxidative stress (Frisard and Ravussin, 2006), whereas increased metabolism from very high oxygen consumption or the return from hypometabolism has been observed to increase oxidative stress markers (Hermes-Lima and Zenteno-Savín, 2002). Oxidative stress is the overproduction of reactive oxygen species (ROS) (free radical production during metabolism), where the reducing and oxidising balance (i.e., redox state) is disrupted (Halliwell, 2007; Jacobs et al., 2021a; Sies, 2015; Speakman et al., 2015). ROS have been shown to mediate other stressful processes in an animal's environment, from reproduction to survival (Costantini, 2019; Dowling and Simmons, 2009; Viblanc et al., 2018). Furthermore, at functional levels, ROS have essential physiological functions, such as signal transduction (Chainy et al., 2016), oxygen sensing (Acker et al., 2006), the immune system (initiator on the autoimmune response) (Vodjgani et al., 2020; Yang et al., 2013), inflammatory response (Giordano, 2005), osmo-protective signalling (Burg et al., 2007), regulation of gene expression (Turpaev, 2002) and cellular functions (Droge, 2002). ROS becomes a problem when levels exceed the levels for normal physiological function, which is a result of either the overproduction of ROS or a compromised antioxidant system (generally referred to as oxidative stress), which can result in damaging proteins, lipids, and DNA affecting survival and reproduction (Costantini, 2008; Finkel and Holbrook, 2000; Sies, 1991). Since ROS production is directly dependant on metabolism, ROS production is dependent on resting metabolic rate (RMR), with an increase of RMR resulting in increased production of ROS and thus increased oxidative stress (Frisard and Ravussin, 2006). Consequently, the implementation of heterothermy, torpor and hibernation is mainly devoid of oxidative stress, likely due to a reduction of RMR and thus reduced ROS production, and could result in a net positive oxidative status (Orr et al., 2009).

Reproduction, as well as heterothermy, can also affect oxidative stress (Agarwal et al., 2012; Costantini, 2016; Metcalfe and Monaghan, 2013). Oxidative stress research regarding the cost to reproduction has garnered attention due its contradictory results of no link or weak support for a life history trade-off (Metcalfe and Monaghan, 2013). Hypotheses such as oxidative shielding (Blount et al., 2016; Viblanc et al., 2018) and hormesis (Costantini, 2014; Oliveira et al., 2018) have been used to explain contradictory findings of this oxidative stress cost trade-off, where several experiments were flawed based on experimental setup (Costantini, 2016; Metcalfe and

Monaghan, 2013; Speakman and Garratt, 2014). One major flaw included the artificial manipulation of reproductive effort, where individuals were forced not to reproduce or required an extra cost to the reproductive effort, which may not occur naturally (Costantini, 2016). Importantly, individuals can set their limits on reproductive effort, where body condition, hormonal profile, behaviour, food availability and glucocorticoid concentration, can all influence reproduction (Costantini, 2014, 2016; Fischer et al., 1995; Norris and Lopez, 2010; Novikov et al., 2015; Palmer, 2010), which is likely to vary between and within species. From this, it has been suggested that social African mole-rats are ideal candidates for investigating oxidative stress between reproductive and non-reproductive individuals, as these animals circumvent most of these complications (Jacobs et al., 2021a). This is largely due to their cooperative breeding and a reproductive division of labour where a single reproductive female (RF) and one to three of the largest males (RMs) monopolise reproduction within the colony (Bennett and Faulkes, 2000; Burland et al., 2004). The remaining colony members (non-reproductive males and females - NRM and NRF, respectively) are, however, reproductively suppressed either physiologically (Bennett et al., 2018; Blecher et al., 2020; Medger et al., 2019) or behaviourally (e.g., incest avoidance) (Bennett et al., 1997; Bennett et al., 1996; Burda et al., 1990; Lutermann et al., 2013) or even the two in unison (Bennett et al., 1996; Hart et al., 2022).

African mole-rats have been used as an ideal model family to investigate the morphological, physiological (including heat-induced heterothermy) and biochemical (including oxidative stress) adaptations to their unique subterranean lifestyle and the associated constraints (Logan et al., 2020; Oosthuizen et al., 2021). Mole-rats live in burrow systems with complex structures (Bennett and Faulkes, 2000; Šumbera, 2019; Šumbera et al., 2008; Thomas et al., 2009; Thomas et al., 2016), which predisposes them to intermittent hypoxic and hypercapnic conditions, high air humidity in the soil and potential heat stress due to complications in heat dissipation and/or poor thermal tolerance (Hart et al., 2021c; Roper et al., 2001; Šumbera, 2019; Wallace et al., 2021). Furthermore, water-clogged soils from rain can result in even poorer gaseous exchange, further exacerbating hypoxic and hypercapnic conditions (Arieli, 1979; Burda et al., 2007; Holtze et al., 2018). Importantly, the excavation of tunnel systems is energetically more expensive than aboveground exploration (Vleck, 1979), with energy requirements of digging directly related to soil moisture and hardness (Lovegrove, 1989; Thomas et al., 2009). These constraints imposed by the burrow

system have resulted in African mole-rats displaying lower RMRs and low body temperatures in comparison to other terrestrial rodent species (Bennett et al., 1994; Bennett et al., 1992; Bennett et al., 1993a; Lovegrove, 1986; McGowan et al., 2020; McNab, 1966). Furthermore, some African mole-rats may be more active during the cooler periods of the day (often at night) to avoid exercise-induced hyperthermia in their humid tunnel systems (Haupt et al., 2017; Šumbera, 2019).

Studies linking oxidative stress and reproduction in the African mole-rats family have focused on species that use some form of physiological reproductive suppression, namely, the Highveld (*Cryptomys hottentous pretoriae*), Damaraland (*Fukomys damarensis*) and naked (*Heterocephalus glaber*) mole-rat, that use some form of physiological reproductive suppression (Jacobs et al., 2021a; Jacobs et al., 2021b). Currently, no study has linked the implementation of heterothermy, heat stress and oxidative stress in an African mole-rat species. As such, this study aimed to address this dearth of knowledge by asking two critical questions. Firstly, is there a blood plasma oxidative cost to reproduction in an aseasonal behavioural suppressed social African mole-rat species with different seasonal climates? Secondly, is there an oxidative consequence involved in the seasonal implementation of heterothermy used to avoid hyperthermia in African mole-rat species? The Natal mole-rats (*C. h. natalensis*) is an ideal species to address these questions as this species possesses many unique behavioural and physiological differences compared to other social African mole-rat species.

The Natal mole-rat, similar to other social mole-rats, shows a reproductive division of labour (Bennett and Faulkes, 2000; Moolman et al., 1998; Oosthuizen et al., 2008), but relies solely on behavioural reproductive suppression through incest avoidance (Oosthuizen et al., 2008). Congruent in other *Cryptomys hottentotus* sp. (Hart et al., 2020b; Malherbe et al., 2003; Spinks et al., 1999; Spinks et al., 1997), the Natal mole-rat is an induced ovulator (Jackson and Bennett, 2005), which breed throughout the year (aseasonal breeder) (Oosthuizen et al., 2008). A specific gestation length for this species is missing, however, the closely related Highveld mole-rat has a gestation period of 63-66 days, where offspring are weaned after 33 days (Bennett and Faulkes, 2000; Malherbe et al., 2003). This duration suggests 3-4 litters are possible per year. Interestingly, no difference in activity is observed between reproductive and non-reproductive individuals, suggesting more equal contributions to burrow maintenance and other cooperative behaviours (Finn et al., 2022). This is in

contrast to the Damaraland mole-rat, where reproductive individuals are less active than non-reproductive individuals (Francioli et al., 2020; Houslay et al., 2020; Oosthuizen and Bennett, 2015). Additionally, the Natal mole-rat is unique in that they demonstrate heterothermy in the wild (Boyles et al., 2012; Oosthuizen et al., 2021). Both the implementation of heterothermy and the reduction of activity has been hypothesised to prevent hyperthermia (even from exercise),_while digging during periods of increased temperature and humidity in the burrow system (Hart et al., 2021c; Oosthuizen et al., 2021). Of all the social mole-rat species, the Natal mole-rat inhabits the most mesic and highest rainfall (Hart et al. 2022b in press), resulting in increased soil moisture and even water logged soils during certain times of the year (Finn, pers. obs/comm.). This increase may result in even higher hypoxic, hypercapnic and humid conditions of their burrow systems (Burda et al., 2007), but burrow conditions in the Natal mole-rat has not been investigated. A recent study on the burrow conditions of the naked mole-rat, which inhabit the dry and arid regions of Ethiopia and Kenya, have revealed burrow temperatures and humidities ranging from 24.6 - 48.8°C and 31.2- 89.7%, respectively (Holtze et al., 2018). Unsurprisingly, the humidity ranges experienced by naked mole-rats within their tunnel systems was vastly higher than those at the surface (6.0 – 42.6 %) (Holtze et al., 2018). Since, the Natal mole-rat lives in a more mesic environment, one could predict that the Natal mole-rat would experience higher humidities than those experienced by the naked mole-rat, which is supported by higher rainfall and by higher air humidities present in that habitat obtained from climate data.

In light of this, we investigated the oxidative balance response to season, reproduction and the implementation of heterothermy (in response to seasonal variation of environmental variables) in male and female Natal mole-rats. We predicted that oxidative markers (total antioxidant capacity-TAC; total oxidant status TOS; OSI- Oxidative stress index; MDA-malondialdehyde) would vary significantly across reproductive status (reproductive vs. non-reproductive) and across the seasons (summer/wet vs. winter/dry) as the result of the inherent cost of reproduction and the seasonal implementation of heterothermy, respectively. We used plasma markers as we did not intend to remove individuals from the population due other longevity studies taking place at this field site.

2. Methods

2.1. Ethics Statement

All protocols were approved by the University of Pretoria ethics committee (permit EC001-19 and NAS128/2020) and complied with regulations stipulated in the *Guidelines for the use of Animals in Research* (Council, 2010). In addition, the Ezemvelo KZN Wildlife Authority provided permits for capturing mole-rats (permits OP27-20 and OP1545-2021).

2.2. Reagents

Unless otherwise stated, all chemicals and reagents used in this study were obtained from Merck (Pty) Ltd (Gauteng, South Africa).

2.3. Animal capture and maintenance

Natal mole-rats were captured using Hickman live traps baited with sweet potato (Hickman 1979) during the summer/wet (February to March 2021) and winter/dry (July to August 2021) period. Captured mole-rats were housed with family members at ambient temperature (15-25°C) in large boxes and provided with wood shavings, a cloth towel to hide under, and sweet potatoes *ad libitum* (Bennett and Jarvis, 1995). At capture, all animals were sexed and weighed (± 1 g; Pelouze SP5, Rubbermaid, USA). In addition, individuals were sexed and assigned a reproductive status, either as reproductive or non-reproductive. Reproductive females were easily recognized by having a perforate vagina and prominent nipples (Bennett and Faulkes, 2000). Breeding male mole-rats possess inguinal testes above or adjacent to the penis, which may sometimes be felt by palpation in reproductive individuals (Bennett and Faulkes, 2000).

2.4. Sample Collection

Blood samples were collected upon animal capture as follows: the animals were handheld, and venous blood samples were collected from the hindfoot. Approximately 300-500ul of blood was collected into heparinised micro-haematocrit tubes and centrifuged for 5 minutes at 2000g. Plasma was separated from the red blood cells, and both fractions were kept at -20°C while in the field, then transferred to a -80°C freezer upon return to the University of Pretoria. Only 1% or less of the total body mass

of the individual of whole blood was allowed to be collected as set out by the University of Pretoria, Faculty of Veterinary Science Animal Ethics Committee. Sample sizes for summer/wet period were ten reproductive females (RFs), ten non-reproductive females (NRFs), nine reproductive males (RMs) and eight non-reproductive males (NRMs). While in winter/dry period, eight RFs, ten NRFs, nine RMs and nine NRMs were sampled.

2.5. Environmental data

Hourly environmental data during the collection period (February to August 2021) was obtained from the global atmospheric reanalysis dataset ERA5-Land (Muñoz Sabater, 2019). The ERA5-Land dataset is a global environmental data analysis model based on satellite and weather station environmental data observations allowing for accurate estimates of environmental data variables at specific locations with a 9 km accuracy ($0.1^\circ \times 0.1^\circ$; Muñoz-Sabater et al. (2021)). Users can specify the boundary GPS points of less than 9 km to query data from the database, and we selected a 1 km radius to encompass the study site. The dataset is freely available from the Copernicus Climate Change Service and holds the intellectual property rights of the raw data. The results contain modified Copernicus Climate Change Service information. We obtained air temperature data (T_{air} : $^\circ\text{C}$), soil temperature ($T_{\text{s}1-3}$: $^\circ\text{C}$), precipitation (T_{p} : - m) and soil moisture ($M_{\text{s}1-3}$: m^3m^{-3}) (Table 1). We used soil temperature and moisture measured at depths of 0 – 7 cm ($T_{\text{S}1}$ & $M_{\text{S}1}$), 7 – 28 cm ($T_{\text{S}2}$ & $M_{\text{S}2}$), and 28 – 100 cm ($T_{\text{S}3}$ & $M_{\text{S}3}$), which correspond to the depths of foraging tunnels ($T_{\text{S}1-2}$ & $M_{\text{S}1-2}$) and the nest ($T_{\text{S}3}$ & $M_{\text{S}3}$) of mole-rats (Bennett and Faulkes, 2000). A temperature logger (DS1922L iButton, Maxim Integrated Products, Dallas, TX, USA) was buried at the study site at 15 cm below the soil surface in an area of direct sunlight and set to record hourly temperatures from August to October 2020. We compared the soil temperatures from the data logger and $T_{\text{S}2}$ from the ERA5-Land dataset. We found that soil temperatures from the data logger were on average 3.67 ± 1.32 $^\circ\text{C}$ higher than $T_{\text{S}2}$, yet the daily fluctuation in temperature was similar between temperature loggers and ERA5-Land values (Supplementary Figure S1). Therefore, we assumed that soil temperatures from ERA5-Land were a fair representation of the actual variability of soil temperatures. It is important to note that the soil temperatures in this study may not accurately represent burrow temperatures

and that ambient temperature in tunnels may be higher than the surrounding soil (*sensu* Holtze et al. (2018)). For the current study, hourly data from the study site for T_{air} , T_s , T_p , and M_s were converted to daily values for the periods between February-March (summer/wet period) and July-August (winter/dry period) corresponding to animal captures, where T_p was converted to represent total rain. Data were analysed using the Welch t-test to compare variables between seasons. Data are presented as mean \pm standard error (s.e.m).

2.6. Total antioxidant capacity (TAC) assay

Plasma TAC levels were 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-ethylbenzthiazoline sulphonate]) by metmyoglobin, which is inhibited by non-enzymatic antioxidants contained in the sample. Oxidised ABTS is measured by spectrophotometry at a wavelength of 750 nm. The capacity of antioxidants in the sample to inhibit oxidation of ABTS is compared with the capacity of known concentrations of Trolox, and the results are expressed as micromole Trolox equivalents per litre (μmol Trolox equivalents/L). Samples were run in duplicate and only once per plate with a repeatability of $r = 0.96$. Intra-assay variability (%CV) was 3.65%.

2.7. Total oxidant status (TOS) assay

Plasma TOS levels were measured through Erel's method (Erel, 2004). Briefly, this method is based on the oxidation of ferrous ion to ferric ion in the presence of various oxidative species. The oxidation reaction is enhanced by glycerol molecules, which are abundantly present in the reaction medium. In an acidic medium, the ferric ion makes a coloured complex with xylenol orange. The colour intensity, measured spectrophotometrically is related to the total amount of oxidant molecules that are present in the sample. The results are expressed in terms of micromole hydrogen peroxide equivalent per litre (μmol H_2O_2 equivalent/L). Samples were run in duplicate and not repeated once per plate with a repeatability of $r = 0.99$. Intra-assay variability (%CV) was 4.5%.

2.8. Oxidative stress index (OSI)

Oxidative stress was determined by the TOS:TAC ratio, which represents the oxidative stress index (OSI) arbitrary unit, which was calculated as follows: $OSI = [(TOS, \mu\text{mol H}_2\text{O}_2 \text{ equivalent/L}) / (TAC, \mu\text{mol Trolox equivalent/L})] * 100$ (Jacobs et al., 2021a; Jacobs et al., 2021b).

2.9. Malondialdehyde (MDA) lipid peroxidation

The concentration of MDA was measured in all plasma samples collected and was quantified using a commercially available kit (Sigma-Aldrich, cat. No. MAK085, A6283, 258105 and 360465), following standard procedures (Halliwell and Chirico, 1993). Polyunsaturated fatty acids (lipids) are susceptible to oxidative attack through ROS, resulting in malondialdehyde (MDA). The kit determines MDA content by reacting with thiobarbituric acid (TBA) to form a colorimetric complex at 532nm. Absorbance was read using Spectramax M2 plate reader (Molecular Devices Corp., Sunnyvale, CA, USA) and compared to a 2mM MDA standard (2-10nmol/ml). Samples were run in duplicate with repeatability of $r=0.93$.

2.10. Statistical analysis for oxidative variables

All statistical analyses were performed in R 4.0.5 (R Development Core Team, 2021). The normality of the response variables (body mass, TOS, TAC, OSI or MDA) was determined using Shapiro Wilk tests. Homogeneity of all dependent variables was confirmed with Levene's test. Log-transformation was attempted to normalise all non-normal data. Normally distributed dependent variables, TAC, TOS and OSI were analysed using a linear model. Body mass and MDA was log-transformed for normality and analysed using a log-link generalised linear model using the *lme4* package (Bates et al., 2015).

Body mass was run as a response variable with sex (male and female), season (wet vs dry) and reproductive state (reproductive (breeder) or non-reproductive (non-breeder)) and all two- and three- way interactions as predictors. Consequently, each oxidative variable (OSI, TAC, TOS or MDA) was used as the response variable and reproductive state, season (wet vs dry) and sex (male or female) as predictors, with all two-way and three-way interactions included. *Post hoc* comparisons were conducted using Tukey's HSD pairwise comparisons using the *emmeans* package (Lenth et al., 2018). Linear regression lines for each sex were fitted to find the

relationship between body mass and each oxidative variable (OSI, TAC, TOS or MDA). Data are presented as mean \pm standard error (s.e.m), and a p-value of ≤ 0.05 was defined as significant.

3. Results

3.1. Environmental data

All environmental data differed significantly between the two seasons (Table 1). Temperatures (both T_{air} and $T_{\text{s1-s3}}$) were higher in the wet season compared to the dry season (Table 1). Likewise, T_p and $M_{\text{s1-s3}}$, were significantly higher in the wet season compared to the dry season (Table 1).

Table 1. Seasonal difference in mean daily climatic variables, consisting of air temperature ($^{\circ}\text{C}$), soil temperature ($T_{\text{s1-Ts3}}$: $^{\circ}\text{C}$), precipitation (m) and soil moisture ($M_{\text{s1-Ms3}}$: m^3m^{-3}), in summer/wet period and winter/dry period. Statistical result of Welch t-test with appropriate F value and p statistic. Data represents mean \pm s.e.m.

Environmental variable	Period		Statistical results	
	Summer/wet	Winter/dry	F	p
Air temperature (T_{air} - $^{\circ}\text{C}$)	16.05 \pm 0,29	7.29 \pm 0,41	311.17	p<0.05*
Soil temperature at 0-7cm (T_{s1} - $^{\circ}\text{C}$)	17.68 \pm 0,22	7.05 \pm 0,37	616.42	p<0.05*
Soil temperature at 7-28cm (T_{s2} - $^{\circ}\text{C}$)	17.67 \pm 0,17	7.33 \pm 0,29	946.9	p<0.05*
Soil temperature at Ts 28-100cm (T_{s3} - $^{\circ}\text{C}$)	17.55 \pm 0,06	8.30 \pm 0,14	3631.1	p<0.05*
Precipitation (T_p - m)	2.93 \pm 0,59	0.61 \pm 0,28	12.598	p<0.05*
Soil moisture 0-7 cm (M_{s1} - m^3m^{-3})	0.46 \pm 0.00	0.41 \pm 0.00	83.703	p<0.05*
Soil moisture 7-28 cm (M_{s2} - m^3m^{-3})	0.47 \pm 0.00	0.41 \pm 0.00	200.49	p<0.05*
Soil moisture 28-100 cm (M_{s3} - m^3m^{-3})	0.48 \pm 0.00	0.41 \pm 0.00	513.85	p<0.05*

Note: An asterisk (*) indicates significance ($p \leq 0.05$).

3.2. Body mass

All statistical results are presented in Table 2. Body mass was significantly affected by reproductive state, sex and the interactions between season*sex and reproductive state*sex, but not by season, reproductive state*season or the three-way interaction between season*sex*reproductive state (Table 2; Fig. 1). Females (77.7 ± 3.0 g) had significantly lower body mass than males (117.1 ± 5.9 g) (Table 2). Reproductive individuals had a significantly higher body mass (106.7 ± 3.9 g) than non-reproductive individuals (86.7 ± 5.8 g) (Table 2; Fig. 1). For post hoc comparisons (sex*reproductive state), RMs (121.3 ± 5.4 g) had a significantly higher body mass than RFs (92.0 ± 3.0 g) ($p < 0.001$). Furthermore, NRFs (64.8 ± 2.6 g) also had significantly lower body mass than NRMs (112.5 ± 8.8 g) ($p < 0.0001$). For within-sex reproductive state comparisons, RFs were significantly larger than NRFs ($p < 0.0001$), but this trend was not significant in males ($p = 0.38$) (Fig. 1). For season*sex comparison, male body mass in the summer/wet period (140.7 ± 2.5 g) was significantly larger compared to females (80.3 ± 4.0 g) ($p < 0.0001$), with a similar trend in the winter/dry period (male: 94.7 ± 5.8 g; female: 74.8 ± 4.4 g) ($p = 0.0054$) (Fig. 1). Male body mass in the summer/wet period was significantly higher than body mass in winter/dry period ($p < 0.0001$), but not in females ($p = 0.79$) (Fig. 1). As a note, body mass analysis was only performed on individuals where oxidative variables were available. An analysis of body mass in a larger dataset on the population ($n = 472$) did not find a difference in body mass between seasons, but body condition (relationship between body mass and body size) was higher in summer (Finn, unpublished data), similar to the results from this study. All remaining comparisons are not significant.

3.3. TAC

All statistical results are presented in Table 2. TAC was unaffected by all primary predictors (season, reproductive state or sex) or interactions (Table 2, Fig. 2A).

3.4. TOS

All statistical results are presented in Table 2. TOS concentrations were significantly affected only by season, but not any other primary predictors (sex and reproductive state) or interactions (Table 2, Fig. 2B). Natal mole-rats captured in the summer/wet period (4.51 ± 0.32 $\mu\text{mol H}_2\text{O}_2$ equivalent/L) possessed lower plasma TOS

concentrations than Natal mole-rats captured in the winter/dry period ($6.06 \pm 0.37 \mu\text{mol H}_2\text{O}_2$ equivalent/L).

3.5. OSI

All statistical results are presented in Table 2. OSI was significantly affected only by season but not any other primary predictors (sex and reproductive state) or interactions (Table 2, Fig. 2C). Natal mole-rats captured in the summer/wet period ($0.34 \pm 0.03\%$) possessed lower plasma OSI values than Natal mole-rats captured in the winter/dry period ($0.49 \pm 0.03\%$).

3.6. MDA

All statistical results are presented in Table 2. MDA was significantly affected by season, sex and sex*season, but reproductive state, and any interactions with reproductive state were not significant (Table 2, Fig. 2D). Overall males had significantly higher MDA ($2752.5 \pm 290.0 \text{ nmol/ml}$) compared to females ($1090.6 \pm 124.6 \text{ nmol/ml}$) (Table 2). The summer/wet period individuals had significantly higher MDA ($2182.0 \pm 310.0 \text{ nmol/ml}$) as compared to winter/dry period ($1584.6 \pm 173.0 \text{ nmol/ml}$) (Table 2). Post hoc comparisons show that within season (season*sex), female MDA during the summer/wet period ($849.4 \pm 151.1 \text{ nmol/ml}$) was significantly lower compared to female MDA during the winter/dry period ($1358.6 \pm 187.0 \text{ nmol/ml}$) ($p=0.04$) (Fig. 2). Contrastingly, male MDA during the summer/wet period ($3749.8 \pm 393.1 \text{ nmol/ml}$) was significantly higher than male MDA during the winter/dry period ($1810.5 \pm 297.0 \text{ nmol/ml}$) ($p=0.007$) (Fig. 2). Lastly, summer/wet period females MDA was significantly lower as compared to summer/wet period males ($p<0.0001$). Contrastingly the winter/dry period MDA did not significantly differ between the sexes ($p=0.78$).

3.7. Oxidative variable-body mass relationship

There was no effect of body mass on any oxidative variable (OSI, TAC, TOS or MDA) in female Natal mole-rats (Table 3, Fig. 3). Similarly, in male Natal mole-rats, no effect of body mass on OSI, TAC or TOS was observed (Table 3, Fig. 3); however, a strong effect of body mass on MDA was observed in males Natal mole-rats (Table 3, Fig. 3).

Table 2. The statistical outputs from the models investigating the effects of season (wet vs. dry), breeding status (reproductive vs. non-reproductive) and sex (male vs. female), their two-way and three-way interactions on the body mass, total antioxidant capacity (TAC), total oxidant status (TOS), oxidative stress index (OSI) and malondialdehyde (MDA) of Natal mole-rats (*Cryptomys hottentotus natalensis*), respectively.

Variable	Body Mass		TAC		TOS		OSI		MDA	
	t	p	t	p	T	p	t	p	t	p
Season	0.093	0.93	-1.093	0.28	2.102	0.04*	2.615	0.01*	2.801	<0.01*
Reproductive state	-3.523	<0.05*	-0.57	0.571	-1.9	0.06	-1.353	0.19	1.746	0.09
Sex	5.017	<0.05*	-1.123	0.27	-0.59	0.55	0.201	0.84	5.495	<0.001*
Season*reproductive state	-1.04	0.30	0.284	0.78	0.529	0.60	0.066	0.95	-1.467	0.15
Season*Sex	-2.572	0.01*	0.008	0.99	-0.838	0.41	-0.945	0.35	-3.601	<0.001*
Reproductive state*Sex	-2.575	0.01*	-0.492	0.62	1.198	0.24	1.417	0.16	-1.065	0.29
Season*Reproductive state*Sex	-0.469	0.64	0.321	0.75	-0.523	0.6	-0.421	0.68	1.059	0.29

Note: An asterisk (*) indicates significance ($p \leq 0.05$).

Table 3. The statistical outputs, using linear regressions between body mass and total antioxidant capacity (TAC), total oxidant status (TOS), oxidative stress index (OSI) and malondialdehyde (MDA), respectively, for male and female Natal mole-rats (*Cryptomys hottentotus natalensis*).

	Male				Female			
	Slope	y-intercept	F	p	Slope	y-intercept	F	p
TAC	1.794	1012.0	1.06	0.31	-1.89	1554	0.48	0.49
TOS	-0.01	5.73	0.26	0.61	0.02	4.11	0.68	0.41
OSI	0.00	0.55	0.78	0.38	0.00	0.25	1.71	0.20
MDA	23.9	-47.9	6.95	0.01*	-5.50	1517.0	0.62	0.43

Note: An asterisk (*) indicates significance ($p \leq 0.05$).

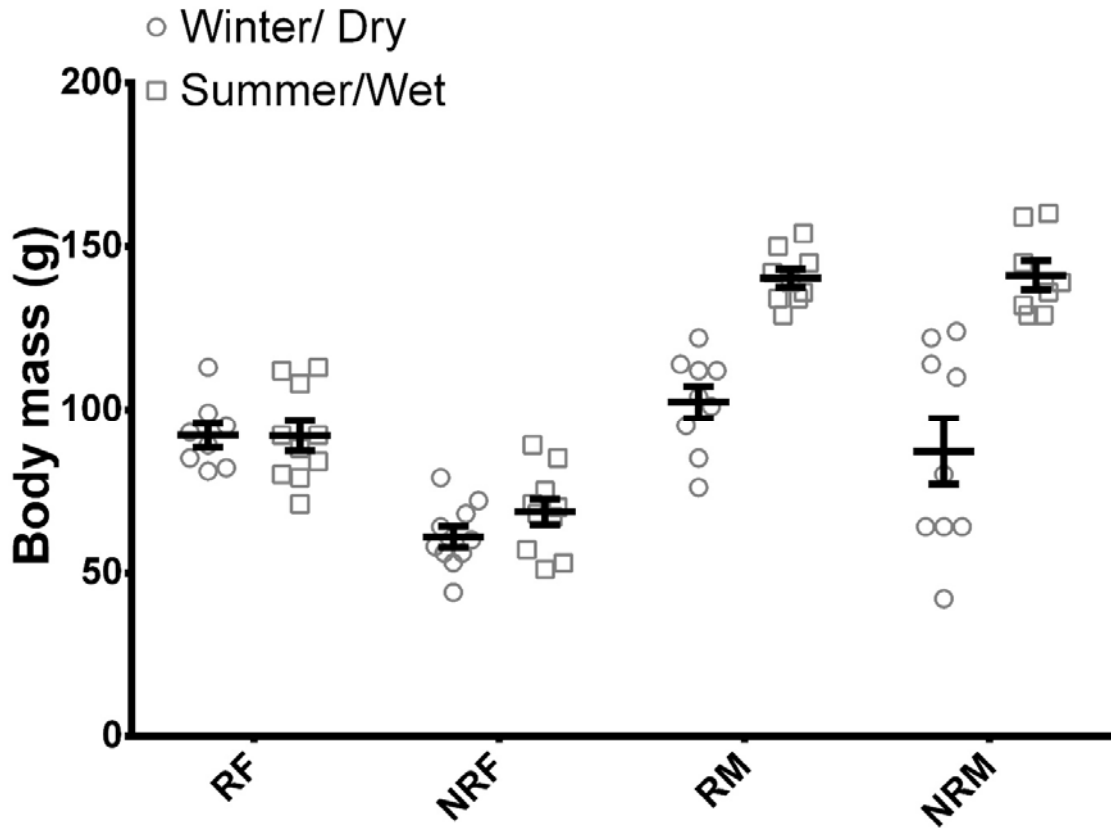


Fig. 1. The variation of body mass (g) in Natal mole-rats (*Cryptomys hottentotus natalensis*) between reproductive (RF) and non-reproductive (NRF) females, reproductive (RM) and non-reproductive males (NRM) across the summer/wet period and winter/dry period. Mean \pm s.e.m.

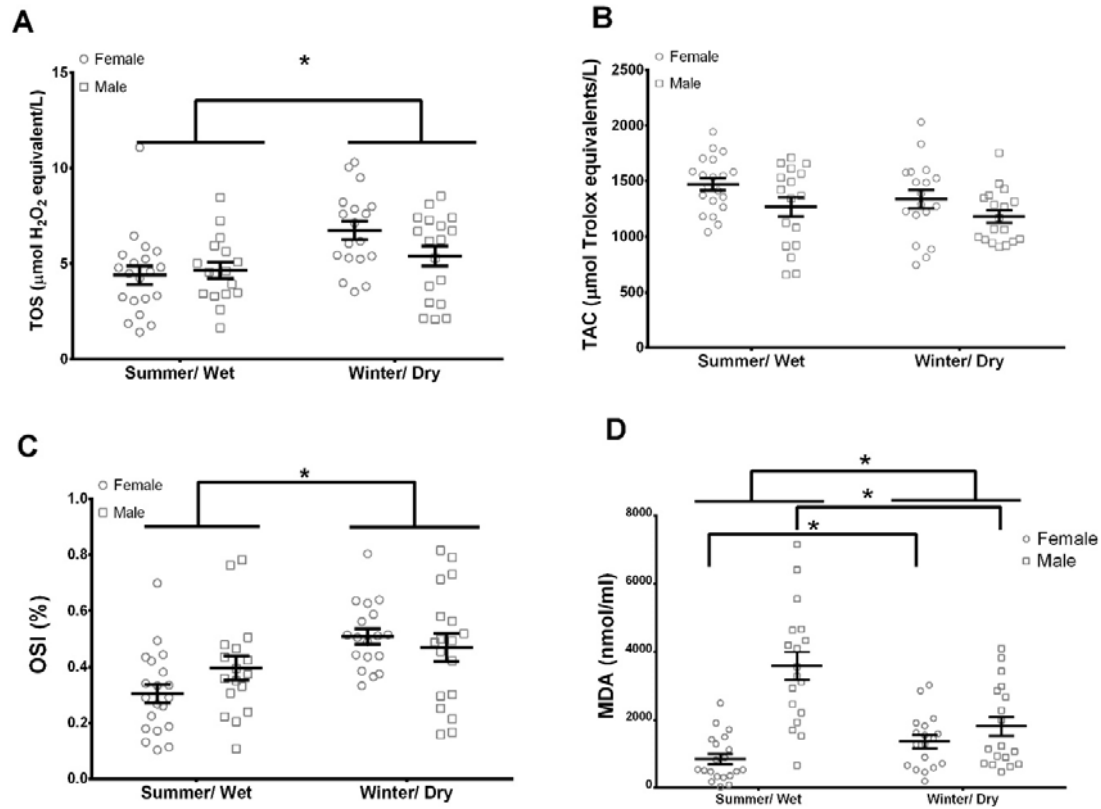


Fig. 2. The variation of A) total antioxidant capacity (TAC- $\mu\text{mol Trolox equivalents/L}$) B) total oxidant status (TOS - $\mu\text{mol H}_2\text{O}_2$ equivalent/L) C) oxidative stress index (OSI - %) and D) Malondialdehyde (MDA - nmol/ml) in Natal mole-rats (*Cryptomys hottentotus natalensis*) between females and males across the summer/wet period and winter/dry season. Mean \pm s.e.m. Note: An asterisk (*) indicates significance ($p \leq 0.05$).

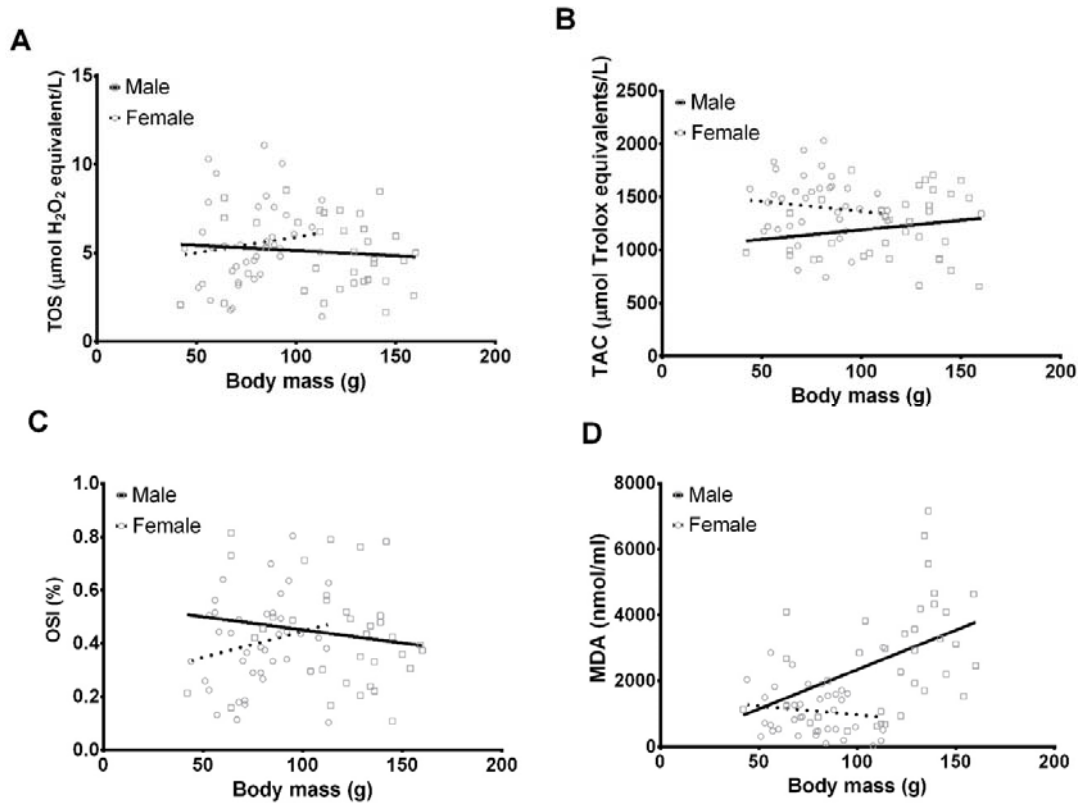


Fig. 3 A) total antioxidant capacity (TAC- $\mu\text{mol Trolox equivalents/L}$) B) total oxidant status (TOS - $\mu\text{mol H}_2\text{O}_2$ equivalent/L) C) oxidative stress index (OSI - %) and D) Malondialdehyde (MDA - nmol/ml) against male and female adult Natal mole-rats (*Cryptomys hottentotus natalensis*) body mass (g). The solid line and open circles (\circ) and dotted line and open squares (\square) represent male and female Natal mole-rats, respectively.

4. Discussion

This study attempted to address two simple, but critical questions. First, whether there is a blood plasma oxidative cost to reproduction in an aseasonally breeding mole-rat, which only utilises behavioural reproductive suppression with different seasonal climates. Secondly, whether there is an oxidative consequence involved in the seasonal implementation of heterothermy in male and female Natal mole-rats. Unlike the social seasonal breeding Highveld mole-rat and the eusocial aseasonal breeding naked mole-rat (Jacobs et al., 2021a; Jacobs et al., 2021b), there was no indication of a direct physiological oxidative cost to reproduction in either sex of Natal mole-rats, as seen by a lack of significant results in TOS, OSI and MDA between reproductive and

non-reproductive individuals. In both female naked and Highveld mole-rats on achieving reproductive activation (i.e. no longer under reproductive suppression), TOS and OSI significantly increased, indicating that the physiological changes associated with breeding and the subsequent loss of reproductive suppression results in oxidative stress (Jacobs et al., 2021b). Both the Highveld and naked mole-rat show a joint physiological and behavioural reproductive suppression mechanism (Bennett et al., 2018; Faulkes et al., 1990; Van der Walt et al., 2001), while the Natal mole-rats exhibits only reproductive inhibition through incest avoidance (Oosthuizen et al., 2008). Physiological reproductive suppression inherently suppresses reproductive hormones such as oestrogen, progesterone and testosterone (Levine and Muneyyirci-Delale, 2018; Nna et al., 2016). Oestrogens promote antioxidant enzyme functions (Miller et al., 2007; Strehlow et al., 2003; Wassmann et al., 2005), whereas progesterone may antagonise the vasoprotective effects of oestrogens amplifying oxidative stress (Yuan et al., 2016). In comparison, the behavioural suppression observed in the Natal mole-rat does not result in drastic decreases in reproductive hormones as seen in the naked and Highveld mole-rat (Oosthuizen et al., 2008). The current study demonstrated that in the absence of physiological suppression, Natal mole-rats do not show an oxidative cost to reproduction as there is almost no discernible physiological difference between reproductive and non-reproductive within in each season. However, the absence of enzymatic antioxidant investigation limits conclusive explanations of whether Natal mole-rats may employ oxidative shielding or hormesis. Female Natal mole-rats have lower levels of oxidative stress than males, possibly due to the increased antioxidant activity and less ROS production in females compared to males, as observed in other mammals (Barp et al., 2002; Miller et al., 2007; Viña et al., 2005). Lastly, the antagonistic nature of high testosterone levels (Alonso-Alvarez et al., 2007; Metcalfe and Alonso-Alvarez, 2010) is unlikely to explain the increased MDA in Natal mole-rat males as testosterone levels have previously been shown to be lower in the summer/wet period as compared to the winter/dry period (Oosthuizen et al., 2008), the opposite trend to what is observed in the present study.

In the current study, season had a more substantial influence on the redox balance than sex and reproductive state in Natal mole-rats. Seasonal variation in ambient temperature, precipitation and soil temperature (Bennett, 1989; Finn et al., 2022; Hart et al., 2021b; Hart et al., 2021c; Herbst et al., 2004; Oosthuizen et al., 2021; Wallace et al., 2021) and soil moisture (Lovegrove, 1989; Okrouhlík et al., 2015;

Vejmělka et al., 2021) play a critical role in the ecology of the African mole-rats. Our results indicate that soil moisture and temperature play a vital role in the thermal physiology and behaviour of the Natal mole-rat. In an effort to reduce the risk of activity-induced hyperthermia due to increased burrow temperatures during the summer/wet period, this species avoids activity during midday and is suspected to implement heterothermy (Finn et al., 2022; Oosthuizen et al., 2021). However, in this case, heterothermy is unlikely to be used for energy conservation, as food resources are likely available in dry and wet seasons due to food caching behaviour of the mole-rats (Bennett and Faulkes, 2000). The food caches are a possible reason why TAC concentrations remained constant across both seasons as Natal mole-rats have access to similar food quality and quantity year-round. Thus, heterothermy is likely used to avoid hyperthermia and possibly the damaging effects of long-term exposure to increased soil temperature and humidity during summer (Bennett and Faulkes, 2000; Finn et al., 2022). The more frequent implementation of heterothermy, results in lowered RMR, which may subsequently result in lower TOS and OSI values (Orr et al., 2009). We found similar decreases in this study during the summer/wet period and this decrease was particularly evident in females

However, the opposite pattern is observed in plasma MDA concentration in Natal mole-rats. Overall, during the summer/wet period, lipid damage (MDA) was higher than during the winter/dry period, a surprising result as the increased TOS and OSI in the winter/dry period would be expected to result in increased MDA. On closer evaluation, female Natal mole-rats followed the expected pattern where MDA increases during the winter/dry period as compared to the summer/wet period, however, males exhibited the opposite trend. Oosthuizen et al. (2021) found that female Natal mole-rats implement heterothermy more often during the summer/wet period, while the male Natal mole-rats implement heterothermy more often during the winter/dry period. Since the Natal mole-rat is an aseasonal breeder, and heterothermy is primarily used for self preservation, reproductive strategies may differ within and between seasons for each sex in order to allow for year round breeding, but this requires further investigation. The sexual dimorphism in the implementation of heterothermy across seasons mirrors MDA values of male and female Natal mole-rats. The sexual dimorphism in body mass may play a significant role in redox balance of Natal mole-rats.

Body mass plays a significant role in thermoregulation, and heat stress (Gardner et al., 2011; Rezende and Bacigalupe, 2015; Sheridan and Bickford, 2011), particularly the larger/ heavier the animal is, the slower it takes to dissipate heat, which may result in several negative consequences (Speakman and Król, 2010). In the current study, the effects of MDA varied within sex, where males, at the upper end of their body mass limit had significantly higher MDA during summer/wet period. In support of this, regression analyses demonstrated a significant positive correlation of body mass with MDA in males but not in females. Since soil temperatures did not exceed thermal neutral zone temperatures (Bennett et al., 1993b), it is therefore unlikely that environmental conditions alone were sufficient for heat and/or desiccation based oxidative stress (Jacobs et al., 2020b). Therefore, we propose that these heavier males may have experienced exercise-induced oxidative damage (Gökbel, 2006; Powers et al., 2016; Vollaard et al., 2005). Firstly, digging and burrow excavation is energetically expensive (Lovegrove, 1989; Zelová et al., 2010). The metabolic output of exercise in soil conditions that are very humid could lead to mild hyperthermia, accentuating oxidative stress and consequently leading to increased oxidative damage (Hillman et al., 2011; McNulty et al., 2005). The Natal mole-rat is better equipped to deal with colder temperatures with high thermal conductivity due to its dense pelage (Kotze et al., 2008; Vejmělka et al., 2021). Heat dissipation capability of these mole-rats could be a factor, as mole-rats are inherently poor at heat dissipation (Hart et al., 2021c; Vejmělka et al., 2021; Wallace et al., 2021), which would be more prominent in larger individuals (Speakman and Król, 2010; Vejmělka et al., 2021). The soil environment of the Natal mole-rat during the summer/wet period is water clogged and would further complicate heat dissipation as evaporative heat loss may not be possible (Bennett et al., 1994; Hart et al., 2021c; McGowan et al., 2020; Wallace et al., 2021; Zelová et al., 2010). However, mole-rats may shed excess heat through their feet and ventral surfaces (Šumbera, 2019; Vejmělka et al., 2021) and/or by passing damp soil (soil cooler than body temperature) under their belly while digging, body temperature can be regulated through conduction and convection (Okrouhlík et al., 2015). Digging activity of wild mole-rats in humidity near saturation or in various soil temperatures has yet to be investigated. Several physiological differences are present between the sexes in the Natal mole-rat, where larger males have a higher sustained metabolic scope, RMR, daily energy expenditure and water turnover rate (Hart et al. 2022b in press; Hart, Bennett and Scantlebury unpublished

results). Furthermore, females are inherently better at dealing with oxidative stress from exercise-induced hyperthermia (Garcia et al., 2018; Gökbel, 2006). Additionally, females have physiological advantages of oestrogen which can upregulate enzymatic antioxidant defenses and produce less hydrogen peroxide in mitochondria (Borrás et al., 2003; Tiidus, 2000; Viña et al., 2005). These factors, along with the smaller body size of females in the current study, may explain why females were not oxidatively damaged as males in the summer/ wet period. We suggest that all these factors together could have contributed to the high oxidative stress observed in male Natal mole-rats.

There are some shortcomings with the research which need to be stated. Firstly, this study involved investigating reproduction under natural conditions, which means we could not account for the oxidative state of the animals before measurement or the age of individuals. Furthermore, the lack of antioxidant enzymatic investigation does not allow us to draw significant direct conclusions about other means of antioxidant defence from being oxidatively stressed (Birben et al., 2012; Habashy et al., 2019; Limón-Pacheco and Gonsebatt, 2009; Sohal et al., 1990) or conclusive evidence of either oxidative shielding (Blount et al., 2016) or hormesis (Alonso-Alvarez et al., 2017; Costantini, 2014; Luna-López et al., 2014; Oliveira et al., 2018). Furthermore, we cannot determine how enzymatic antioxidant defences were affected by heat stress. Lastly, despite preventing terminal endpoints for animals, plasma markers is limiting when providing information about tissue oxidative stress (Nussey et al., 2009; Oldakowski et al., 2012). The benefits of using plasma include the non-destructive (no euthanasia of the animals and tissue harvesting) sampling, where studies on rare animals, or the removal of individuals from a population are not feasible, leaving blood sampling as the only option (Christensen et al., 2015). Lastly, previous research has shown that some oxidative markers measured in plasma can reflect tissue (heart, liver and kidney) oxidative stress (Margaritelis et al., 2015; Veskoukis et al., 2009). Plasma values however, do not reflect long term oxidative stress due to the fast turn over rate of plasma (Nussey et al., 2009). Lastly, oxidative damage can vary between tissue types depending on the type of oxidative stress investigated (Costantini, 2008; Jacobs et al., 2021c; Jacobs et al., 2020b; Schmidt et al., 2014). Since we expect the source of the male oxidative damage to be a consequence of exercise-induced stress and/or heat related due to lack of heat dissipation, the tissues most vulnerable to heat stress are the brain (Chen et al., 2013;

Hsu et al., 2006), liver (Zhang et al., 2004; Zhang et al., 2003), kidneys (Jacobs et al., 2021c; Jacobs et al., 2020b) and the small intestine (Henle and Leeper, 1982), whereas for exercise-induced oxidative damage, tissue related changes are lacking (Powers et al., 2011; Powers et al., 2016). It is speculated that tissues such as heart and lungs are also greatly affected by increased ROS generation during exercise, which could lead to oxidative damage (Powers et al., 2011). Additionally, exercise-induced hyperthermia as a result of digging in warm wet soils could exacerbate the possible environmental oxidative damage experienced even further (Georgescu et al., 2017; Hillman et al., 2011; McAnulty et al., 2005). However, skeletal muscle tissue has a high heat tolerance and is unlikely to experience significant oxidative damage from exercise induced hyperthermia or whole-body hyperthermia (King et al., 2016). However, heavy exertional exercise can induce oxidative stress in skeletal muscle (Davies et al., 1982), and since digging is energetically expensive and costly (Lovegrove, 1989), skeletal muscle damage could likely be observed despite the high heat tolerance of skeletal muscle, as oxidative damage is primarily predicted to be caused by exercise and not heat stress (King et al., 2016). Since we do not know the antioxidants available to these tissues or the concentration of enzymatic antioxidants, we can only predict that oxidative stress would reflect plasma values and that oxidative damage would be elevated in these tissues. Lastly, another shortcoming in the current study, is the reliance on satellite reanalysis climate dataset for soil temperatures. While climate reanalysis models (e.g., ERA5-Land used in this study) provide ease of data access, some variables may be under estimated compared to actual local conditions. For example, soil temperatures collected from data loggers at the study site were higher than the soil temperatures from the climate dataset (Finn et al., 2022; Oosthuizen et al., 2021). Maximal soil temperatures observed can be as high as 29.4°C closer to the surface and 22-24°C at depths between 25-30cm (Oosthuizen et al., 2021). Thus, soil temperatures at the study site may be much higher than suspected, in addition to great fluctuation to these soil temperatures depending on year or vegetation cover. Furthermore, soil humidity would be high in the summer/wet period due to the higher moisture soil content and air humidity from climate data regularly exceeding 90%. This study highlights the need to collect accurate atmospheric conditions of burrows and soil types from different African mole-rat species to better understand their evolutionary adaptations to their unique habitat type and soil conditions.

In conclusion, this study brings to light two important hypotheses. Firstly, the means of reproductive suppression (i.e solely behavioural vs. physiological and behavioural) that plays a significant role in a cooperative animal redox balance. Secondly, implementing heterothermy may be used to avoid the detrimental oxidative consequences of environmental and/or exercise-induced hyperthermia. Further research is required to increase our knowledge of these two critical hypotheses. Lastly, sex specific differences of exercise-induced oxidative stress is the first observed for an African mole-rat species, and may be a relevant sex difference in the longevity of females compared to male social mole-rats.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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