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# The stress of being alone: Removal from the colony, but not social subordination, increases fecal cortisol metabolite levels in eusocial naked mole-rats

Phoebe D. Edwards[a,b,c,*], Skyler J. Mooney[a], Curtis O. Bosson[c], Ilapreet Toor[b], Rupert Palme[d], Melissa M. Holmes[a,b,e], Rudy Boonstra[b,c]

[a] Dept. of Psychology, University of Toronto Mississauga, Mississauga, ON L5L 1C6, Canada
[b] Dept. of Ecology & Evolutionary Biology, University of Toronto, Toronto, ON M5S 3B2, Canada
[c] Centre for the Neurobiology of Stress, Dept. of Biological Sciences, University of Toronto Scarborough, Toronto, ON M1C 1A4, Canada
[d] Dept. of Biomedical Sciences, University of Veterinary Medicine, 1210 Vienna, Austria
[e] Dept. of Cell & Systems Biology, University of Toronto, Toronto, ON M5S 3G5, Canada

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ABSTRACT

In many social species, hierarchical status within the group is associated with differences in basal adrenocortical activity. We examined this relationship in naked mole-rats (*Heterocephalus glaber*), eusocial rodents with arguably the most extreme social hierarchies of all mammals. This species lives in colonies where breeding is restricted to one socially dominant 'queen' and her male consorts, and all other individuals are reproductively suppressed 'subordinates'. The relationship between cortisol and social status in naked mole-rats has not fully been elucidated, as prior results on this topic have been contradictory. We used non-invasive feces sampling to measure baseline cortisol levels in eight laboratory colonies of naked mole-rats, to either replicate or reject rank differences. First, we successfully validated an assay to measure fecal cortisol metabolites (FCMs). Removal from the colony for the validation experiment, either alone or with an opposite sex conspecific, induced prolonged elevation of FCM levels on a scale of days to weeks. This increase in cortisol did not prevent the removed animals from sexually maturing. In colony-housed animals, we found no relationship between rank in the social hierarchy and FCM levels. Further, queens, breeding males, and reproductively suppressed subordinates all had equivalent FCM levels. We conclude that this species shows little evidence of the 'stress of dominance' or 'stress of subordination' and that reproductive suppression in naked mole-rats is not driven by elevated cortisol levels.

## 1. Introduction

Animals living in groups are faced with a variety of social challenges, including the trade-off between costs of attaining and then maintaining social status and the benefits that status confers. Such costs are often assessed through activity of the hypothalamic-pituitary-adrenal axis and the concentration of its final output, glucocorticoids (cortisol or corticosterone). Early field endocrine studies of baboon social groups demonstrated that socially subordinate individuals tend to have higher basal cortisol levels than dominant individuals, which were attributed to the physical and psychological costs of aggression and harassment they received from the dominants (Sapolsky, 1982). This 'stress of subordination' has been replicated in a variety of other species of primates, rodents, and carnivores (e.g. Goymann et al., 2001; Hackländer et al., 2003; Zhang et al., 2018). However, it has become clear that the relationship between social status and glucocorticoid levels is largely social-system dependent (Creel, 2001; Sapolsky, 2005; Creel et al., 2013). For many species that live in cooperatively breeding social groups - where only a few, dominant individuals reproduce and the others assist in rearing offspring - dominants tend to have higher glucocorticoid levels than subordinates (Creel, 2001; Creel et al., 2013). This has been ascribed to lower levels of within-group violence experienced by the subordinates (Abbott et al., 2003; Creel et al., 2013), though a few cooperatively breeding species do have high violence and higher subordinate cortisol levels at certain times (e.g. meerkats during the dominant female's pregnancy; Young et al., 2006). Finally, there are species where no consistent differences in glucocorticoid levels between dominant and subordinate individuals have been found, and levels are influenced instead by hierarchy stability (e.g. Setchell et al., 2010; Preis et al., 2019).

* Corresponding author at: Dept. of Psychology, University of Toronto Mississauga, Mississauga, ON, Canada
*E-mail address:* phoebe.edwards@mail.utoronto.ca (P.D. Edwards).

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Naked mole-rats (NMRs; *Heterocephalus glaber*) are on the extreme end of the spectrum of cooperative social behavior. NMRs are small, subterranean rodents native to East Africa. They are unusual among mammals in that they are eusocial, living in large colonies (up to 300 individuals) with strict social and reproductive hierarchies. Within each colony, reproduction is usually restricted to a single breeding female, called the queen, and 1–3 breeding males (Jarvis, 1981). These breeders are typically socially dominant over all other members of the colony, the subordinates, which are essentially suppressed into a permanent pre-pubertal adult stage. Further, not all subordinates are socially equal: individual subordinates also maintain a particular position within the overarching social hierarchy. These ranks are quite stable, though changing colony demands or removal/death of the queen can cause animals to shift their roles and ranks (Clarke and Faulkes, 1997; Mooney et al., 2015). Individuals of this species do not live solitarily, though a subset of individuals can disperse to find a mate and form new colonies (O'Riain et al., 1996; Braude, 2000).

Though NMRs are one of the most pronounced examples of a social hierarchy system in mammals, the relationship between social rank and cortisol is not clear. In intact colonies, non-pregnant queens have been found to have lower urinary cortisol than subordinates, contrary to the expectation for cooperatively breeding mammals (Faulkes and Abbott, 1997). Although the queen is the most aggressive animal in an NMR colony, subordinate cortisol levels are not associated with the rate of queen aggressive behavior (Clarke and Faulkes, 2001). Further, breeding males and subordinate males had no differences in urinary cortisol levels (Clarke and Faulkes, 1998). When assessing cortisol levels across linear dominance ranks within colonies, socially dominant individuals were found to have higher urinary cortisol levels in one colony, but not in two others (Clarke and Faulkes, 1997). This cortisol pattern did not change when the queens were removed - social instability did not increase cortisol levels in subordinates in the two colonies where they had been low (Clarke and Faulkes, 1997). Within subordinates, no differences in plasma cortisol levels were found between presumed 'workers' and 'soldiers', though soldiers were classified as larger, more high-ranking, and more aggressive (Hathaway et al., 2016). Taken together, these results are highly variable, potentially due to the limited availability of NMR colonies, blood samples, and urine samples inherent in these studies.

Our objective was to clarify the relationship between rank and cortisol levels in NMR, and either replicate or reject rank differences using fecal cortisol metabolites (FCMs) collected from 8 independent captive NMR colonies. Quantifying hormone levels in feces is an appealing approach for measuring baseline cortisol in NMRs. Feces sampling is non-invasive and allows repeated, longitudinal sampling in species that are difficult to bleed (Sheriff et al., 2011; Palme, 2019). It is extremely challenging to obtain blood samples from live NMRs, and certainly not without inducing significant additional stress. Furthermore, FCMs represent the integrated average of the relatively short-term fluctuations in cortisol seen in blood, and hence provide a more representative picture of chronic cortisol levels (Palme, 2019). However, native cortisol or corticosterone is typically not excreted in feces, meaning that only their metabolites can be measured (Palme et al., 2005). Validating methods to measure FCMs and the time course of their excretion is essential given the variability in these details across species (Touma and Palme, 2005; Palme, 2019). We used an enzyme immunoassay (EIA) to quantify FCMs, which measures total immunoreactivity of any FCMs that cross-react with the antibody. While liquid chromatography tandem mass spectrometry (LC-MS) is in some ways more ideal as individual metabolites can be measured, a single sample requires much more time and expense to process, limiting the use of this approach for studies with larger sample sizes, such as this one (Palme, 2019). There are also almost no reference standards for exotic FCMs available, and for this reason it would be much more complicated to set up such a measurement in a new species (Palme, 2019). Therefore, an EIA was used to quantify FCM levels in NMRs. We first assessed cortisol metabolism and excretion in NMRs, and then used the validated assay to compare FCM levels across social rank.

## 2. Materials & methods

### 2.1. Animals and housing

All experimental animals were bred and reared at the University of Toronto Mississauga and colonies were housed as previously described (Toor et al., 2015). In brief, colonies were housed in polycarbonate cages of various sizes (small: 30 × 18 cm × 13 cm; medium: 46 × 24 cm × 15 cm; large: 65 × 45 cm × 23 cm) connected by plastic tubing and lined with corncob bedding. All animals were housed on a 12:12 light/dark cycle at 28–30 °C and had ad libitum access to a diet of sweet potato and wet protein mash (Harlan Laboratories, Inc.). We created three experimental groups of NMR. The first was used for the validation experiments for FCMs, the second for assessing the impact of social pairing on FCMs, and the third for assessing the relationship between FCMs and social rank in established colonies. All animals in Experiments 1 and 2 were non-reproductive subordinates older than 1 year and were, therefore, all young- to middle-aged adults. Animals in Experiment 3 included both breeders and subordinates, and animals ranged from 4 months to 18 years of age. All procedures were in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the Animal Care Committees at both the University of Toronto Mississauga and the University of Toronto Scarborough.

### 2.2. Experiment 1. Validation experiment

The animals used in Experiment 1, consisting of 7 male and 3 female adult subordinates (32–48 g), were removed from their home colonies at the University of Toronto Mississauga vivarium and transferred to the vivarium at the University of Toronto Scarborough. After arrival, animals were individually housed in polycarbonate radiometabolism cages (47 cm × 26 cm × 20 cm) that had a floor comprised of wire mesh with a tray below. This system enabled urine to pass through to the cage bottom, while the feces were caught on the wire mesh, allowing independent collection of excreta. Any feces that were visibly contaminated with urine was not collected. Total transportation time (home cage to radiometabolism cage) was approximately 1 h.

To evaluate how removal from the colony affected cortisol levels, we collected baseline fecal samples at the time of removal from colony at University of Toronto Mississauga. After animals were transferred to

**Table 1**
Experimental timeline for the validation study.

| Date | Treatment | Description | Sample intervals (hours post) |
|---|---|---|---|
| Oct 20–23 | Baseline and Transport | Removal from colony and transfer to new facility | 0, 12, 24, 36, 48, 60 |
| Oct 24–25 | Radiometabolism study | Injection of $^{3}$H-cortisol | 0, 2, 4, 8, 10, 12, 14, 16, 18, 22, 26, 30, 34, 38 |
| Oct 26–27 | Physiological validation | Injection of ACTH | 0, 12, 24, 36 |
| Oct 28–29 | Biological validation | Restraint stress | 0, 12, 24, 36 |

Experiments began at 7:00 h (hour 0); h post = time after initiation of experiment. ACTH = synthetic adrenocorticotropic hormone.

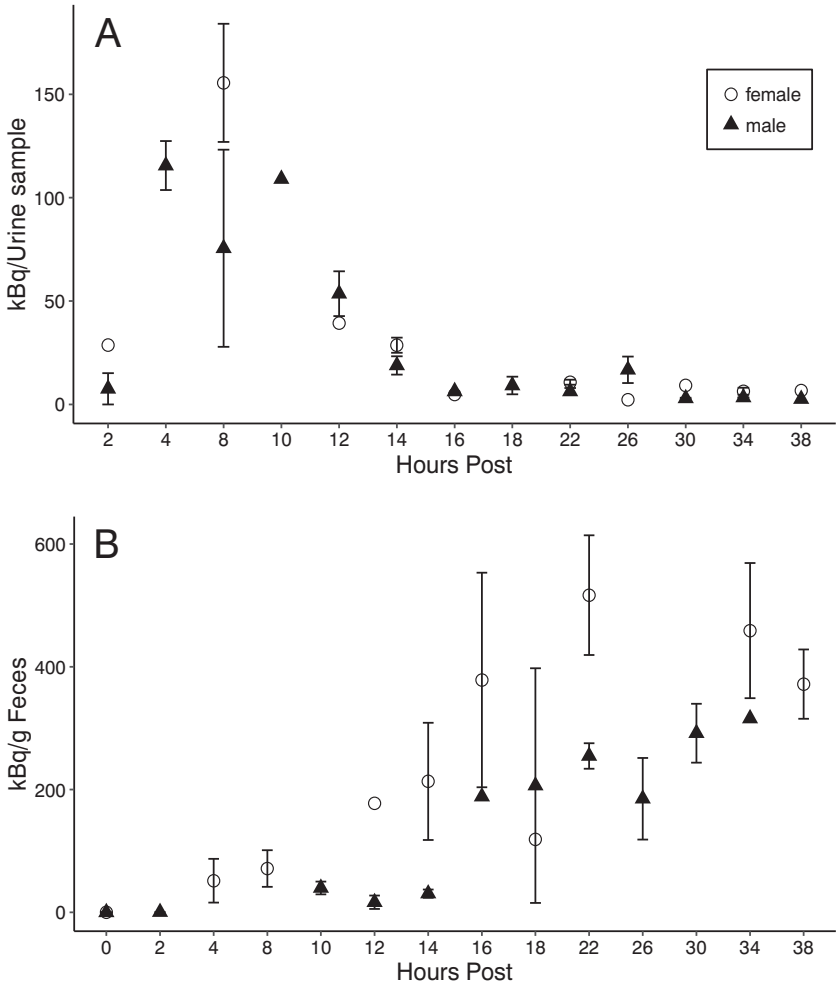


**Fig. 1.** Time-course of excretion of radioactivity in urine (A) and feces (B) in naked mole-rat females ($n$ = 3; open circles) and males ($n$ = 6; triangles). Times represent hours post $^{3}$H-cortisol injection. Points represent the mean excretion ± SE. Not all individuals provided samples at each collection time point, and therefore missing points are due to lack of excretion. When no confidence interval is given, points represent the single sample collected for that sex.

their radiometabolism cages, we collected fecal samples every 3 h for 60 h (Table 1). Most individuals did not defecate every 3 h. To reduce the percentage of missing data points, data were pooled into 12 h intervals (e.g. all data from 3 h, 6 h, 9 h, and 12 h were averaged into one data point for each animal). Sample values were averaged if an animal provided more than one sample in a given 12 h interval. One male died after transport and was therefore excluded from the experiment.

Radiolabeled cortisol was then used to determine the time course and route of excretion (feces vs urine), as well as the metabolic products of cortisol. The dominant glucocorticoid in NMRs is cortisol; studies using a corticosterone radioimmunoassay have detected no corticosterone in NMR plasma (Ganem and Bennett, 2004) and studies with LC-MS have reported very low levels of corticosterone relative to circulating cortisol in NMR plasma (Lewis et al., 2018). Animals were injected with 1110 kBq (30 μCi) of a tritiated cortisol solution (1,2,6,7-[$^{3}$H]; Amersham Biosciences, Buckinghamshire, UK) containing 14.2% ethanol (*v*/v) and 85.8% saline. We collected urine and feces at 2, 4, 8, 10, 12, 14, 16, 18, 22, 26, 30, 34, and 38 h post-injection (Table 1). We collected urine by first aspirating from the surface of the pan with a 1 mL pipette. The pan was then rinsed with 4 mL of 80% methanol and this rinse was aspirated and added to the urine sample. Between sampling periods, pans were twice rinsed with a radioactive decontamination solution.

To determine whether adrenocortical stimulation caused increased FCM levels, all 9 animals were next injected intraperitoneally with 4.0 IU/kg (40 μg/kg) of synthetic adrenocorticotropic hormone (ACTH; Synacthen Depot, CIBA, Ontario, Canada). Synacthen is comprised of the first 24 amino acids in ACTH, which are conserved across species, and does not include amino acids 25–33, which tend to be species-specific (Synacthen Depot Product Monograph, 2012). This synthetic ACTH has been successfully used to trigger the stress-axis in other rodent species (e.g. Touma et al., 2004; Bosson et al., 2009; Dantzer et al., 2010) as well as in more distantly related small mammals such as the snowshoe hare (Boonstra et al., 1998). We collected fecal samples every 2–3 h for 36 h post-injection. Samples were pooled into 12 h intervals as described above, to compensate for low levels of excretion. Following the ACTH challenge, all 9 animals were placed individually in a small restraint chamber (Broome Style Rodent Restrainer [Noryl Plastic], Unifab Corp. Kalamazoo, MI) for 10 min. These chambers were 3.8 cm in diameter x 14 cm long and restricted movement. We then collected fecal samples every 2–3 h for 36 h post-restraint, with samples pooled into 12 h intervals as above.

### 2.3. Experiment 2. Social pairing effects on FCM levels

This experimental group was composed of 6 male and 6 female adult subordinates (29–60 g), which were removed from their home colonies and housed in new polycarbonate cages (46 cm × 24 cm) lined with corncob bedding with one unfamiliar opposite-sex animal in the University of Toronto Mississauga vivarium. Fecal samples were collected from each individual at baseline (pre-pairing), and then weekly for 4 weeks of pairing. NMRs were observed until an animal defecated,

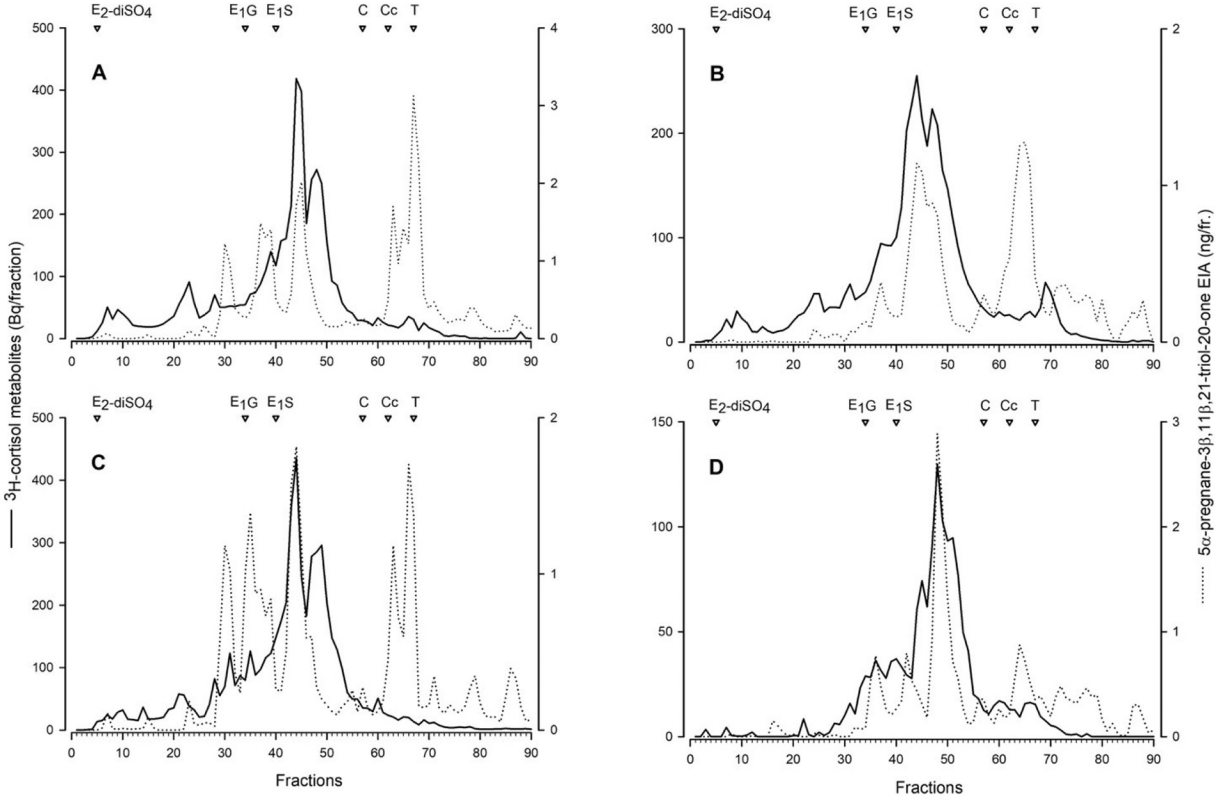


**Fig. 2.** Reverse-phase high-performance liquid chromatographic radioimmunograms for naked mole-rats. Fecal extracts (containing peak radioactivity) were analyzed from two females (A + B) and two males (C + D) injected with $^{3}$H-cortisol. In each fraction, the presence of $^{3}$H-metabolites (solid line; left axes) and metabolites reacting with the 5α-pregnane-3β,11β,21-triol-20-one EIA (dotted line; right axes) are shown. The approximate elution times of standards are marked with triangles ($E_2$-di$SO_4$: estradiol disulphate; $E_1$G: estrone glucuronide; $E_1$S: estrone sulfate; C and CC: cortisol and corticosterone, respectively; T: Testosterone).

and then the sample was immediately collected with forceps and placed in a 0.5 mL vial.

### 2.4. Experiment 3. In-colony social rank and FCM levels

Fecal samples were collected from 8 unmanipulated colonies in the University of Toronto Mississauga vivarium in the manner described above. After collection, animal ID was immediately recorded, so all samples were from known individuals. In total, fecal samples were collected from 165 individuals with 1 sample per individual. At the time of collection, animal weight (g) and age (months) was recorded. Fecal sample collection for each colony took up to two days, resulting in approximately two weeks total of sample collection.

Linear hierarchical rank within colony was assigned as described in Toor et al. (2015). In summary, when two NMRs meet head-on, one will climb over the other ('pass-over'). The dominant animal in the interaction is the one that passes on top of the other, whereas the subordinate animal crawls under. This is a common dominance interaction paradigm used in NMR behavioral research to assess rank (e.g. Clarke and Faulkes, 1997; Clarke and Faulkes, 1998; Toor et al., 2015). For all individuals in these colonies, status in the linear hierarchy is known based on the percent of pass-overs scored in tests against the other colony members. The individual in the colony with the highest percent of pass-overs is given a rank of 1 (most dominant), the individual with the second highest percent of pass-overs is given a rank of 2, and so on. These tests were conducted in 6 out of the 8 colonies. Only these colonies were used to assess the relationship of linear rank with FCM levels.

Queen aggression and the relationship to subordinate FCM levels was also assessed. Queen aggression was quantified using 30 min behavior videos of each colony, where duration (seconds) of queen agonistic behavior was assessed. Agonistic behavior included shoving, biting, dragging, and incisor fencing. Total duration of queen aggression in each colony was calculated as the summed total duration (in seconds) of all agonistic behaviors.

### 2.5. Urine and fecal sample processing

Urine samples were stored at 4 °C until processing and fecal samples were stored at −20 °C. Fecal samples were freeze dried using a lyophilizer (LabConco, Missouri, USA) overnight to remove moisture. Dried fecal samples were then homogenized using a mortar and pestle. Due to the small sizes of NMR fecal samples, the entire sample for each individual was weighed and extracted in 1 mL of 80% methanol. Sample weights were accounted for in the final calculation of hormone concentration (ng hormone per g feces). Samples were shaken (30 min at 15,000 rpm) on a multi-vortexer and then centrifuged (15 min at 2500*g*) to separate the supernatant from fecal material. Urine was dried down under air to control for volume.

### 2.6. Radioactivity and characterization of fecal cortisol metabolites

To measure $^{3}$H radioactivity in excreta, the 200 μl extract aliquots of each fecal sample and each urine sample were mixed with 5 mL ACS scintillation fluid (Amersham, USA) and measured on a liquid scintillation counter (Packard Tri-Carb 2900TR, Boston, MA). The excretion of radioactivity in both the feces and urine was then calculated for each animal. Fecal extracts from 2 males and 2 females were dried down and sent to the University of Veterinary Medicine (Vienna, Austria) to characterize the metabolites. Radioactive cortisol metabolites in these samples were separated according to their polarity by reverse phase high performance liquid chromatography (RP-HPLC). To determine which of two antibodies cross-reacted with fecal cortisol metabolites, fractions were analyzed using a 11-oxoetiocholanolone EIA, which

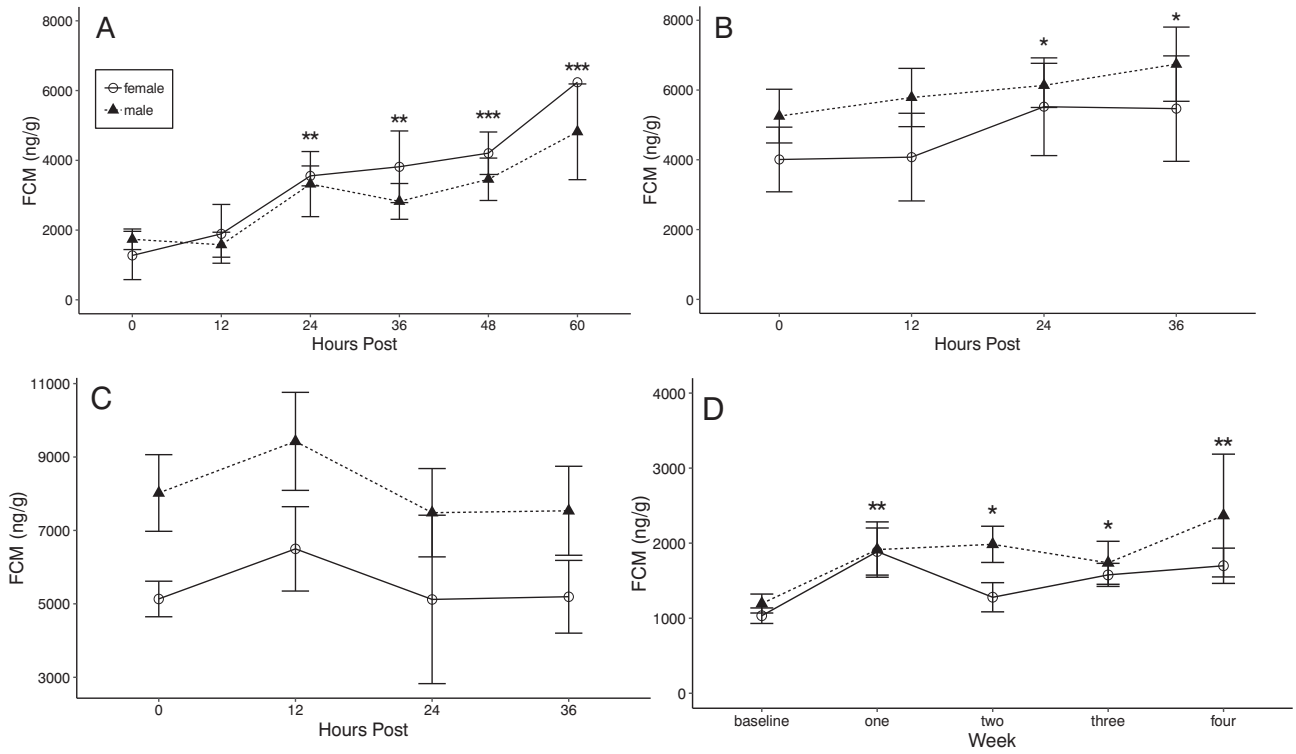


**Fig. 3.** Concentration of fecal cortisol metabolites (FCMs) represented as ng/g feces ± SE in naked mole-rats in response to: A. removal from the colony and transport to a new facility; B. injection of synthetic adrenocorticotropic hormone (ACTH); C. a restraint paradigm in a plastic chamber restricting body movement; and D. removal from the colony and social pairing with a novel, opposite sex conspecific. Significance is indicated by * for $p < .05$, ** for $p < .01$, and *** for $p < .001$ relative to the baseline in each (0 h post). Females (n = 3) are represented by open circles and males (n = 6) are represented by triangles. Different individuals were used in the social pairing experiment (Fig. 3D; n = 6 females and 6 males).

measures metabolites with a 5β-3α-hydroxy-11-one structure (Möstl et al., 2002), and a 5α-pregnane-3β,11β, 21-triol-20-one EIA, which measures a 5α-3β,11β-diol structure (Touma et al., 2003).

After determining that 5α-pregnane-3β, 11β, 21-triol-20-one EIA was the better assay for this species (described below), all fecal sample extracts were analyzed with this assay. It is described in detail in Touma et al. (2003), but in brief, it is a double antibody assay where biotinylated steroid labels are utilized, and thus the enzyme does not contact the sample matrix. The cross-reactivity to gonadal steroids and their reduced metabolites is < 1%. The minimum detection limit of the assay is 2 pg per well. NMR fecal extracts were diluted 1:500 in assay buffer and run in duplicates. The average intra-assay coefficient of variation (CV) calculated between pools run at the beginning and end of each plate for the validation and social pairing experimental samples was 6.8% and the inter-assay CV was 9.6% ($N$ = 7 plates). The average intra-assay CV for the in-colony fecal samples was 15.1% and the inter-assay CV was 9.1% ($N$ = 8 plates).

### 2.7. Statistical analyses

All FCM data were log-transformed for normality and are expressed as log ng FCM/g feces. For Experiment 1 (validation: separation from colony, $^3$H-cortisol injection, ACTH challenge, restraint paradigm) and Experiment 2 (social pairing), the change in FCM levels over time were assessed with a linear mixed effect model. Log FCM was the response variable, with collection time (h post each treatment), sex, and the sex/collection time interaction as fixed effects and animal ID as a random effect. Sex and the sex/collection time interaction effect were not significant in any of the models, and sexes were subsequently analyzed together. Effect sizes (β) were estimated using the linear models. Effect sizes for pairwise comparisons in radioactive excretion between the sexes are reported as Cohen's d (d).

For Experiment 3 (in-colony social rank) eight FCM outliers (> 2 SD from their colony mean) were removed, which resulted in a normal distribution in log FCM data for all colonies based on the Shapiro-Wilk test. This left a sample size of 157 animals for the comparison of caste and FCM levels and 138 animals for the comparison of linear rank and FCM levels. To understand the relationship between FCM levels and social status within the colonies, we first assessed the relationship among hierarchical rank, weight, and age to test if they were redundant factors or if weight and age were separate factors that could potentially explain additional variation in the FCM data outside of rank alone. After finding weight and rank were highly related, but not age and rank (across colonies and within all colonies except for 1, see results), we built a linear mixed effect model with log FCM as the response variable and linear hierarchical rank, age, sex, and the sex/rank interaction as fixed effects and colony as a random effect. In order to compare our results with those in the literature, suggesting between colony variability, we also looked at each of the 6 colonies independently using a generalized linear model with log FCM as the response variable and linear hierarchical rank as the fixed effect. Each individual was only sampled once, and thus animal ID was not included as an effect in any of the colony models.

Finally, we compared FCM levels among castes: queens, breeding males, and subordinates using a nonparametric Kruskal-Wallis test to account for highly different sample sizes and lack of homogeneity of variance for each caste. The effect size is reported as the epsilon squared ($\varepsilon^2$). This comparison was conducted across all 8 colonies. One colony had 2 queens, so the total sample size of queens was 9, and not all colonies had an active breeding male, so the total sample size of breeding males was 6. We also tested the effect of duration of queen aggression and queen FCM on colony average FCM and colony standard deviation of FCM using a linear model with colony average FCM as the response variable, and either queen FCM or queen duration of

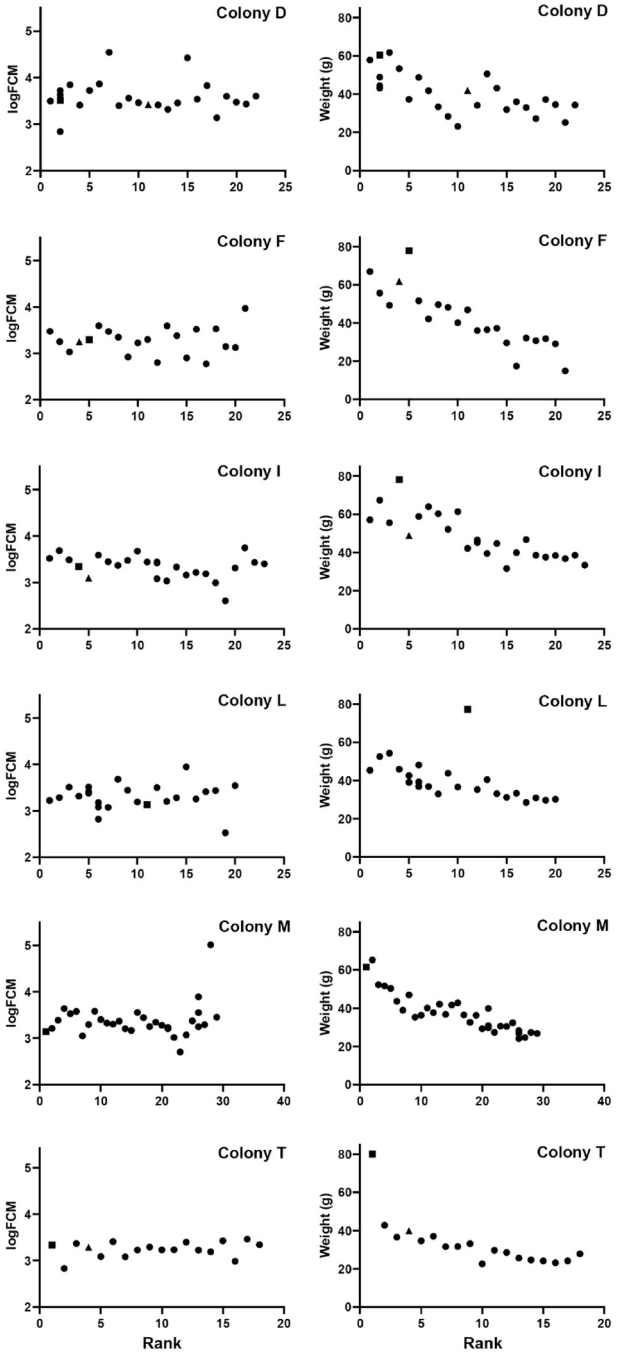


**Fig. 4.** Individual FCM levels (log ng/g) by hierarchical dominance rank (left) and individual weight (g) by hierarchical dominance rank (right). Low rank numbers represent the most dominant individuals. Squares represent the queen, triangles are breeding males, and circles are subordinates. There was no effect of rank on FCM levels in any colony ($n$ = 138 animals total), though dominant animals were heavier.

aggression (seconds) as the fixed effect. Analyses were done in R version 3.5.1 (R Core Team, 2018). Models were built using the package 'nlme' (Pinheiro et al., 2018).

## 3. Results

### 3.1. Experiment 1. Validation experiment.

#### 3.1.1. Radiometabolism study

Of the $^{3}$H-cortisol injected, 16% ± 1.9% was recovered in excreta (14.6% ± 0.3% in urine and 1.4% ± 0.4% in feces). The average time lag to peak radioactivity excretion was 7.8 ± 1.1 h in the urine and 27.8 ± 3.1 h in the feces (Fig. 1). Time lag of excretion was similar between males and females in urine (d = 0.72, $t$ = 1.19, $p$ = .28). Females took longer to display peak radioactivity excretion in feces (d = 1.47, $t$ = 2.89, $p$ = .03), with peak excretion at 35.3 ± 1.3 h post-injection, compared to males 24.0 ± 3.0 h post injection (Fig. 1).

RP-HPLC revealed that $^{3}$H-cortisol was virtually absent in the feces, and only its metabolites were excreted. In both sexes the main radioactive peaks eluted around fraction 43 to 48. The 5α-pregnane-3β, 11β, 21-triol-20-one EIA reacted with several radiolabelled metabolites and revealed a highly similar pattern of peaks across fractions in both sexes (Fig. 2). The 11-oxoetiocholanolone EIA did not detect significant amounts of immunoreactive substances in the fractions. Therefore, all subsequent analyses of FCMs were carried out with the 5α-pregnane-3β, 11β, 21-triol-20-one EIA.

#### 3.1.2. Colony removal, ACTH challenge, and restraint

Removal from colony and transport to a new facility caused a significant, sustained increase in FCM levels (main effect of collection time: $F_{5,38}$ = 13.89, $p$ < .001; Fig. 3A). Compared with baseline levels in the colony, FCM levels were not elevated in feces collected 12 h post-removal (β = −0.07 ± 0.07, $t_{38}$ = −0.99, $p$ = .33), but were significantly elevated by 24 h (β = 0.23 ± 0.07, $t_{38}$ = 3.08, $p$ = .004) and remained elevated at 36 h (β = 0.25 ± 0.07, $t_{38}$ = 3.40, $p$ = .002), 48 h (β = 0.35 ± 0.07, $t_{38}$ = 4.67, $p$ < .001), and 60 h (β = 0.43 ± 0.08, $t_{38}$ = 5.31, $p$ < .001) post-removal. Injection of synthetic ACTH caused an increase in FCM levels (main effect of collection time: $F_{3,22}$ = 3.50, $p$ = .03; Fig. 3B). Compared to baseline samples taken at the time of ACTH injection, FCM levels were not elevated at 12 h post-ACTH injection (β = 0.02 ± 0.04, $t_{22}$ = 0.56, $p$ = .58), but were significantly elevated at 24 h (β = 0.10 ± 0.04, $t_{22}$ = 2.28, $p$ = .03) and 36 h (β = 0.11 ± 0.04, $t_{22}$ = 2.64, $p$ = .02) hours after injection. The 10 min restraint paradigm failed to change FCM levels (main effect of collection time: $F_{3,17}$ = 0.96, $p$ = .43, Fig. 3C) and no differences between baseline and any post-restraint collection time were detected (12 h, 24 h, or 36 h; all $p$ > .05).

### 3.2. Experiment 2. Social pairing effects on fecal cortisol metabolites

Removal from colony and pairing with an opposite sex animal caused a significant, sustained increase in FCM levels (main effect of collection time: $F_{4,44}$ = 3.29; $p$ = .02; Fig. 3D). Compared to baseline (in-colony) levels, FCMs were significantly elevated after pairing for 1 (β = 0.21 ± 0.07, $t_{44}$ = 3.07, $p$ = .004), 2 (β = 0.15 ± 0.07, $t_{44}$ = 2.18, $p$ = .03), 3 (β = 0.16 ± 0.07, $t_{44}$ = 2.36, $p$ = .03), and 4 weeks (β = 0.22 ± 0.07, $t_{44}$ = 3.18, $p$ = .003) post-removal, with levels remaining high and similar over time.

### 3.3. Experiment 3. In-colony social rank and fecal cortisol metabolites

A strong negative relationship between hierarchical rank and weight was detected (β = −0.30 ± 0.03, $t_{130}$ = −11.53, $p$ < .001; Fig. 4), with dominant animals being heavier, as the top hierarchical rank is 1. However, age did not affect rank across all animals treating colony as a random effect (β = 0.01 ± 0.01, $t_{130}$ = 0.99, $p$ = .33), nor within any colony aside from one, "Colony L" (β = 0.36 ± 0.12, $t_{20}$ = 3.03, $p$ = .007). Therefore, age was included as an effect in all subsequent models, but not weight as it was highly redundant with rank. There was no effect of rank on FCM levels seen across all colonies, treating colony as a random effect (β = −0.002 ± 0.005, $t_{128}$ = −0.35, $p$ = .73). There was additionally no effect of age (β = −0.0003 ± 0.0007, $t_{128}$ = −0.41, $p$ = .68) nor sex (β = 0.06 ± 0.07, $t_{128}$ = 0.81, $p$ = .42) on FCM levels in this model. Pooling the sexes by removing sex as an effect did not bring the effect of rank to significance (β = −0.006 ± 0.004, $t_{130}$ = −1.38, $p$ = .17)

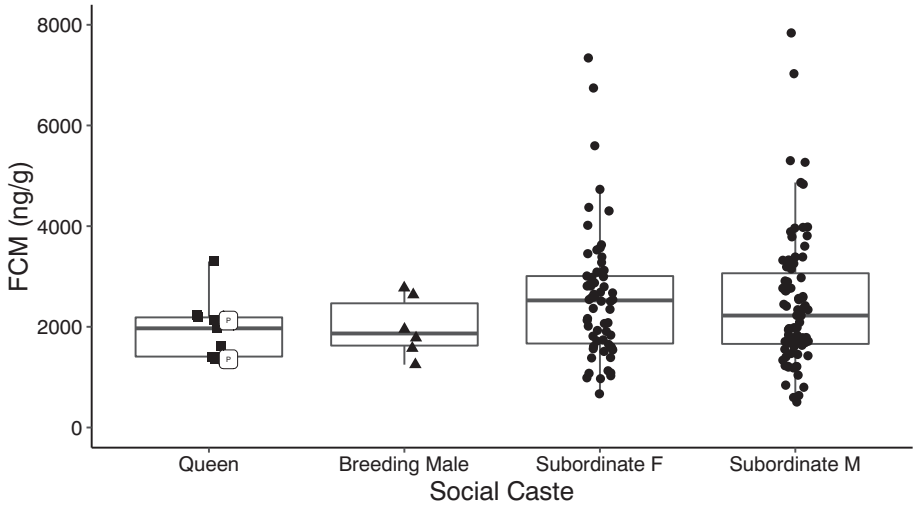


**Fig. 5.** Comparison of fecal cortisol metabolite (FCM) levels represented in ng/g feces across social castes: queens ($n$ = 9), breeding males (n = 6), subordinate females ($n$ = 62), and subordinate males ($n$ = 80). Pregnant queens are marked with a "P" directly to the right.

nor that of age ($\beta = -0.0003 \pm 0.0007$, $t_{130} = -0.51$, $p = .61$). When assessing the same relationship within each colony individually, no colony showed a significant effect of rank on log FCM level (all $p > .05$; Fig. 4).

Comparing the social castes, the Kruskal-Wallis test showed there were no differences in log FCM levels among queens, breeding males, subordinate females, and subordinate males ($\epsilon^2 = 0.02$, $\chi^2_3 = 2.95$, $p = .40$; Fig. 5). There was extremely high variation in subordinate FCM levels, potentially due to the increased sample size relative to the other groups (Fig. 5).

We tested whether queen aggression levels (duration of aggressive behavior) and queen FCM levels were predictors of subordinate FCM levels within their colony, and might explain subordinate FCM variation. There was no effect of queen duration of aggression on colony average FCM levels ($\beta = -0.001 \pm 0.01$, $t_4 = -0.11$, $p = .92$) nor on colony FCM standard deviation ($\beta = -0.007 \pm 0.006$, $t_4 = -1.3$, $p = .27$). We also found that queen FCM levels had no effect on colony average FCM levels ($\beta = 0.68 \pm 0.47$, $t_4 = 1.46$, $p = .22$).

## 4. Discussion

We have characterized cortisol metabolism in NMRs, validated an assay to measure FCMs, and demonstrated in two independent experimental groups that removal from colony resulted in increased FCM levels, persisting on the order of days to weeks. We also found no FCM differences associated with hierarchical rank across all colonies or within any of the six colonies studied. Further, no significant differences in FCM levels were detected among the social castes (queens, breeding males, and subordinates).

As in other mammals, no native cortisol was detected in NMR feces, indicating that glucocorticoids are metabolized by the liver prior to excretion (Palme et al., 2005; Touma and Palme, 2005). The time course of peak excretion of $^3$H cortisol in NMRs is long (8 h in urine and 28 h in feces) and our total recovery of radioactivity was low (16.1%) relative to other small mammals (Table 2). However, these values are not impossible and do not reflect a failure of methods. Six of the species reported in Table 2 were done in our lab with identical or highly similar experimental setups, with variation in recovered radioactivity ranging from 38% to 71%. Hence, variation in radioactive recovery is driven by actual species differences, not methodological differences. Generally, we had low yield of fecal samples in NMRs and our window of collection (38 h) may not have covered the amount of time it could take to excrete the majority of the radioactive metabolites if these animals had a later peak, or a second peak, in excretion. Pigs (*Sus scrofa*) show peak radiolabeled cortisol excretion in the feces 48 h post injection (Palme et al., 2005) and bank voles (*Myodes glareolus*) had peak excretion at 6 h, and a second peak at 20 h with 21% total recovery in feces and urine combined (Sipari, 2015). Notably, fecal radioactivity was still very high in our NMRs at 38 h (Fig. 1), indicating that there was likely still more $^3$H cortisol to excrete. Such species differences in excretion rate and recovery or radiolabeled injections are likely due to differences in metabolism, digestive rates, and gastrointestinal function. The generally slow excretion in NMRs is likely related to their extremely low metabolic rate relative to other mammals (Buffenstein and Yahav, 1991; Goldman et al., 1999) and their suite of digestive adaptations to maximize resources extracted from fibrous, low quality food, and cope with periods of limited food availability. These adaptations include increased retention times in the gut and extensive microbial fermentation in the cecum (Buffenstein et al., 2012). Further, NMRs lack a gall bladder (Buffenstein et al., 2012), and as glucocorticoid metabolites are passed from the liver to the gut in bile (Palme et al., 2005), this may impact the speed of transfer of metabolites from the liver to the excreta. Therefore, we conclude that the low and slow excretion levels of radiolabeled metabolites are likely another aspect of the unusual metabolism and digestion of this species.

However, the low portion of $^3$H-cortisol metabolites excreted in the feces does not preclude measurement of FCM. While these metabolites reflect only a portion of circulating cortisol, they are still highly concentrated in the feces and cross-react with the antibody as indicated by HPLC. Importantly, we were able to successfully detect an increase in FCM levels in response to the ACTH challenge, and an increase in FCM levels in response to colony removal, consistent with blood cortisol data (Peragine et al., 2016). Therefore, while the FCMs quantified in this assay may only represent a small portion of circulating cortisol, this portion is representative of biologically meaningful changes in blood cortisol levels.

While the ACTH injections successfully triggered an increase in FCMs, our use of the restraint paradigm to increase FCM levels was not successful. There are two likely reasons for this. One is that, because FCM levels remained elevated after separation from the colony and throughout the ACTH challenge, they never returned to true baseline levels (Fig. 3). Thus, following the ACTH challenge, the increases in FCM may have reached a ceiling. A second possibility is that a 10 min restraint paradigm might not actually be a relevant stressor for these animals given their natural subterranean burrow habitat and behavioral ecology. For example, they spend a large portion of their time in densely-packed huddles and are therefore used to having their movement restricted. To differentiate between these possibilities, a control group

**Table 2**
Species comparison of $^{3}H/^{14}C$-cortisol or corticosterone excretion in small mammals.

| Species | Time to peak radioactivity in feces (hours) | Time to peak radioactivity in urine (hours) | Radioactivity recovery (urine + feces) | Greater portion excreted via feces or urine? | Reference |
|---|---|---|---|---|---|
| European hare *Lepus europaeus* | 33 | 13 | 90% | Urine | Teskey-Gerstl et al., 2000 |
| Chinchilla *Chinchilla lanigera* | 30 | 5–10 | 45.5% | Urine | Ponzio et al., 2004 |
| Naked mole-rat *Heterocephalus glaber* | 28 | 8 | 16% | Urine | |
| Eastern grey squirrel *Sciurus carolinensis* | 24–40 | 8–16 | 71% | Urine | Bosson et al., 2013 |
| Laboratory rat *Rattus norvegicus* | 15 | 2–6 | 87% | Feces | Lepschy et al., 2007 |
| North American red squirrel *Tamiasciurus hudsonicus* | 11 | 7 | 69% | Urine | Dantzer et al., 2010 |
| Guinea pig *Cavia aperea f. porcellus* | 8 | 2.5 | 88.1% | Urine | Keckeis et al., 2012 |
| Eastern chipmunk *Tamias striatus* | 8 | 4 | 48.3% | Urine | Montiglio et al., 2012 |
| Columbian ground squirrel *Urocitellus columbianus* | 7 | 4.5 | 38% | Urine | Bosson et al., 2009 |
| Snowshoe hare *Lepus americanus* | 6 | 3.5 | 67% | Urine | Sheriff et al., 2009 |
| Laboratory mouse *Mus musculus* | 4–10 | 2 | 95% | Feces | Touma et al., 2003 |
| Bank vole *Clethrionomys glareolus* | 4–6 | 2 | 21% | Feces | Sipari, 2015; Sipari et al., 2017 |

would be useful in future studies.

We report elevated FCM levels in two independent groups of NMRs removed from their colony. This is consistent with our previous report of elevated plasma cortisol following colony removal and social pairing (Peragine et al., 2016) and might reflect psychosocial stress associated with separation from their natal social group. A similar effect was observed in tamarins and marmosets: social, cooperatively breeding primates, in which removal from the group resulted in increases in urinary cortisol levels (Ziegler et al., 1995; Smith and French, 1997). However, it is important to acknowledge that the increased FCM seen in NMRs after removal from colony might be triggered by another factor aside from psychosocial stress. Subordinate NMRs begin to activate reproduction within days/weeks after removal from colony (Faulkes et al., 1990; Swift-Gallant et al., 2015), and our animals also showed evidence of such an activation. Reproductive activation in NMRs is a period of intense, irreversible development, and the transition to breeder status entails ovarian development in the females (Jarvis, 1981), increased secretion of sex hormones (Faulkes et al., 1990), skeletal development (Henry et al., 2007; Pinto et al., 2010), changes in neural morphology (Holmes et al., 2007; Holmes et al., 2011), and changes in neural gene expression (Faykoo-Martinez et al., 2018). Such marked physiological changes could induce elevation of glucocorticoids, through changing developmental and energetic demands. Another consideration is that NMRs are poikilothermic and regulate their body temperature by huddling with colony members (Buffenstein and Yahav, 1991). Thus, increased cortisol in isolated or pair housed NMRs could reflect an adaptive response to thermoregulatory challenges. Of course, psychosocial stress, reproduction, and metabolic alterations need not be mutually exclusive.

We found no evidence that social status, in both the linear hierarchy and reproductive caste analyses, was related to FCM levels in NMRs (Fig. 4 and Fig. 5). This is in agreement with several earlier studies (Clarke and Faulkes, 1997; Clarke and Faulkes, 1998; Hathaway et al., 2016). Furthermore, in another eusocial mole-rat, the Damaraland mole-rat (*Fukomys damarensis*), no difference in FCM levels was found between queens and subordinates (Medger et al., 2018). However, we must consider the cases where our results differ and why. One colony (out of three) studied by Clarke and Faulkes (1997) was reported to have higher urinary cortisol levels in more highly ranked individuals. Interestingly, the authors reported that this colony (colony “NN”) had the highest frequency of agonistic behaviors among subordinates, and was also the only colony where subordinate females showed increases in body weight even prior to the removal of the queen. After the removal of the queens from all three colonies, multiple females in colony NN began to reproductively activate and to compete for dominance, whereas in the other colonies a single female became queen uncontested. It may be that colony NN was sampled at a critical moment where several of the more dominant females were becoming larger, more aggressive, and reproductive in anticipation of usurping the role of queen. This transition to the reproductive state, along with the increased aggression reported in the colony, may have driven the higher cortisol levels in the dominant animals in colony NN. Thus, there may only be a relationship between hierarchy and cortisol in NMRs when several subordinates are competing to take over the breeding role. In our study, we found no evidence that queen aggression levels were correlated with colony FCM levels. While this result is consistent with prior work (Clarke and Faulkes, 2001), our measure of queen aggression was very brief (30 min) and may not have captured the true scope of aggressive behavior or variation in targeted individuals. A more in-depth classification of queen aggression on individual FCM levels would be useful in the future.

Our results also diverge from prior evidence that non-pregnant queens have lower cortisol levels than subordinates (Faulkes and Abbott, 1997). While pregnant NMR queens were previously found to have higher cortisol levels, an increase in cortisol during pregnancy is typical of most mammals and unrelated to psychosocial stress (Edwards

and Boonstra, 2018). Our results in the current study were not confounded by queen pregnancy, as only two out of nine queens were pregnant (Fig. 5), and removing these two queens from the analysis did not alter results. The most plausible explanation for these differences is that both our study and Faulkes and Abbott (1997) had a low sample size of queens ($n = 9$ and $n = 3$, respectively), though ours had a much larger sample size of subordinates ($n = 142$ animals compared to $n = 15$). The high individual variation in cortisol levels may have allowed the queens sampled in Faulkes and Abbott (1997) to fall lower than the subordinate NMRs. Similarly, the same study (Faulkes and Abbott, 1997) reported no difference in urinary cortisol levels in two individuals removed from the colony and housed singly. Again, however, the sample size was smaller and hormonal measures can be highly variable.

Our data further support that reproductive suppression in NMRs is not driven by social stress in the dogmatic sense, as subordinates do not have higher FCM levels than dominants, and animals also showed elevated FCMs and reproductive activation simultaneously (Clarke and Faulkes, 1997; Clarke and Faulkes, 2001; Swift-Gallant et al., 2015; Peragine et al., 2016). However, this does not necessarily mean that glucocorticoids definitively have no role in regulating reproduction in this species. Subordinate NMRs have higher levels of glucocorticoid receptor mRNA expression in the hippocampus than do paired and breeding NMRs (Faykoo-Martinez et al., 2018), implying there may be differential sensitivity to the effects of circulating glucocorticoids across castes. Additionally, in utero or early life exposure to glucocorticoids could affect aspects of phenotype related to propensity for reproductive maturation. Such an effect has been found in cooperatively breeding meerkats, where treatment of pregnant females with cortisol results in offspring that grow more slowly and are more likely to engage in cooperative alloparental behavior (Dantzer et al., 2019). Similar mechanisms may exist in NMRs, where maternal cues may predispose elements of reproductive and social development, but this remains to be determined.

## 5. Conclusions

In summary, we have validated the 5α-pregnane-3β, 11β, 21-triol-20-one EIA for detection and measurement of FCMs in NMRs. We found that the assay successfully detected an increase in FCM following injection with ACTH. However, the restraint paradigm triggered no increase in FCM in this species. Removal of a NMR from its colony, whether alone or paired with a novel conspecific, triggered prolonged increases in FCMs, though the psychosocial versus physiological correlates of the increased FCMs are unknown. Finally, we found no FCM differences associated with social rank, either driven by caste (breeding versus subordinate) or by linear dominance hierarchy.

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