Effect of colony disruption and social isolation on naked mole-rat endocrine correlates

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Highlights

•Non-breeding naked mole-rat sexes differ in hormone responses to isolation.Females show elevated plasma cortisol concentrations during isolation.
•Colony disruption causes an increase in cortisol concentrations in resident females.
•Male cortisol concentrations do not change with long-term isolation or colony disruption.
•Colony disturbance may cause a disruption in reproductive suppression of females.

Declarations of interest: none

Abstract

The social environment of animals can have profound implications on their behaviour and physiology. Naked mole-rats (*Heterocephalus glaber*) are highly social with complex dominance hierarchies that influence both stress- and reproduction-related hormones. Homeostasis may be affected by aggressive interactions, colony instability and social isolation. Furthermore, naked mole-rat colonies are characterised by a marked reproductive skew; a single female and few males are reproductively active while other colony members are reproductively suppressed. Thus, there are distinct differences in related hormone concentrations between reproductively active and non-active animals; however, this changes when non-reproductive individuals are removed from the colony. We investigated the effects of social isolation and colony disruption on plasma cortisol and progesterone concentrations in non-breeding naked mole-rats. During colony disruption, we found a significant increase in cortisol concentrations in females removed from the colony for social isolation (experimental) as well as in females that remained in the colony (control). Cortisol concentrations were reduced in both groups after experimental animals were paired up. No changes in cortisol concentrations were observed in control or experimental males after removal from the colony or pairing. This suggests that the females, but not the males, found colony disruption and social isolation stressful. Upon removal from the colony, both control and experimental females showed a small increase in progesterone, which returned to basal levels again in the control animals. Experimental females showed a dramatic spike in progesterone when they were paired with males, indicating reproductive activation. The sex difference in the stress responses may be due to the stronger reproductive suppression imposed on females, or the increased likelihood of dispersal for males. It is clear that the social environment reflects on the endocrine correlates of animals living in a colony, and that the colony structure may affect the sensitivity of the animals to changes in their environment.

1. Introduction

Sociality is defined as the tendency of animals to live in groups and forms the basis of many social interactions between these animals (Lacey and Sherman, 1997). Sociality is adaptive and occurs when the benefits of group living exceeds the costs (Silk, 2007). It is a complex phenomenon and may be driven by a multitude of selective pressures (Lacey and Sherman, 1997). Groups may, therefore, vary considerably in size, structure and the degree of cooperation (Krause and Ruxton, 2002). The social environment of an animal is complex and can either be perceived as a major source of stress or it can serve to reduce the impacts of stressors (Beery and Kaufer, 2015; Creel et al., 2013). Therefore, social interactions, group stability, but also social isolation are factors that may influence the stress experienced by individuals of a social species.

The naked mole-rat (NMR; *Heterocephalus glaber*) is a highly social, subterranean rodent that has evolved a cooperative breeding system similar to that of eusocial insects (Jarvis, 1981). A characteristic of these societies is an extreme reproductive division of labour and a skew in lifetime reproductive success. Breeding is restricted to a single female (the 'queen') and one or two males, while more than 90% of individuals in a colony never have the opportunity to reproduce (Jarvis and Bennett, 1993). Breeding individuals are larger than the non-breeding individuals and queens are considerably longer (Jarvis, 1981; O'Riain et al., 2000). Non-breeding NMRs of both sexes are physiologically inhibited from reproducing (Faulkes et al., 1990a, 1991; Faulkes et al., 1994). Non-breeding females are anovulatory and typically have low plasma luteinising hormone (LH) and progesterone concentrations (Faulkes et al., 1990a; Faulkes et al., 1990b; Zhou et al., 2013), while non-breeding males have low testosterone concentrations (Faulkes et al., 1991; Zhou et al., 2013). Recent evidence suggests that reproductive inhibition in both non-breeding females and males may result from hyperprolactinaemia (Bennett et al., 2018). The reproductive suppression is lifted upon removal of the non-breeders from the colony and from the influence of the queen, such as during a dispersal event, where after females start to ovulate, as indicated by an increase in progesterone concentrations and normal reproductive behaviours can arise (Faulkes and Abbott, 1991; Faulkes et al., 1990a).

In a group setting, social interactions can be a cause of social stress leading to increased glucocorticoid secretion (Beery and Kaufer, 2015; Blanchard et al., 2001; Sloman et al., 2001; Tamashiro et al., 2005; Verdejo-Garcia et al., 2015). Social hierarchies may also influence hypothalamo-pituitary-adrenal (HPA) axis activity, reflected in varying levels of glucocorticoid secretion (Creel et al., 2013). Elevated glucocorticoid levels have been observed in subordinate female meerkats (*Suricata suricatta*) (Young et al., 2006) and Alpine marmots (*Marmota marmota*) (Hackländer et al., 2003). Wild NMR colonies comprise of 40 to 90 individuals, but can sometimes reach up to 300 members (Brett, 1986; Faulkes et al., 1994). Dominant (breeding) NMRs assert their dominance by using aggressive shoving behaviour (Clarke and Faulkes, 2001; Margulis et al., 1995). This aggression may lead to increased glucocorticoid secretion in either dominants or subordinates. It has been suggested that raised glucocorticoid levels may be the cause of reproductive suppression in subordinate individuals of cooperatively breeding species (Creel, 2001; Creel et al., 2013), but it does not seem to be the case in NMRs (Bennett et al., 2018; Clarke and Faulkes, 1997, 1998, 2001; Edwards et al., 2020).

Group stability can also play a role in HPA axis activity and thus glucocorticoid secretion (Chamove and Bowman, 1978; Sapolsky, 1992). In NMRs, glucocorticoid levels increase along with aggressive behaviours arising during queen succession or upon removal of a queen from a colony (Clarke and Faulkes, 1997; Medger et al., 2019). In their natural environment, group stability may be disrupted following dispersal events, although a limited number of individuals would leave the colony at a time (Braude, 2000; O'Riain et al., 1996). It is unknown how the removal of subordinate NMRs from a colony affects the glucocorticoid levels of the remaining resident animals.

Social isolation of socially-bonding species can also be stressful (Beery and Kaufer, 2015; Duarte et al., 2018). Increased glucocorticoid levels in individuals removed from their social group have been observed in several species (Castro and Matt, 1997; Duarte et al., 2018; Lyons et al., 1999; Woodruff et al., 2013; Ziegler et al., 1995) including NMRs (Edwards et al., 2020). It is, however, unclear whether the short-term increase as observed by Edwards et al. (2020) persists over extended isolation periods. Furthermore, glucocorticoid levels rise in individuals following the removal of their mates or group members (Adrian et al., 2008; Ferland and Schrader, 2011) and reunion with the

removed individuals often leads to a reduction in glucocorticoid levels (Duarte et al., 2018; Ferland and Schrader, 2011; Smith and French, 1997). In nature, dispersing NMRs would be temporarily socially isolated whilst they search for breeding partners, but the majority of the animals remain resident in a colony for the duration of their lifetimes (Buffenstein et al., 2012; O'Riain et al., 1996).

This study investigated the stress response of NMRs following the removal of non-reproductive animals from a colony by measuring plasma cortisol concentrations. We examined the effects of the colony disruption on animals remaining in the colony and the effects of long-term social isolation on the removed animals. We also assessed the effect of pairing isolated animals on plasma cortisol concentrations. We hypothesised that cortisol concentrations would increase in NMRs that were removed from their colonies, but that the concentrations would decrease again once the animals were paired. We also predicted that cortisol concentrations would remain constant in animals that remained in the colonies. No sex difference was expected.

Secondly, we measured progesterone concentrations in females that remained in the colonies and females that were removed from the colonies and subsequently paired. We predicted that progesterone concentrations of individuals in the colonies would remain unchanged, whereas females that were removed from the colony would show an increase in progesterone concentrations that would peak when the animals are paired with males.

2. Materials and Methods:

2.1. Animals:

Experimental animals were obtained from two captive, laboratory colonies comprised of 63 and 15 animals respectively. They were housed in Perspex burrow systems in a temperature-controlled room (±30 ºC) with fresh food (fruit, vegetables and Pronutro™ as a cereal supplement) supplied every second day. No water was supplied as naked mole-rats obtain enough moisture from their food. The pantry and toilet areas of the burrow systems were cleaned every second day and new wood shavings were added. Body masses of the chosen animals ranged from 30.2 g to 74.3 g for

males and 27.5 g to 71.0 g for females. All experimental procedures were approved by the University of Pretoria ethics committee (number: EC001-18).

2.2. Experimental protocol:

Twelve non-breeding males were chosen from the smaller and twelve non-breeding females were chosen from the larger colony, respectively. All 24 animals were tagged with Passive Integrated Transponder (PIT) tags (Biomark, Boise, ID, USA) for identification and allowed a two-day recovery period before the start of the experiment. The experiment consisted of three phases: 1) a baseline, 2) a separation, and 3) a pairing phase. During the baseline phase, all animals remained in their respective colonies for three weeks. For the separation phase, six of the twelve females and males, respectively, were removed from their colonies while the other 12 animals remained in their respective colonies to serve as controls. Thus, two treatment groups were formed: a) a control and b) an experimental group. Upon removal, the NMRs were placed individually in plastic boxes with wood shavings and paper towelling as nesting material and were fed and cleaned at the same time as the colonies. Individual NMRs were housed in the same room as the colonies for three weeks. During the final or pairing phase, separated females were added to the boxes containing the separated males and their behaviour and interaction with each other were monitored. When copulatory behaviour was observed, individuals were left together to form breeding pairs. Fighting was only observed in one pair with the female showing aggression towards the male. Therefore, a mesh-divider was placed into their box, separating the female from the male, but still allowing some interaction. The divider was removed three days later and no further fighting was observed. The experimental protocol was completed four weeks after pairing of the experimental animals.

2.3. Blood sampling:

Blood samples were collected from all 24 animals in each of the three experimental phases. During the baseline phase, samples were collected three times at one-week intervals, while in the separation phase, blood samples were collected twice, one and three weeks after the removal of the experimental animals. Finally, blood was collected twice, two and four weeks after pairing of the experimental animals.

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Blood sampling took place in the morning between 08h00 and 11h00 after the animals were pre-warmed under a 40 Watt lamp. Animals were weighed and then restrained by hand while approximately 0.1 ml of blood were collected with a heparinised capillary tube from the saphenic vein in the hind foot. Samples were taken within a 3-minute period (Romero & Reed, 2005; Sheriff et al., 2011). Blood was transferred to heparinised Eppendorf tubes and centrifuged for five minutes. Finally, the blood plasma was decanted and stored at -80 ºC until hormone assays could be completed. Animals were returned to their respective housing after the blood sample was taken.

2.4. Plasma cortisol and progesterone analysis:

Collected blood plasma was used to determine cortisol and progesterone concentrations using competitive enzyme-immunoassays (EIA). The EIA for cortisol was first described by Palme and Möstl (1997) and sensitivity was recorded at 0.02 ng/ml. The coefficients for intra-assay variance, determined by repeated measurements of high- and low-value controls, were 5.67% and 6.90%, respectively, and those for inter-assay variance were 11.47% and 11.58%, respectively. The progesterone EIA used was described by Schwarzenberger et al. (1996). The coefficients for intra-assay variance, again determined by repeated measurements of high- and low-value controls, were 4.07% and 5.33%, respectively, while those for inter-assay variance were 8.30% and 8.79%, respectively. The sensitivity of the progesterone EIA was 0.32 ng/ml. All EIAs were performed at the Endocrine Research Laboratory, University of Pretoria, as described by Ganswindt et al. (2002).

2.5. Statistical analyses:

Cortisol and progesterone concentrations were compared between the two treatments (control and experimental) as well as across the three experimental phases (baseline, separated and paired). Steroid concentrations for males and females were analysed separately for cortisol, and plasma progesterone concentrations were only determined and analysed in females. The means of the different samples (three samples for baseline and two each for separated and paired) were calculated for each of the three phases. We analysed almost all data using generalized linear mixed models with gamma distributions and log-link functions. Only plasma cortisol concentrations for males were analysed using a general linear mixed model as it was normally distributed. All analyses

were done using the R statistical software (R Core Team, 2018, https://www.R-project.org/). Analyses were performed with the R package lme4 (Bates et al., 2015). *P*-values were subsequently generated using the R package car (Fox and Weisberg, 2011). The fixed factors included in the models were treatment (control and experimental), phase and their interaction and animal ID was included as a random factor to account for repeated measurements. Tukey's HSD post hoc multiple comparisons were generated using the R package emmeans (Lenth, 2019). A correlation of progesterone with cortisol was evaluated using Spearman's rank test (females). *P*-values of ≤ 0.05 were considered to be significant and results are presented as mean ± standard error (SE).

3. Results:

3.1. Cortisol:

Plasma cortisol concentrations of female NMRs were similar between control and experimental animals (Wald χ^2 = 0.02, df = 1, p = 0.90), but differed significantly between the three experimental phases (Wald χ^2 = 60.7, df = 2, p < 0.001). Significantly higher cortisol concentrations were measured when the experimental females were separated from the colony compared to when they were still part of the colony (baseline) and the concentrations decreased again once they were paired up with a male (Tukey HSD: p < 0.001; Figure 1). Cortisol concentrations were similar during the baseline and the paired phases (Tukey HSD: p = 0.94). Interestingly, this same pattern was found for both control and experimental females (Treatment*Phase: Wald χ^2 = 0.81, df = 2, p = 0.67; Figure 1).

Plasma cortisol concentrations of male NMRs did not differ between the control and experimental animals (Wald χ^2 = 2.35, df = 1, p = 0.13) or the three phases of the experiment (Wald χ^2 = 2.75, df = 2, p = 0.25). There was also no interaction effect between treatment and experimental phase (Wald χ^2 = 4.08, df = 2, p = 0.13; Figure 1).

3.2. Progesterone:

Overall mean progesterone concentrations for the combined treatments continuously increased through the course of treatment with the lowest concentrations measured during baseline (35.91 ± 1.63 ng/ml), a moderate but significant increase during the following separation phase (48.87 ± 4.53

Figure 1: Plasma cortisol concentrations (ng/ml) of female and male non-breeding naked mole-rats while in a colony (baseline), when singly housed (separated) and when paired with an individual of the opposite sex (paired) for experimental animals and while in a colony for 10 weeks for control animals. The dots represent individual data points. *** $p < 0.001$
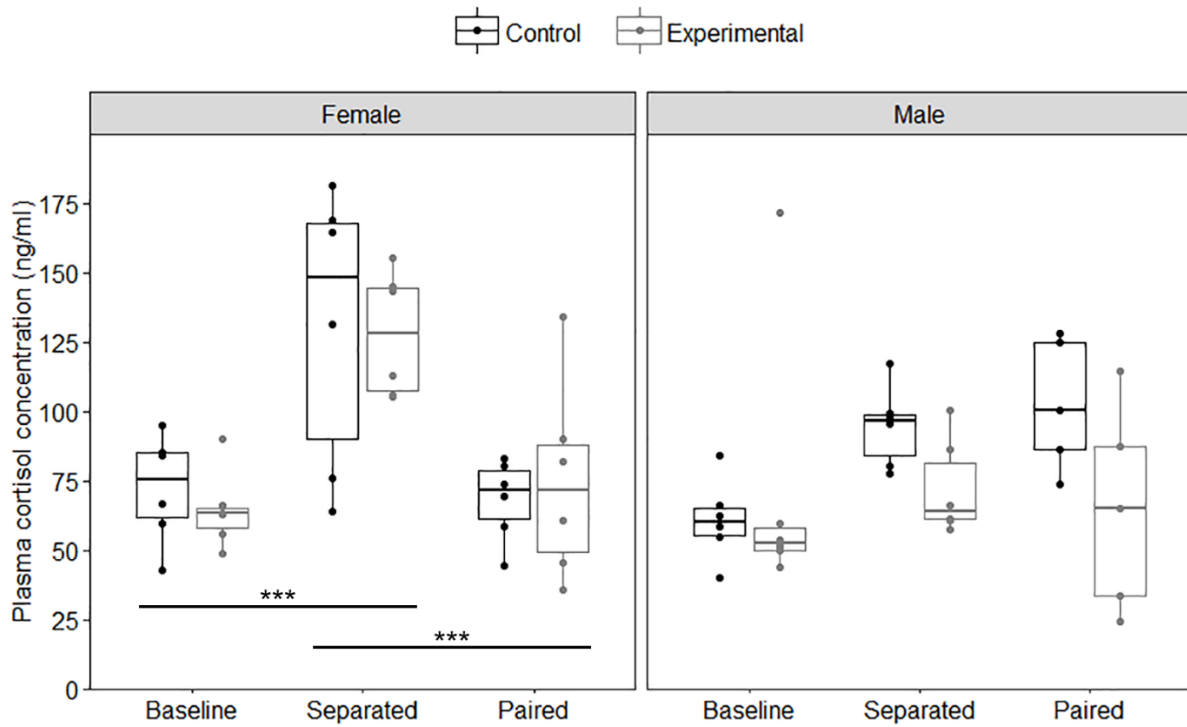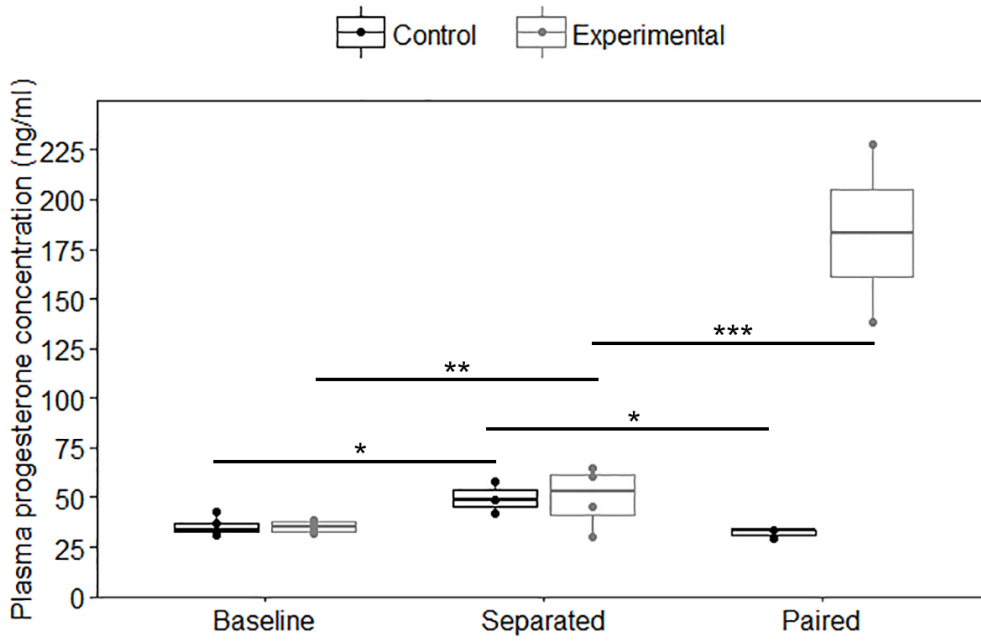
Figure 2: Plasma progesterone concentrations (ng/ml) of female non-breeding naked mole-rats while in a colony (baseline), when singly housed (separated) and when paired with an individual of the opposite sex (paired) for experimental animals and while in a colony for 10 weeks for control animals. The dots represent individual data points. $*p < 0.05$, $**p = 0.01$, $*** p < 0.001$

ng/ml), and significantly higher concentrations measured during the pairing phase (87.45 ± 33.89 ng/ml; Wald χ^2 = 82.80, df = 2, p < 0.001), which were likely caused by the large increase in progesterone concentrations in experimental females (see interaction results below). There was no overall effect of treatment on progesterone concentrations (Wald χ^2 = 1.24, df = 1, p = 0.27), but the interaction of treatment and experimental phase was significant (Wald χ^2 = 134.04, df = 2, p < 0.001). For the control females, progesterone concentrations were moderately but significantly higher during the separation phase (Tukey HSD: p ≤ 0.03; Figure 2) compared to the baseline and pairing phases while progesterone concentration during the baseline and pairing phases was similar (Tukey HSD: p = 1.00; Figure 2). Similar to control females, progesterone concentrations of experimental females also showed an increase during the separation phase in comparison to baseline (Tukey HSD: p = 0.01), but the concentrations continued to increase further during the pairing phase (Figure 2). The progesterone concentrations of females paired with males were higher than any of the other concentrations measured for control and experimental females (Tukey HSD: p < 0.001; Figure 2). There was no difference in progesterone concentrations between control and experimental females during the baseline and separation phases (Tukey HSD: p = 1.00). Progesterone concentrations were not correlated with cortisol concentrations (ρ = 0.36, p = 0.10).

4. Discussion:

NMRs live in groups with complex social structures where most colony members never reproduce or leave their natal colony. Therefore, individuals are never isolated from their group unless they disperse to set up new colonies. Additionally, the social structure of the colony may be disrupted when specific individuals disperse, so-called dispersive morphs (O'Riain et al., 1996). The impact of this disruption and social isolation, as well as the underlying physiology, particularly of non-breeding NMRs, has not been well studied. Thus, we investigated the effect of a 'forced' dispersal event, orchestrated by the removal of individuals from an intact colony, on circulating stress hormones in non-breeding male and female NMRs, and reproductive hormones in non-breeding female NMRs.

In female NMRs, cortisol concentrations of experimental animals significantly increased after removal from the colony, suggesting that social isolation is perceived as a stressful event. Similar

results were observed in female black tufted-ear marmosets (*Callithrix penicillata*) (Duarte et al., 2018; Smith and French, 1997), cotton-top tamarin monkeys (*Saguinus oedipus*) (Ziegler et al., 1995) and tuco-tucos (*Ctenomys sociabilis*) (Woodruff et al., 2013). Additionally, a recent study that measured glucocorticoid metabolites in faecal samples of NMRs also found an increase in glucocorticoid concentrations following isolation (Edwards et al., 2020). This increase was observed within one day of removal and continued to increase until 60 hours of isolation (Edwards et al., 2020). The present study provides evidence that cortisol concentrations remain high in females even after three weeks of isolation. Plasma cortisol concentration decreased again to baseline levels when the NMR females were paired with males, a trend that was also observed in marmosets (Duarte et al., 2018; Smith and French, 1997). In contrast, Edwards et al. (2020) show an increase in faecal glucocorticoid concentrations in both sexes after pairing, although that increase was not as pronounced as that observed with isolation in the same study. The differences between our and the Edwards et al. (2020) study may be explained by the lack of isolation of the animals before pairing in the latter. Females may be stressed because of removal from their natal colonies regardless of whether they are solitary or paired after removal; however, immediate pairing appears to be less stressful than isolation for female NMRs.

Interestingly, control females remaining in the colony showed the same pattern of increase and decrease in cortisol concentrations as the experimental animals. During queen succession or after removal of a NMR queen from a colony, the group becomes unstable and cortisol levels rise (Clarke and Faulkes, 1997; Medger et al., 2019). We hypothesise that the removal of several subordinate females from a colony could have had a similar effect by disrupting the dominance hierarchy and leading to elevated cortisol concentrations. Once the hierarchy in the group was re-established, the colony stabilised again, and cortisol concentrations decreased back to baseline. A similar decrease in cortisol concentrations after a new queen has been established was observed by Medger et al. (2019).

Disruption to the colony hierarchy may also explain the slight increase in progesterone concentrations of control females after the removal of experimental animals. The colony disruption may have interrupted the reproductive suppression exerted on the non-breeding females by the

queen, resulting in a slight increase of reproductive hormone secretion. However, full reproductive activation as seen in some non-breeding females after queen removal (Clarke and Faulkes, 1997; Margulis et al., 1995) was not achieved and progesterone concentrations returned to basal levels as colony hierarchy was re-established.

Plasma progesterone concentrations of experimental females continuously increased over the experimental period. A small, but significant increase was observed when the females were separated from the colony. Faulkes et al. (1990a) observed the first increases in progesterone concentrations of females eight days after removal from their colonies. When the females were subsequently paired with males (three weeks after removal), a marked increase in progesterone concentration was observed similar to observations in NMRs by Swift-Gallant et al. (2015). Swift-Gallant et al. (2015) also demonstrated that progesterone concentrations of female NMRs increased as late as one month post-removal from a colony. Thus, measurements of plasma progesterone concentrations during the separation phase (one and three weeks after removal) may have been taken too close to removal in our study to see large increases in plasma progesterone concentration. Sampling during the pairing phase (5 weeks after removal) may have allowed enough time after removal for increased progesterone secretion and the sharp increase is an indicator of reproductive activation in these animals.

Male NMRs showed different trends in cortisol concentrations compared to females as no significant differences were evident between any of the three experimental phases. Control males in the colony did not show changes in circulating cortisol concentrations when experimental animals were removed, as also demonstrated for Siberian hamsters (Castro and Matt, 1997). Clarke and Faulkes (1998) have previously shown that non-breeding males in a NMR colony show no changes in cortisol concentrations upon breeding male removal. Thus, the non-breeding males remaining in the colony do not appear to find removal of several other non-breeding colony members stressful.

Experimental males showed no change in cortisol concentrations after removal or subsequent pairing with females. This result differs from that of Edwards et al. (2020) where separation from a group lead to a significant increase in faecal glucocorticoid concentration of male NMRs. We may not have observed an increase in cortisol levels with isolation in males as we measured it much later

(after 1 week) than Edwards et al. (2020), who sampled over a period of 12 to 60 hours post isolation. Contrary to females, in which isolation may have a long-term effect on glucocorticoid levels (see above), male NMRs may show only short-term effects of isolation on glucocorticoid concentrations. Additionally, the colony from which we removed the males consisted mainly of males and very few females. Thus, the males may have been predisposed to disperse and removal from the colony may affect them to a lesser extent. Contrary to the present study, Edwards et al. (2020) also observed a significant increase in faecal glucocorticoid metabolite concentrations in males associated with immediate pairing. The lack of an isolation period in their compared to our study may again explain the differences observed between the studies, as discussed for females.

The differences between male and female NMR responses to colony disruption and social isolation may be explained by their differing reproductive characteristics. Non-breeding males within a colony experience reproductive suppression imposed by the queen (Faulkes and Abbott, 1991, 1997); however, spermatogenesis still takes place and some non-breeders are capable of producing motile spermatozoa (Faulkes and Abbott, 1991; Faulkes et al., 1991; Faulkes et al., 1994). Furthermore, males do not compete for breeding opportunities, their reproductive skew is lower than that of females and cortisol levels are similar in non-breeders and breeders (Clarke and Faulkes, 1998, 2001). Thus, social hierarchies in a colony might be less rigid for males than for females and removal from a colony may not serve as a large change to a males' lifestyle resulting in the lack of change in cortisol concentrations observed in the present study.

In contrast, female non-breeding NMRs are subjected to complete physiological suppression of reproduction. They are anovulatory and have low plasma LH levels resulting in a socially induced reproductive block (Clarke and Faulkes, 1997; Faulkes et al., 1990a; Faulkes et al., 1990b). Therefore, the hierarchies within the colony enforced by the queen may be stricter for females than for males and the removal of several females together may have had a larger effect on the stress levels of females, resulting in the observed sex differences in the effect of colony disruption and social isolation on NMRs. Females experience removal from their group and a change to their colony structure as stressful, whereas males do not. Sex differences in the physiological stress response to

social isolation and group instability have also been shown in other rodent species (Beery and Kaufer, 2015; Hatch et al., 1965; Palanza, 2001; Palanza et al., 2001).

The different responses of males and females could also relate to the dispersal strategy of NMRs. Dispersal behaviour differs between genders where males move away to join new groups more often than females (Clutton-Brock, 2016; Torrents-Ticó et al., 2018). Little is known about NMR dispersal. Dispersers in laboratory colonies are mainly males of a specific phenotype (O'Riain et al., 1996); however, dispersers in wild colonies belong to both sexes (Braude, 2000). Braude (2000) suggested that males and females possibly use different environmental cues for dispersal and those of females may be absent in lab conditions, thus O'Riain et al. (1996) only observed male disperser morphs. The related Damaraland mole-rat (*Fukomys damarensis*) also displays a male-biased dispersal in which males join established groups (Hazell et al., 2000; Torrents-Ticó et al., 2018). Therefore, male NMRs may be more likely to disperse than females and males may thus be less affected by removal from the colony or removal of others in comparison to females.

In conclusion, female NMRs showed the anticipated rise in cortisol concentrations associated with the stress perceived due to social isolation and colony disruption, and a subsequent reduction following the pairing of the single animals. In contrast, no changes were observed in stress-associated hormone levels in isolated males or the remaining control animals. Progesterone levels of both control and experimental females increased when animals were removed from the colony, where after it returned to basal levels in control animals and spiked in the paired experimental animals. It appears that female NMR experience social isolation and changes to the colony structure as stressful while males appear to be less affected. This study highlights the physiological variation that underlies the different behaviours of male and female NMR and emphasises the importance of the social environment in the physiology and behaviour of social animals.

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