



# Hormonal and behavioral variation during release from reproductive suppression in an extreme cooperative breeder

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## Abstract

Naked mole-rats (*Heterocephalus glaber*) are cooperatively-breeding rodents that exhibit strong reproductive suppression within their natal colonies but can sexually mature once separated from the colony's dominant breeders. We removed subordinate animals from their natal colony and examined hormonal and behavioral changes during sexual maturation, their variation, and their association with reproductive success. We present the results from two small-cohort, year-long studies: 1) Changes in fecal hormone metabolites in newly introduced pairs of naked mole-rats ( $n=9$  pairs), the individual hormonal variation during reproductive maturation, and association with subsequent reproductive success; 2) Social behavior of paired animals ( $n=26$  pairs) upon meeting and the association with subsequent reproductive success. Within Study 1, we found that only half of the paired subordinates successfully reproduced over the course of one year. Reproductively successful females had higher estradiol metabolite concentrations while they were still in their natal colonies, before they were removed and paired. This suggests that pre-pubertal (within colony) conditions may be related to ultimate reproductive success. While progesterone metabolite concentrations significantly increased in removed females, the magnitude and patterns of change suggest that only one female ovulated during the first three weeks of pairing. In Study 2, 10 out of 26 pairs successfully reproduced within the first year. Upon meeting, there were no overall differences in durations of social behavior between pairs that would go on to be reproductively successful or unsuccessful. Our work indicates that there is clear individual variation in the onset of reproduction and ultimate reproductive success of these social subordinates.

**Keywords** Naked mole-rat · Progesterone · Cortisol · Estradiol · Ovulation · Sexual maturation

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## Introduction

In many species that breed cooperatively, reproduction is monopolized by dominant individuals, while subordinate individuals in the group have limited or no reproduction (Clutton-Brock 2002, 2006). Although, in some cooperatively breeding insects, subordinates can be sterile (e.g. Roisin and Korb 2011; Warner et al. 2017), permanent sterility of subordinates has not been described in mammals, meaning that all subordinates should have the potential to reproduce once they are separated from social suppressive cues. However, the timeline to reproductive activity varies by species. In cotton-top tamarins (*Saguinus oedipus*), subordinate females do not begin cycling until removed from their group and paired with a male, whereafter they reproduce within three ovarian cycles (Ziegler Te Savage et al. 1987). Subordinate female Damaraland mole-rats (*Fukomys damarensis*) do not cycle within their natal colony (Molteno

and Bennett 2000), but when they are removed from the colony and paired with an unrelated individual, females are impregnated within one year (4/4 females; Bennett et al. 1996). In contrast, in common marmosets (*Callithrix jacchus*), about half of subordinate females are cyclic within their group, and these females are more likely to display sexual behaviors when they meet an unrelated male than are acyclic subordinates (Saltzman et al. 1997a, b). Similarly, in cooperative meerkats (*Suricatta suricatta*), certain subordinate females are more prepared for reproduction than others: older/heavier females and those from groups with more unrelated males have higher luteinizing hormone levels (Carlson et al. 2004).

Naked mole-rats (*Heterocephalus glaber*) are extreme cooperative breeders with very high reproductive skew relative to other mammals (Jarvis 1991; Holekamp and Engh 2009). These subterranean rodents live in colonies of dozens to hundreds of individuals, where breeding is monopolized by a reproductive female called the “queen” and her breeding male consort (Jarvis 1981). All other individuals, “subordinates”, are non-reproductive and do not demonstrate sexual behavior within the colony (Jarvis 1981; Goldman et al. 2006). Within the colony, female subordinate naked mole-rats are generally anovulatory with underdeveloped ovaries and uteri (Faulkes et al. 1990, 1991; Swift-Gallant et al. 2015). This reproductive suppression occurs at least at the level of the pituitary, with subordinate naked mole-rats being less responsive to administration of gonadotropin-releasing hormone (GnRH) than breeders (Van Der Westhuizen et al. 2002). Higher in the brain, subordinates have increased RFamide-related peptide-3 (the mammalian orthologue of gonadotropin-inhibitory hormone) relative to breeders (Peragine et al. 2017). Reproductive suppression occurs in the physical presence of the queen, and olfactory cues alone are not sufficient to maintain suppression (Faulkes and Abbott 1993; Smith et al. 1997). However, there is hormonal variation among subordinates, with studies finding that some high-ranking females have higher circulating progesterone (Toor et al. 2022) and fecal androgen metabolites (Majelantle et al. 2024) than lower ranking female subordinates, albeit with high individual variation.

When subordinate naked mole-rats are removed and housed separately from the colony, they begin to undergo endocrine changes that are likened to puberty, including increases in sex steroid hormones (Faykoo-Martinez et al. 2021). Naked mole-rats are spontaneous ovulators and can ovulate within one week from the onset of follicular growth (Faulkes et al. 1990). In mammals, the ovarian cycle consists of: 1) The follicular phase, which is a period of growth and maturation of a subset of ovarian follicles. Circulating estradiol levels increase during this time, ultimately triggering the luteinizing hormone surge that results

in ovulation; 2) Ovulation, where the mature oocyte is released from the ovarian follicle; 3) The luteal phase, where the ruptured ovarian follicle forms the corpus luteum and produces high levels of progesterone (reviewed in Brown 2018). In naked mole-rats, the follicular phase is estimated to be six days long, and post-ovulatory increases in progesterone can be detected on day seven, with the luteal phase lasting for an additional ~27 days (Faulkes et al. 1990). In addition, increases in circulating estradiol concentrations have been detected in female naked mole-rats one month after removal from the colony (Swift-Gallant et al. 2015), which may be related to follicle maturation and the onset of ovulation. Similarly, increases in cortisol levels in naked mole-rats recently removed from their colonies have been detected in females (Blecher et al. 2020) or in both sexes (Edwards et al. 2020). It is unclear if this change is related to the psychosocial stress of separation from the colony, the widespread physiological changes that accompany sexual maturation, or both.

Following removal from the natal colony, we and others have observed considerable individual variation in the timing and degree of sexual maturation in naked mole-rats, with some of these separated or “reproductively activated” individuals never actually reproducing even when provided the opportunity (Toor et al. 2020; Smith and Buffenstein 2021, T. Park personal communications). The extent of variability in how quickly subordinates become reproductively active after separation from the colony is unclear, as well as how this compares to other cooperative breeders. In these two studies, we examined the release from reproductive suppression and subsequent reproductive success in naked mole-rats. Our objective in Study 1 was to document hormonal changes as animals progressed from within-colony reproductive suppression to reproductive activation, and to determine whether hormonal levels during this time are associated with which pairs ultimately go on to reproduce over the course of the next year. To do this, we separated individuals from their natal colony and paired them with an opposite-sex mole-rat from a different colony. We collected fecal samples to quantify sex steroid hormone and glucocorticoid metabolites, which allows for repeated, non-invasive sampling of individuals. Our objective in Study 2 was to examine if the behavioral interactions of pairs upon meeting are predictive of whether they successfully reproduce. We documented different types of social behaviors (sexual, prosocial, and aggressive; see Table 1) and how much time pairs spent not interacting (non-social). We used behavior recordings collected from the pairs in Study 1 described above, as well as behavior recordings from pairs published in an earlier study (Toor et al. 2020), which have not previously been analyzed in this manner.

**Table 1** Descriptions of behaviors scored (duration in seconds) in naked mole-rat pairs

Behavior	Description
Aggression	Biting, open mouth gaping and incisor fencing (animals have their mouths open displaying or locking incisors), dragging, thrashing, head-butting, shoving.
Sexual	Sniffing or licking genitalia, copulation or attempted copulation (mounting, lordosis).
Prosocial	Social behaviors that are not aggressive or sexual: sniffing the body or face, huddling.
Non-social	Calculated for each video by subtracting the sum of the durations of all social behaviors above from the video length. Includes self-directed behaviors, inactivity, and environmental manipulation behaviors such as digging and gnawing.

## Materials and methods

Naked mole-rat colonies were housed at the University of Toronto Mississauga in a manner previously described for this species (Edwards et al. 2020; Toor et al. 2020). All animal work was done in accordance with the guidelines of the Canadian Council on Animal Care and approved by the Animal Care Committee at the University of Toronto. We considered pairs to be reproductively successful if the female became visibly pregnant within one year of pairing. Statistical analysis was conducted in R, version 4.2.2 (R Core Team 2022).

### Study 1

In Study 1, 20 naked mole-rats from five different origin colonies were initially selected for pairing. Selection criteria were that animals were subordinates (non-breeding) and of adult body size (body mass range: 46–72 g). The exact ages of the paired subordinates were unknown, but based on the years when their natal colonies were established, animals were a maximum of 11 years old. Naked mole-rats are known to be able to reproduce from 7.5 months old to >30 years old (Buffenstein 2005; Buffenstein and Craft 2021). Animals were marked on the back with an ID and then paired in a clean, new polypropylene cage with an opposite-sex animal from a different colony. If introduced animals fought to the point of drawing blood, they were separated, placed alone in a cage for a few minutes, and then paired with a different animal. Two individuals (1 male, 1 female) both attacked every animal with which they were paired and hence could not be included in the study, resulting in 9 pairs total. Fecal samples were collected while the animals were within their natal colony (within three days prior to removal from the colony) and subsequently every 2–3 days over the next two weeks of pairing.

Samples were collected during brief handling, in a manner previously described (Edwards et al. 2021) and stored at  $-20^{\circ}$  C until analysis. Consistent with previous efforts for feces collection in this species, defecation rates were low. In total, 60 fecal samples were collected. Because only a subset of animals defecated at each collection, time points were pooled. Thus, sampling times in the statistical analysis are in-colony (samples collected before animals were removed from the colony or on the day that animals were paired; ~24 hour gut passage time in this species; Edwards et al. 2020), samples from days 2–3, day 5, days 9–11, day 14, and days 19+ (Table S1).

Wet fecal samples were weighed and extracted for 1 hour in 80% methanol, at a ratio of 0.05 g feces to 1 mL methanol. Fecal sex steroids were analyzed at the Toronto Zoo using estradiol, pregnane, and testosterone enzyme immunoassays (EIAs). These assays measure native hormones and cross-react with their metabolites (Kummrow et al. 2011). We have previously biologically validated the estradiol EIA for this species (Edwards et al. 2021). Fecal cortisol metabolite levels were analyzed at the University of Toronto Scarborough using a  $5\alpha$ -pregnane- $3\beta,11\beta$ , 21-triol-20-one EIA, which measures metabolites with a  $5\alpha$ - $3\beta,11\beta$ -diol structure (Touma et al. 2003), that we have previously validated for this species (Edwards et al. 2020). The average inter-assay coefficients of variation (CVs) were as follows: pregnane assays – 10.61%, estradiol assays – 16.98%, testosterone assays – 13.95%, cortisol metabolite assays – 13.28%.

To determine how hormone levels changed by time point across reproductive activation, we used linear mixed effect models (LMMs) in the package nlme (Pinheiro et al. 2023) with each hormone as the response variable, sampling time as a fixed effect, and animal ID as a random effect. Distributions of model residuals were assessed using histograms and Q-Q plots, and hormone data was subsequently log transformed so that residuals would meet assumptions of normality. Hormone data was analyzed separately for each sex, based on the a priori expectation that males and females will differ in their overall amounts and changes in steroid hormone concentrations, and an interest in examining reproductive changes within each sex. To detect progesterone peaks in females after colony removal, which might suggest that ovulation has occurred, we used the iterative method (Brown et al. 1994, 1996) which determines hormonal peaks against baseline values, using the package hormLong (Fanson and Fanson 2015). This process removes samples with concentrations over  $n$  standard deviations from the mean ( $n$  specified by user, we used 1 in this case), then recalculates the mean and again removes samples over  $n$  standard deviations from the mean, and so on, until no outlying values are detected. The remaining values are considered baseline values, and points that exceed the baseline are

considered peaks (Brown et al. 1994, 1996). Two females (Female E – successful breeder; Female O – unsuccessful breeder) had too few samples for this iterative analysis and were hence excluded. To test differences in within-colony or post-pairing hormone levels in successful and unsuccessful pairs, we used LMMs with each hormone as the response variable, reproductive success as a fixed effect, and animal ID as a random effect (in the post-pairing analysis with multiple samples per ID).

Potential body mass at pairing differences in successful and unsuccessful reproducers were examined using a linear model with pair type (successful or unsuccessful) as a fixed effect. We tested overall body mass at pairing (g) and pair differences in body mass (female mass (g) – male mass (g)) as the response variables.

## Study 2

In Study 2, behavior videos were recorded from the pairs set up for Study 1. Immediately when animals were introduced, 30 min of behavior were recorded using GoPro Hero 3 cameras. Duration (s) of social behaviors per video (described in Table 1) were scored using BORIS software (Friard and Gamba 2016). These data were coupled with behavior data collected from 18 additional naked mole-rat pairs set up for a prior study assessing the effect of social phenotype on reproductive success (Toor et al. 2020), which has never been analyzed in the manner described here. While durations of the same behaviors were assessed in Toor et al. (2020), behavior videos upon pair introduction were recorded for 10 min instead of 30 min. These differences in recording time were accounted for by dividing behavior durations by the total recording time in seconds. Across all 26 pairs (18 from Toor et al. 2020, 8 from Study 1 after a separated pair was not included, see below), 10 were reproductively successful within one year of pairing, and 16 were not.

We examined summed pair behavior duration for each pair at meeting, e.g. female aggression (s) + male aggression (s) per pair. We used generalized linear models (GLMs) with a binomial distribution to assess the effects of behavior on reproductive success as the response variable. Summed behavior durations (rounded to the nearest whole second) were divided by video length (s) and used as fixed effects in the model. To test pair similarity and reciprocity of behavior, we used the absolute value of the within-pair difference in duration for each social behavior (prosocial, aggressive, and sexual behavior) divided by video length (s).

## Results

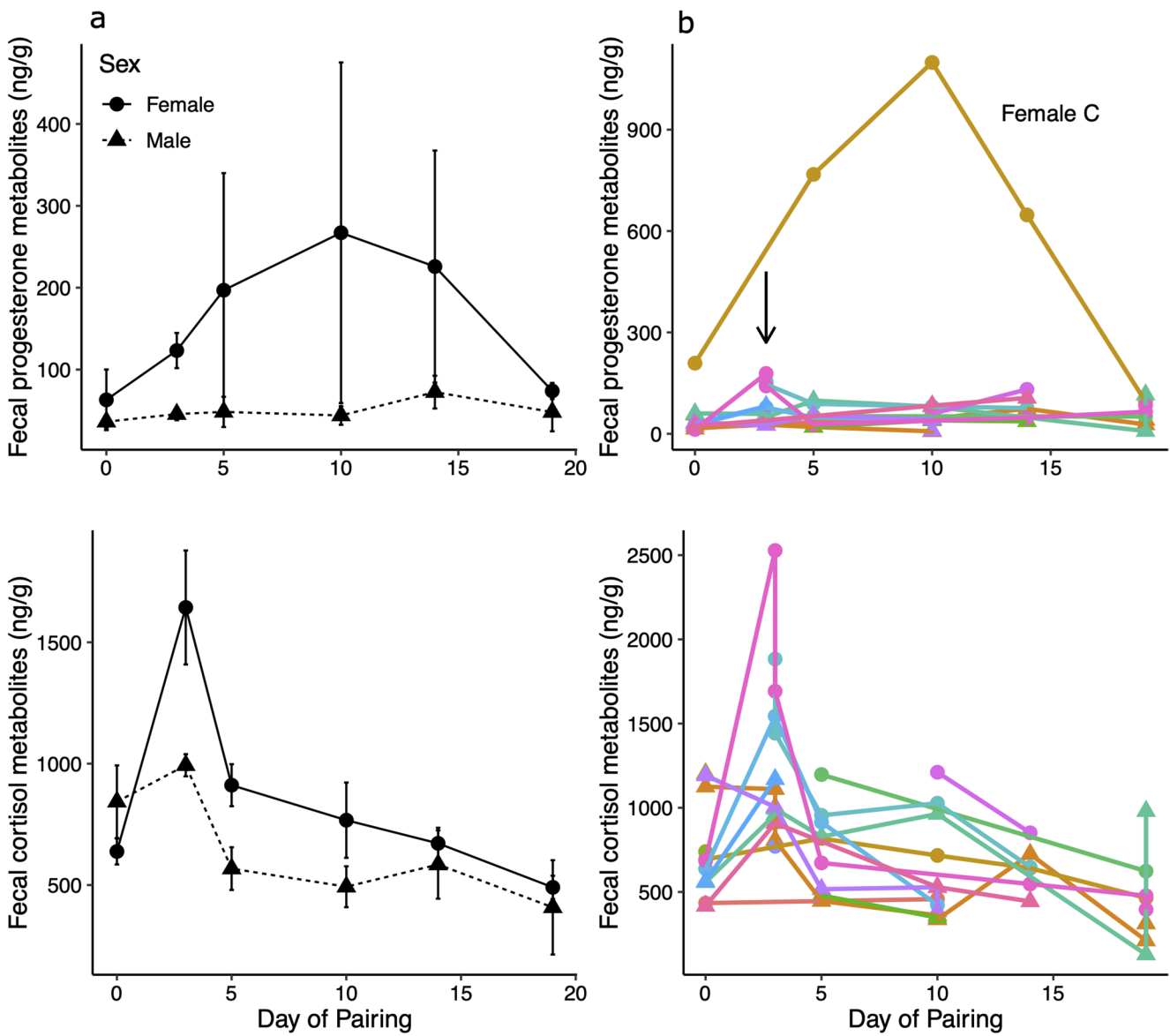
### Study 1

Out of the nine pairs, four were successful (1–2 litters that year), four pairs did not reproduce during the course of the study, and one pair had an individual removed by vivarium staff in error after six months. This last pair was not included in the comparison of successful and unsuccessful pairs but was included in the longitudinal hormone analysis after colony removal.

Relative to their within-colony baseline levels, females displayed increased estradiol, progesterone, and cortisol metabolite concentrations post-pairing, whereas males showed no hormonal changes during this time (Fig. 1a; full model output in supplementary material Table S2). Using the iterative method with a setting of one standard deviation above the mean, three females showed progesterone peaks after pairing (Females K, M, and S on day 2–3 of pairing). Female C had no detectable peak, and instead had elevated progesterone metabolite concentrations for a sustained period: from days 5 to 14 of pairing (Fig. 1b). These same four females displayed cortisol peaks after pairing (Females K, M, and S on day 2–3 and Female C on day 5). The other three females (Females A, I, and Q) displayed neither fecal progesterone nor cortisol metabolite peaks, however Q provided no fecal sample days 2–5. Although there is clear hormonal variation among individuals, only half of the females with the progesterone/cortisol metabolite peaks were reproductively successful.

Females that went on to successfully reproduce had higher fecal estradiol metabolite levels while they were still within their natal colony compared to unsuccessful females ( $\beta = -0.26 \pm 0.07$  SE,  $p = 0.028$ ; Fig. 2). Within colony, the mean and SD fecal estradiol metabolite concentrations were  $54.93 \pm 10.13$  ng/g in successful females and  $29.59 \pm 3.02$  ng/g in unsuccessful females. Successful and unsuccessful females had no differences in any other within-colony or post-pairing hormone concentrations (all  $p > 0.100$ ). Similarly, males that were ultimately successful or unsuccessful in reproduction did not differ in any fecal hormone metabolite concentrations within-colony or post-pairing (all  $p > 0.418$ ; supplementary material Table S3).

Body mass at pairing (g) did not differ between successful or unsuccessful breeders. Relative to successful females, unsuccessful females did not differ in body mass at pairing ( $\beta = -5.25 \pm 6.56$  SE,  $p = 0.454$ , successful females mean and SD =  $58.00 \pm 10.27$  g, unsuccessful females =  $52.75 \pm 8.30$  g). Relative to successful males, unsuccessful males did not differ in body mass at pairing ( $\beta = -5.00 \pm 2.64$  SE,  $p = 0.107$ , successful males mean and SD =  $58.75 \pm 2.50$  g, unsuccessful males =  $53.75 \pm 4.65$  g). Body mass difference at pairing



**Fig. 1** Fecal progesterone metabolites and fecal cortisol metabolite concentrations (ng/g) across the first 19 days of pairing. **(a)** average values for females (circles;  $n=9$ ) and males (triangles;  $n=9$ ). Error bars indicate standard error of the mean. Day 0 reflects within-colony hormone levels. **(b)** individual variation in patterns of change in fecal

progesterone and cortisol metabolites. Female C, labeled in the top right, displayed an extended surge in progesterone metabolites. The black arrow indicates where progesterone peaks were detected in other females using the iterative method

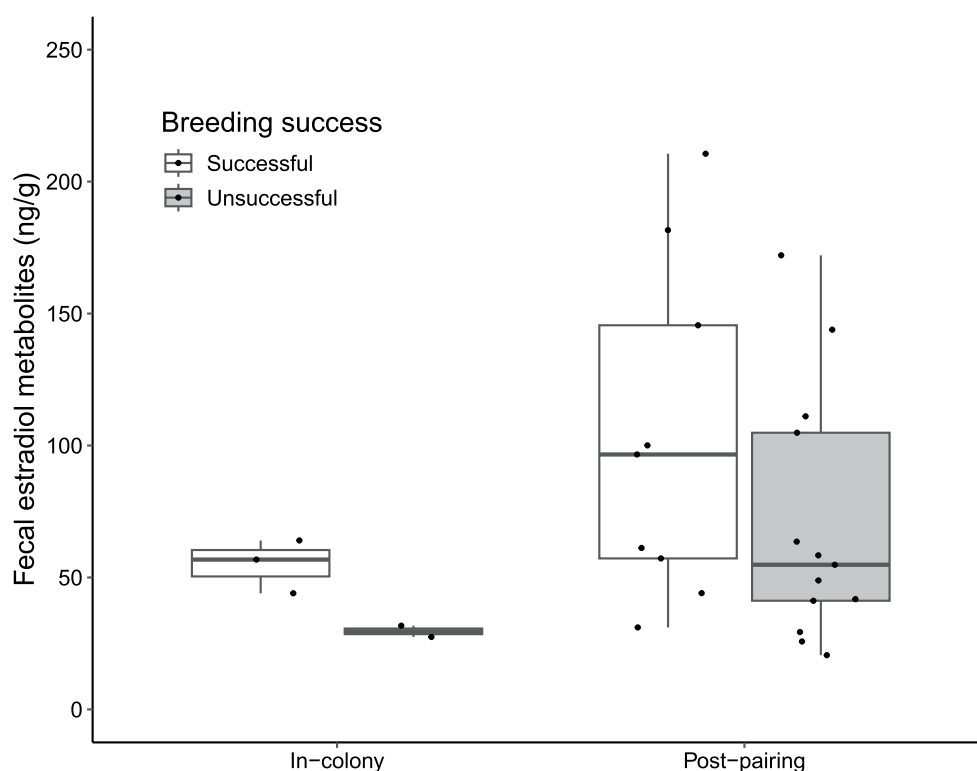
(female mass (g) – male mass (g)) did not differ by breeding success ( $\beta = -0.25 \pm 6.65$  SE,  $p = 0.971$ ).

### Study 2

Out of the 26 pairs pooled from Study 1 and from the study of Toor et al. (2020), 10 were successful. In the first introductions among pairs, durations of aggressive, sexual, prosocial, or non-social behaviors did not predict reproductive success (full model output in supplementary material Table S4). Further, pair similarity in aggressive, sexual, and prosocial behavior did not predict reproductive success.

Overall, aggression duration was low in the first meeting, with an average of 3.8 seconds of total aggression summed between both members of the pair (range 0–19 s), although it is important to point out that pairing in a laboratory setting selects for an absence of extreme aggression. Sexual behavior and prosocial behavior generally occurred for longer and were variable with average durations (s) and standard deviations of  $92.9 \pm 156.2$  and  $307.6 \pm 300.6$ , respectively.

**Fig. 2** Fecal estradiol metabolite concentrations (ng/g) in female naked mole-rats, with in-colony values on the left ( $n=5$ ) and post-pairing values on the right ( $n=8$  females, 24 samples). White bars represent females that reproduced within one year of pairing, and gray bars represent females that did not reproduce during the course of the study



## Discussion

We found that subordinate naked mole-rats do not have uniform trajectories of sexual maturation nor success at reproducing and establishing new colonies themselves. Reproductive success is variable among these individuals that have theoretically been released from reproductive suppression and appears to be associated potentially with pre-pairing, in-colony conditions rather than hormonal changes immediately following colony removal.

The general hormonal increases detected in females after separation from the natal colony are consistent with prior work (Faulkes et al. 1990; Swift-Gallant et al. 2015; Blecher et al. 2020; Edwards et al. 2020). Overall, our work supports the perspective that cortisol and sex steroid hormones all increase in females in the first weeks following separation from the colony, as opposed to being inversely related. These findings are aligned with recent work in naked mole-rats demonstrating that circulating cortisol concentrations are positively associated with luteinizing hormone concentrations (Hart et al. 2024). However, the lack of change in male hormone levels after removal from the colony are inconsistent with prior findings of elevated testosterone (Faulkes and Abbott 1991; Swift-Gallant et al. 2015; Faykoo-Martinez et al. 2018) and cortisol metabolites (Edwards et al. 2020; cf. Blecher et al. 2020), and this difference may have been due to our shorter sampling period post removal, generally small

sample size across studies, and high individual variation in fecal metabolite data.

Within our study, we detected peaks in fecal progesterone metabolite concentrations post-pairing in some, but not all, females. Further, the increase in fecal progesterone metabolite concentrations in a particular female (Female C) was orders of magnitude higher, and sustained longer, than in the other females (Fig. 1b). This suggests that Female C had ovulated and entered the luteal phase of the ovarian cycle during the time course of the study, while the other females had not. The progesterone peaks detected in other females were so comparably small and so soon after colony removal (day 2–3 of pairing) that we speculate that these smaller peaks are not related to ovulation (Fig. 1b). These smaller increases in progesterone, while statistically significant changes (Table S2) and considered peaks by the iterative method, were not sustained at elevated levels, as would be observed in the luteal phase post-ovulation (Faulkes et al. 1990). We therefore caution that statistically significant increases in progesterone immediately following colony removal are not necessarily an indication that females have begun cycling. We suggest that these immediate small increases in progesterone may be adrenal in origin, as they are generally concomitant with cortisol increases (Fig. 1b). Adrenal production of estradiol has been previously detected in naked mole-rats (Zhou et al. 2013), and adrenal production of progesterone is known to occur in other rodents (Piva et al. 1973).

Fecal hormone concentrations following pairing did not differ between successful and unsuccessful reproducers. However, successfully reproducing females appear to have had higher estradiol concentrations while they were in-colony, relative to unsuccessful females (Fig. 2). We caution that this sample size is very small and exploratory, as only five of the females from the eight pairs that could be assessed for reproductive success provided fecal samples during the in-colony collection timepoint. Potentially, higher estradiol levels could be associated with later stages of maturation of ovarian follicles (reviewed in Brown 2018), if particular subordinate females are “less suppressed” than others within-colony. A larger sample size may find increased variation in this measure; while median fecal estradiol concentrations post-pairing are higher in successful females, variation in this measure is pronounced and differences between successful and unsuccessful females are not statistically significant.

Behavioral measures at first meeting were not predictive of successful or unsuccessful reproduction. We suggest that reproductive success in this species may rely on multiple factors, where both individuals in a pair need to be physiologically ready and behaviorally receptive. Dispersing naked mole-rats likely have little mate-choice in the wild, as the above-ground journey to find mates can be long and risky and may rely on chance encounters with conspecifics (Braude 2000). The observation of male-male sexual behavior in this species (Gilbert et al. 2022) is consistent with the idea that lack of available mates favors indiscriminate mating. On the other hand, within the colony, male breeders are sexually solicited by the queen during estrus (Jarvis 1991), indicating that female receptive behavior is important in the context of successful reproduction. It is possible that, in artificial pairs created in a laboratory setting, one or both individuals in the pair may not undergo the physiological and behavioral changes to transition from subordinate to breeder, and either of these scenarios can result in reproductive failure, creating inconsistent physiological and behavioral differences among successful and unsuccessful pairs. In a natural setting, new breeders would arise from either dispersal (O’Riain et al. 1996; Braude 2000) or from high-ranked individuals within the colonies replacing the original breeders (Clarke and Faulkes 1997), and these animals are uniquely primed for the onset of puberty-like changes, whereas other animals within the colony may be far from reproductive competence, even when social conditions are ideal. These data support the concept that subordinate individuals in this species are highly variable, and pre-existing differences in subordinates influence which individuals are reproductively successful when the opportunity arises.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s42991-025-00528-5>.

**Author contributions** Phoebe Edwards: Formal Analysis, Investigation, Writing – Original Draft, Funding Acquisition. Juweriah Munir: Investigation. Ilapreet Toor: Investigation. Bruce Goldman: Conceptualization, Writing - Review & Editing. Rudy Boonstra: Resources, Writing - Review & Editing. Rupert Palme: Methodology, Resources, Writing - Review & Editing. Gabriela Mastromonaco: Methodology, Resources, Writing - Review & Editing, Supervision, Funding Acquisition. Melissa Holmes: Conceptualization, Resources, Writing - Review & Editing, Supervision, Funding Acquisition.

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**Data availability** The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

## Declarations

**Animal ethics** All animal work was done in accordance with the guidelines of the Canadian Council on Animal Care and approved by the Animal Care Committee at the University of Toronto (AUP numbers 20011632 and 20011695).

**Conflict of interest statement** On behalf of all authors, the corresponding author states that there is no conflict of interest.

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