Integrative and Comparative Biology

Integrative and Comparative Biology, volume 61, number 5, pp. 1841–1851
doi:10.1093/icb/icab106

Society for Integrative and Comparative Biology

INVITED PAPER

Queen Pregnancy Increases Group Estradiol Levels in Cooperatively Breeding Naked Mole-Rats

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Synopsis For cooperative species, there can be great value in the synchronization of physiological states to coordinate group behavioral states. This is evident in naked mole-rats (*Heterocephalus glaber*), which have the most extreme form of cooperative breeding in mammals. Colonies have a single reproductive female, "the queen," and 1–3 breeding males. These breeders are supported by adult "subordinates," which are all socially suppressed into a pre-pubertal state. Subordinates cooperate in colony maintenance, defense, and alloparental care. Prior work has reported that there may be social sharing of hormones among individuals in the colony because when the queen is pregnant, subordinates of both sexes develop enlarged nipples and female subordinates can develop vaginal perforation. We sought to document the hormonal changes and mechanisms behind these observations. We found that subordinate estradiol levels were elevated during the queen's pregnancy and were correlated with queen levels. To determine if this occurs by direct hormone-sharing, where group members uptake the hormones of conspecifics through excreta or the skin, we then tested whether treating a single subordinate in the colony with estradiol would induce the same effect in other colony members. It did not, which indicates that the influence on group estradiol levels may be specific to cues from the queen. These queen cues may be behavioral in nature, as we found that queens were less aggressive during pregnancy, which prior work has suggested may relax reproductive suppression of subordinates. Yet, levels of queen aggression alone were not associated, or were weakly associated, with their colony's estradiol levels, though our sample size examining this particular relationship was low. This is suggestive that additional queen cues of reproductive status, beyond just aggression, may be relevant in influencing the subordinate hormonal change, or that the relationship between aggression and colony estradiol levels is more subtle and would need to be elucidated with a larger sample size. These results have implications for how cooperative breeders coordinate reproduction and alloparental care, and how social cues can influence individual and group physiology.

Introduction

Naked mole-rats (NMRs; *Heterocephalus glaber*) are a cooperatively breeding species with the highest known reproductive skew in mammals. These subterranean rodents live in large colonies which average 70–80 individuals in the wild (Jarvis 1991; Jarvis et al. 1994). In each colony, there is a single breeding female, "the queen," and 1–3 breeding males. All other members of the colony are socially subordinate and reproductively suppressed into a prepubescent state. The reproductive suppression is induced by the physical presence of the queen, not by queen urinary pheromones or contact with other colony members (Faulkes and Abbott 1993; Smith et al. 1997). It has been proposed that the aspect of queen presence that suppresses reproduction is her aggressive behavior toward subordinates (Reeve and Sherman 1991; Faulkes and Abbott 1997). However, subordinates are not sterile. If they are removed from the colony they can become reproductive in 1–2 weeks, and if a breeder dies a same-sex subordinate can become reproductive and take

Advance Access publication 28 May 2021

their place (Faulkes and Abbott 1997). A subset of subordinate NMRs appear prone to dispersal, showing motivation to leave their natal colony (O'Riain et al. 1996; Braude 2000; Toor et al. 2020) and potentially form new colonies as breeders. However, the vast majority of subordinate NMRs (estimated as high as 99%) will never reproduce throughout their entire lives (Jarvis et al. 1994).

There is nonetheless a time period in which subordinates within the colony show visible changes associated with reproductive tissues. During the second half of the queen's pregnancy, subordinate females, males, and juveniles can all begin to develop enlarged nipples, and subordinate females can develop vaginal perforation, but do not reproduce even though breeding males are present (Jarvis 1991). There are two proposed hypotheses for this phenomenon. The first is a consequence of altered queen behavior during pregnancy. Jarvis (1991) suggested that the queen's reproductive dictatorship is relaxed during pregnancy, permitting subordinates to begin to sexually mature. Queen shoving behavior, an aggressive act, does decrease during pregnancy (Reeve and Sherman 1991), but there has been little subsequent investigation into this mechanism. Further, this hypothesis may not explain why males and juveniles exhibit these changes.

A second possibility is that these presumed hormonal changes in the subordinates are an adaptive coordination with queen hormonal state. The queen could be giving certain cues that trigger hormonal changes in colony members, possibly to prime them for alloparental behavior. Many subordinate NMRs engage in alloparental behavior by huddling with pups in the nest, grooming them, and participating in pup retrieval (Jarvis 1991). Watarai et al. (2018) proposed that subordinates uptake the pregnant queen's hormones by consuming her feces (coprophagy), which stimulates estrogen-driven alloparental behavior. To demonstrate how this would work, Watarai et al. fed pellets of mashed banana mixed with either pregnant queen feces, nonpregnant queen feces, or estradiol to NMR subordinates. They found that subordinates fed the pregnant feces pellets or estradiol pellets were more responsive to pup playback calls. However, there is little evidence that this consumption of queen feces actually occurs in practice. Though NMRs do engage in coprophagy, they have not been documented consuming queen feces specifically, and coprophagy is mostly documented in pups at the end of weaning (likely for a transitional source of food and seeding the gut microbiome). The extent to which subordinate adults engage in coprophagy, particularly consuming queen feces, is still under debate (Jarvis 1991; Braude et al. 2021; Buffenstein et al., manuscript under review). As the queen would also need to produce a large volume of feces that would have to be distributed among subordinates and widely consumed, this mechanism currently lacks strong support. However, the queens could still be soliciting a colony-wide increase in estradiol that primes pup care using other cues besides, or in addition to, feces. Another mechanism of hormonal transfer between NMR individuals could be urinary and percutaneous transfer (absorption through the skin). This has been documented in group-living laboratory mice (Guzzo et al. 2013) and big brown bats (*Eptesicus fuscus*; deCatanzaro et al. 2014; Greville et al. 2017). The latter is particularly relevant because female big brown bats naturally cohabitate like NMRs. The authors concluded that this hormone transfer was due to direct percutaneous and intranasal absorption of hormones excreted by group-mates, most likely through their urine. This could be a potential mechanism of direct hormonal transfer in NMRs. Although queen urine does not appear to be important in reproductive suppression (Faulkes and Abbott 1993; Smith et al. 1997), NMRs differentiate between soiled bedding from their own colony and that from other colonies, indicating that excreta does carry some relevant cues for this species (O'Riain and Jarvis 1997; Toor et al. 2015, 2020).

The aim of this study was to understand the hormonal changes in queens and subordinates during queen pregnancy, and the mechanisms that elicit hormonal changes in the subordinates within intact colonies. We documented estradiol levels in NMR colonies during and after queen pregnancy using noninvasive fecal sampling. Inherent in direct hormonal transfer (be it by absorption through feces, urine, or the skin) is the implication that it should be able to occur among all individuals in the colony. To test this possibility, we manipulated estradiol levels in a single subordinate individual in five different colonies to examine the effect on estradiol levels of other colony members. If direct transfer of estradiol is present, the other colony subordinates should show an increase in estradiol levels in response to the focal animal's treatment. We additionally recorded queen behavior during and after pregnancy. We tested whether there were differences in aggression during and after pregnancy, in accordance with the findings of Reeve and Sherman (1991). We also examined if queen behavior was associated with subordinate hormonal changes. If queens are losing reproductive control of subordinates during pregnancy, we should see a negative relationship

between queen aggression and subordinate estradiol levels.

Methods

Pregnant queen and colony monitoring

All NMRs were bred and housed at the University of Toronto Mississauga. Separate colonies were housed in polycarbonate cages connected by plastic tubing and lined with corncob bedding. All animals were housed in a 12:12-h light/dark cycle at 28–30°C and had *ad libitum* access to a diet of sweet potato and wet protein mash (Teklad Global 19% protein extruded rodent diet). 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. Five colonies with pregnant queens were used to document longitudinal changes in queen and subordinate estradiol levels during and after pregnancy (colonies: B, E, M, U, and Y). Colony sizes (number of adult individuals) were as follows: B—17, E—30, M—17, U—9, and Y—13. The gestation length of this species is estimated as 66–76 days (Jarvis 1991) or 72–77 days (Lacey and Sherman 1991), and the queen appears heavily pregnant in the last ~35 days of pregnancy, and so the queens in this study classified as pregnant were in the second half of gestation. Samples from nonpregnant queens are from these same individuals but with sample collection occurring >7 days postpartum.

Estradiol treatments

Five different colonies were used for the estradiol manipulations (colonies: D, H, MA, N, and Q). Colony sizes (number of adult individuals, including the manipulated animal) were as follows: D—11, H—5, MA—10, N—16, and Q—18. In these colonies, all individuals were given subcutaneous microchips for identification prior to the start of the experiment, allowing us to record individual ID as a repeated measure. None of these colonies had visibly pregnant queens during the experiment. One large, adult subordinate female in each colony was chosen for treatment. The average weight of the treated females was 61 g (range 48–74 g); for reference, the average weight of adult NMRs overall in these colonies was 55 g (range 20–80 g). Each of these five focal females was treated with both estradiol and vehicle injections in a counterbalanced design. The five females were injected subcutaneously with the first injection type (either estradiol or vehicle) every day at approximately noon for 1 week. Then, they were left unmanipulated for 1 week. During the third week, the second injection type was given (either estradiol or vehicle, whichever had not been received previously). Fecal samples were collected from focal individuals and colony members during both the estradiol injections and the vehicle injections. Fecal samples were collected every day, though not all animals defecated during sample collection every day. Estradiol injections consisted of 40 μg/kg estradiol (Sigma-Aldrich, St. Louis, MO) in 0.1 mL sesame oil. Vehicle injections consisted of 0.1 mL sesame oil or 0.1 mL saline. While the original intent was for each focal animal to get 1 week of sesame oil and 1 week of saline (a control for a planned second experiment) due to COVID-19-related campus shutdowns, this was not achieved and we treated either saline or sesame oil injections as control injections. After injection, the injection site was dabbed dry to make sure that no fluid was secreted from the injection site that could contaminate other colony members.

Hormone analysis

Fecal sample collection occurred 3–4 times per week for the pregnancy monitoring study, and daily for the hormone treatment study; however, not all animals produced samples at each time point. To collect feces, samples were either collected opportunistically (the animal was observed defecating) or during brief handling. For the latter, the animal was briefly restrained by the scruff and a gentle massaging pressure was applied to the stomach area and lower back (~30 s). The individual was then placed in a small (30 × 18 × 13 cm) clean polypropylene cage for up to 5 min. This procedure was moderately successful in inducing the animals to defecate in the clean cage, and the sample was then collected. If the individual did not defecate after 5 min in the small cage, it was returned to the colony. In between individuals, the small cages were wiped down with Prevail disinfectant. Fecal samples were collected with clean forceps and placed in a 1.5 mL vial. Any sample contaminated with urine was not collected. Samples were stored in a small cooler on ice packs during collection for a maximum of 2–3 h, and then transferred to a −20°C freezer until further processing.

Fecal samples were transported on ice and processed in the reproductive endocrinology laboratory at the Toronto Zoo. Samples were weighed and extracted in 80% methanol at a ratio of 0.05 g feces to 1 mL methanol. The mixture was placed on an orbital shaker (Heathrow Scientific, Vernon Hills, IL) for 1 h. To test that 1 h was sufficient to extract all hormones from the feces, aliquots taken following

1 h extraction and overnight extraction were compared. They showed no differences in hormone concentrations, indicating that 1 h extraction is sufficient for these samples. The extraction supernatant was transferred to a new vial and diluted 1:10 in phosphate buffer. Samples were run in duplicate using an estradiol enzyme immunoassay (EIA; described in Kummrow et al. 2011). A serially diluted pool sample was found to be parallel to the standard curve. To validate that this EIA was able to detect changes in estradiol metabolites in NMR feces, we used the five focal subordinates that had been treated with daily estradiol injections and compared fecal estradiol levels during estradiol injections and during control injections. In total, we ran 14 assay plates, with an inter-assay coefficient of variation of 13%.

Behavior scoring

Videos of whole-colony behavior were recorded using GoPro Hero 3 cameras during queen pregnancy and postpartum (>7 days after parturition). An average of five videos were taken per colony across pregnancy and postpartum (range 3–6), with each video lasting 30 min. Queen behavior was scored using BORIS software (Friard and Gamba 2016). Duration (seconds) of queen aggression and frequency of aggression per video were scored, with aggression defined as biting, thrashing, dragging, headbutting/shoving, and incisor fencing (both animals have their mouths open displaying or locking incisors). Videos were scored by two observers (P.E. and D.A.), one of whom (D.A.) was blind to the hypothesis that queens may have altered aggression levels during pregnancy. The three videos from colony E were scored by only P.E. due to time constraints; the other 19 videos were scored by both observers. Observer scores were similar ($R^2 = 0.92$ for duration of aggression, $R^2 = 0.94$ for frequency of aggression), and the averages between the two observers for each video were used in the analysis.

Statistical analysis

Analyses were performed in R version 6.3.6 (Pinheiro et al. 2020) and models built using the packages nlme (Pinhiero et al. 2020) and lme4 (Bates et al. 2015). All hormone data were log transformed for normality. For the monitoring of estradiol levels during the queen's pregnancy, we first tested whether queen reproductive status (pregnant or non-pregnant) was associated with differences in her fecal estradiol levels. We used linear mixed models (LMMs) with queen reproductive status as a fixed effect, queen ID as a random effect, and queen fecal estradiol level as the response variable. Then, we tested whether queen reproductive status was associated with differences in colony fecal estradiol levels. Though the identities of the subordinates were known on the days of fecal sample collection, the majority of these animals did not have permanent identifiers (subcutaneous microchips or tattoos). Hence, we could not use individual subordinate ID as repeated measures from day to day, and instead used whole colony subordinate daily average estradiol levels as the response variable across collection timepoints. We used LMMs with queen reproductive status as a fixed effect, colony ID as a random effect, and colony subordinate average estradiol levels as the response variable.

For the colony response to estradiol treatment, to first check that estradiol manipulations were successful and that the assay could detect these changes, we used a LMM with treatment (estradiol or control injections) as a fixed effect, animal ID as a random effect, and focal animal fecal estradiol levels as the response. We also tested the time course of estradiol increase in the focal animals during treatment, with an LMM using day as a fixed effect, animal ID as a random effect, and focal animal fecal estradiol levels as the response. To test whether other colony members responded to a subordinate being treated with estradiol, we used LMMs with treatment type as a fixed effect, individual ID as a random effect, and individual fecal estradiol level as the response variable, not including the focal animals. We also tested models with sex and the sex by treatment interaction as fixed effects, as well as weight and the weight by treatment interaction as fixed effects, to determine if, for example, only females responded or only larger or smaller individuals responded to the treated animal.

To examine if queen pregnancy affects queen aggression levels, as well as examine other queen factors associated with aggression, we used a generalized linear mixed effect models with a Poisson error structure and a log-link function. Queen reproductive status (pregnant or not pregnant) was used as a fixed effect, and queen identity as a random effect. Because there was high individual variation in queen aggression, we also examined queen qualities that may be predictive of her aggression levels as fixed effects. The queens in this study were very close in age (queen dates of birth: B = unknown, E = 2/2015, M = 6/2015, U = 8/2014, and Y = 9/2014). They were also relatively close in size (queen nonpregnant weights: B = 62 g, E = 72 g, M = 66 g, U = 66 g, and Y = unknown, weights collected after the study concluded). Therefore, age and weight were not used.

However, the queens differed in the number of previous successful litters (any offspring surviving past weaning) they had produced, which we considered to be a measure of the establishment and reproductive success of a queen. Hence, number of previous successful litters was also used as a fixed effect. Queen duration of aggression (seconds per video) or frequency of aggression (counts per video) was used as the response variable. Not all behavior videos were precisely 30 min, as some had to be prematurely truncated because of a disruption. To account for this, an offset of log video duration in seconds was used in both models. Overdispersion was tested for using the package DHARMa (Hartig 2020).

Finally, we tested whether queen aggression levels were predictive of colony estradiol levels. We first used LMMs with queen duration of aggression/video length or queen frequency/video length as a fixed effect, colony ID as a random effect, and subordinate estradiol levels within 24 h of the video recording as the response, to reflect the closest estimation of hormonal status at the time of the videos. However, this model does not account for the effect of queen reproductive status. Because queen aggression levels and queen reproductive status are collinear, they cannot both be used as predictors in the same model. Hence, we then used LMMs with the residuals of each of the aggression models (described in the paragraph above) and queen status as fixed effects, colony ID as a random effect, and log subordinate estradiol levels as the response variable. The aggression residuals fixed effect should represent variation described by aggression alone while accounting for queen reproductive status. We have a small sample size of colony fecal samples that were obtained on the days videos were recorded ($N = 18$), so these particular tests should be considered preliminary. However, given the challenges associated with obtaining large sample sizes in this nontraditional laboratory species, we present all data and analyses for potential comparison with other research groups and future work.

Results

Pregnant queen and colony monitoring

We collected 21 fecal samples from the five queens during pregnancy and postpartum. During pregnancy, queens had higher fecal estradiol levels than when they were not pregnant ($\beta = 0.81 \pm 0.21$, $t_{15} = 3.78$, $P < 0.002$; Fig. 1). High variation in estradiol levels existed even within reproductive status (Fig. 1). Pregnant queens had a range of 45–3872 ng/g fecal estradiol, and nonpregnant queens had a range of 62–425 ng/g. We compared colony-wide estradiol levels (queens not included in the dataset, but female and male subordinates and breeding males included) when queens were pregnant and after they had given birth. We collected 117 fecal samples from colony members across the five colonies. The daily average colony estradiol levels ($N = 34$ averages) were higher when the queens were pregnant than when the queens were not pregnant ($\beta = 0.33 \pm 0.09$, $t_{28} = 2.83$, $P < 0.001$; Fig. 1). Subordinate fecal estradiol ranges were 22–5194 ng/g when the queen was pregnant and 18–134 ng/g when she was not pregnant. Further, the queen's estradiol levels were positively associated with their colony's daily average estradiol levels ($\beta = 0.31 \pm 0.13$, $t_{16} = 2.41$, $P = 0.03$, $R^2 = 0.52$), though we had a small sample size of day-matched queen and subordinate samples ($N = 18$ days; Fig. 2).

Estradiol treatments

In the five focal individuals treated with estradiol ($N = 39$ fecal samples), estradiol injections resulted in increased fecal estradiol levels relative to control injections ($\beta = 0.52 \pm 0.08$, $t_{33} = 6.43$, $P < 0.001$). By the second day of estradiol treatment (24 h after the first estradiol injection), focal animal fecal estradiol levels had not yet significantly increased relative to the first day ($\beta = 0.22 \pm 0.14$, $t_{11} = 1.57$, $P = 0.14$), but by the third day of estradiol treatment, levels had detectably increased relative to the first day ($\beta = 0.44 \pm 0.14$, $t_{11} = 3.19$, $P = 0.009$). During estradiol treatment, focal individuals had a 3.8× increase in average fecal estradiol levels (mean: 191 ng/g, range: 45–604 ng/g) relative to during control treatment (mean: 50 ng/g, range: 20–132 ng/g). From this, we concluded that the estradiol injections were successful in increasing individual estradiol levels and that the assay was successful at detecting this change in the feces of NMRs.

Though the estradiol treatments were successful in increasing estradiol levels in the focal individuals, no differences in fecal estradiol levels were detected in the nonfocal colony members during this period (effect of estradiol treatment, relative to control group as the intercept: $\beta = -0.04 \pm 0.03$, $t_{135} = -1.30$, $P = 0.20$; Fig. 3). All colony members, including queens (all of which were not visibly pregnant), were included in that analysis. When the queens were then removed from the analysis, so that we were only comparing nonfocal, nonqueen colony members, the results were very similar to those that included queens (effect of estradiol treatment, relative to control group as the intercept:

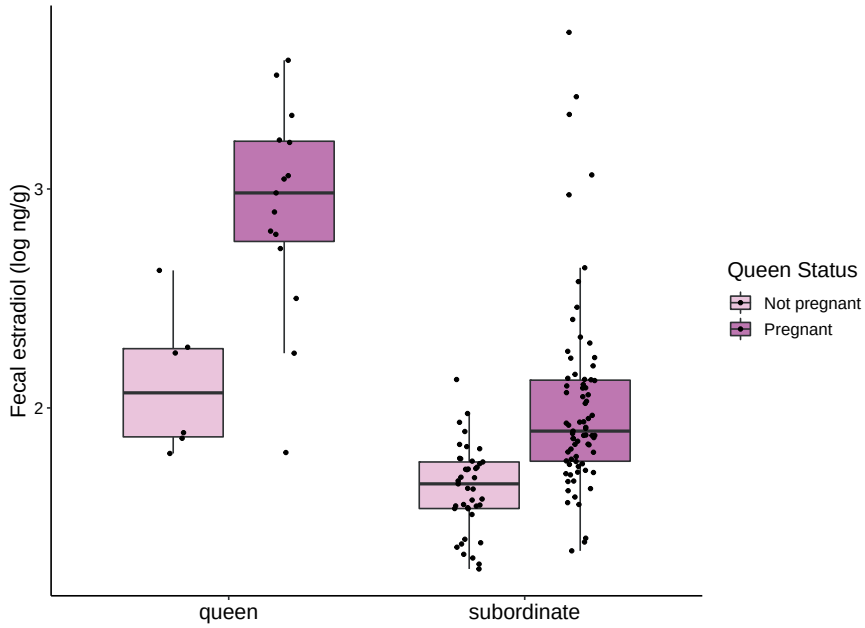

Fig. 1. Queen and subordinate fecal estradiol levels (log ng/g) as a function of queen reproductive state. Both the queens and the colony subordinates had higher fecal estradiol levels when the queen was pregnant than when she was not pregnant (queens $P = 0.002$ by reproductive state; subordinates $P < 0.001$ by queen reproductive state).

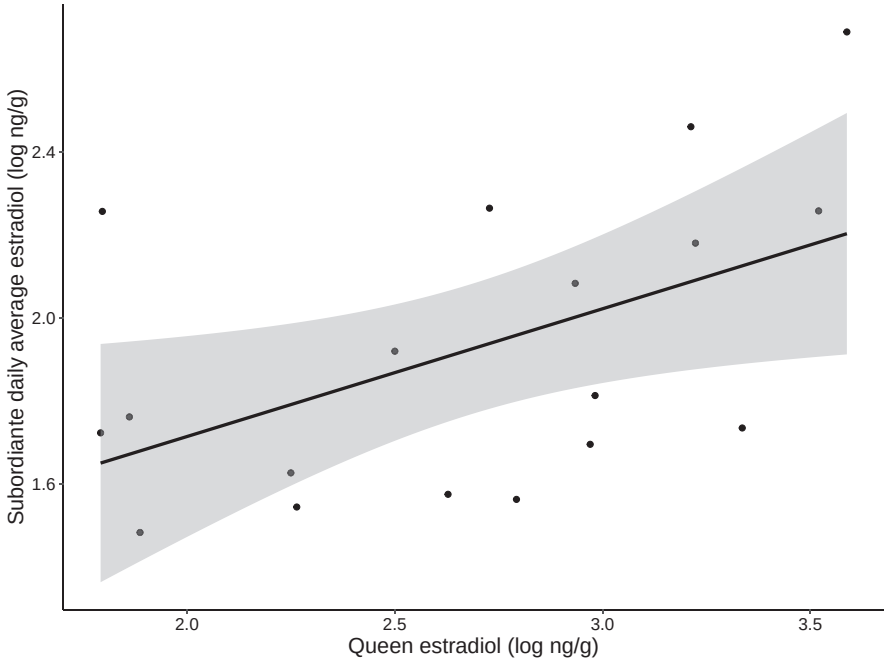

Fig. 2. The relationship between queen estradiol levels on a given day and colony average estradiol levels on the same day. A positive association is detected between queen fecal estradiol and her colony's subordinate average fecal estradiol collected on the same day ($R^2 = 0.52$, $P = 0.03$).

$\beta = -0.04 \pm 0.03$, $t_{121} = -1.52$, $P = 0.13$). We then tried two additional models incorporating sex and the sex by treatment interaction, and weight and the weight by treatment interaction to evaluate if a response occurred in only particular colony members. However, there was neither a significant sex by treatment interaction ($\beta = -0.07 \pm 0.07$, $t_{120} = -1.09$, $P = 0.28$) nor weight by treatment interaction ($\beta = -0.003 \pm 0.002$, $t_{120} = -1.29$, $P = 0.20$), and no significant main effects of treatment, sex, or weight in either of the models (all $P > 0.37$).

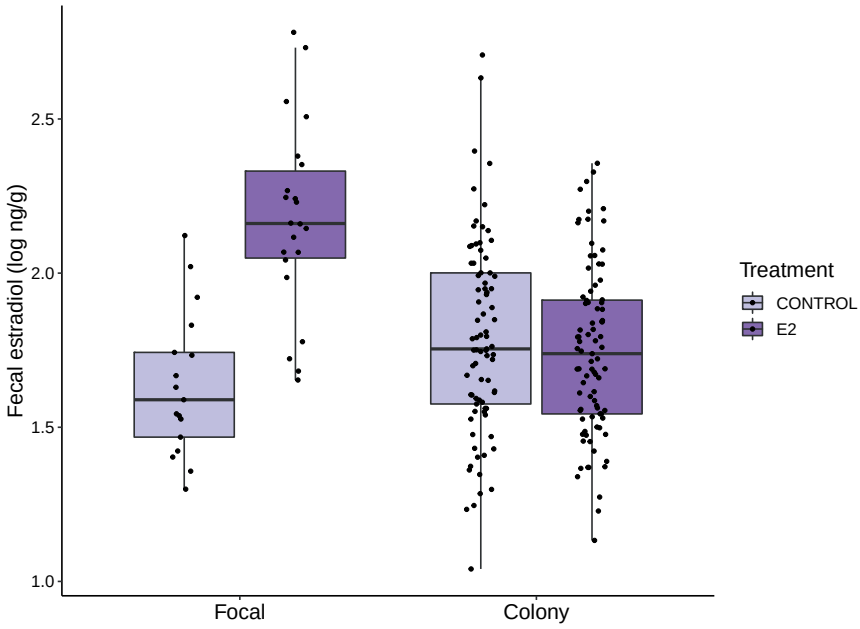

Fig. 3. Subordinate estradiol manipulation and colony response. Estradiol treatment successfully increased estradiol levels in the focal animals ($P < 0.001$), but no effect of treatment was detected on the estradiol levels of other individuals in the colony ($P = 0.93$). In the statistical analysis, individual ID was included as a random effect to account for repeated measures.

Queen behavior

Queen aggression varied by reproductive state, with pregnant queens having lower durations of aggression than nonpregnant queens ($\beta = -0.81 \pm 0.11$, $z_{18} = -7.49$, $P < 0.001$; Fig. 4). Similarly, pregnant queens had lower frequencies of aggression than nonpregnant queens ($\beta = -0.68 \pm 0.17$, $z_{18} = -4.05$, $P < 0.001$; Fig. 4). In both of these models, the number of previous successful litters was also included as a fixed effect, and there was a negative relationship between number of previous litters and duration of queen aggression ($\beta = -0.24 \pm 0.10$, $z_{18} = -2.30$, $P = 0.02$), as well as between number of previous litters and frequency of queen aggression ($\beta = -0.20 \pm 0.10$, $z_{18} = -2.02$, $P = 0.04$).

Finally, we had inconclusive results on whether queen aggression levels were predictive of colony estradiol levels. In the LMMs testing the effect of queen aggression alone on subordinate estradiol levels, there was no relationship between duration of queen aggression and subordinate estradiol levels ($\beta = -15.76 \pm 10.96$, $t_{16} = -1.44$, $P = 0.17$) nor frequency of queen aggression and subordinate estradiol levels ($\beta = -37.99 \pm 28.59$, $t_{16} = -1.33$, $P = 0.20$). When we accounted for queen reproductive status by taking the residuals of the aggression models in the above paragraph, and include them as a fixed effect along with queen status, there was a marginal negative association between the aggression residuals and log subordinate estradiol levels (duration aggression: $\beta = -0.29 \pm 0.04$, $t_{12} = -2.26$, $P = 0.04$; frequency aggression: $\beta = -0.10 \pm 0.05$, $t_{14} = -1.85$, $P = 0.08$). These models additionally do not accurately fit the random effect due to sample size.

Discussion

Based on the observations that subordinate NMRs can undergo physical changes during the queen's pregnancy, we tested social hormone sharing within colonies. We found that subordinates have elevated estradiol levels during the queen's pregnancy. Queen fecal estradiol levels were generally correlated with their colony's subordinate average fecal estradiol levels collected on the same day. However, this effect appeared to be specific to changes in the queen, because treatment of a single large female in the colony with estradiol injections only raised estradiol levels in the injected focal individual, not in the colony as a whole. This suggests that the subordinate hormonal changes during the queen's pregnancy are most likely related to cues from the queen specifically, and does not support general hormone sharing within NMR colonies. These cues from the queen may be aggression levels, as we found that queens were less aggressive during pregnancy than when they were not pregnant. However, our analyses examining the relationship between queen aggression and subordinate estradiol levels directly were inconclusive. It is possible that there is a relationship between queen

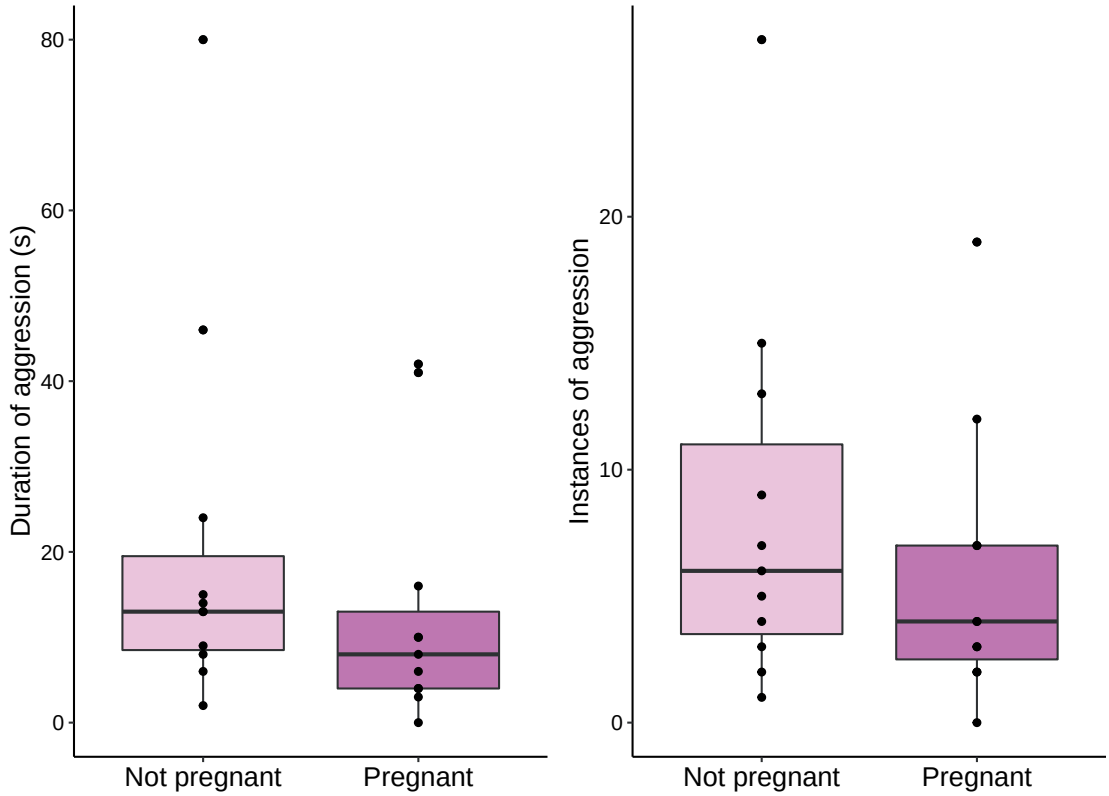

Fig. 4. Queen duration of aggression (seconds per video) and queen frequency of aggression (instances per video) by reproductive status. For both measures, queens were less aggressive when visibly pregnant ($P < 0.001$). In the statistical analysis, queen ID was included as a random effect to account for repeated measures and aggression was offset by video length total seconds.

aggression and subordinate estradiol levels, but this relationship is more subtle than that of queen reproductive status and subordinate estradiol levels and cannot be demonstrated with this sample size. It is also possible that there are other cues of queen status beyond aggression that may carry relevant information for the rest of the colony.

Our results generally do not support the hypothesis that increased estradiol in the colony is due to NMRs ingesting colony-mates' hormones through coprophagy (as proposed by Watarai et al. 2018). Treatment of the focal animals with estradiol resulted in elevated estradiol levels in their own feces, but no change in other colony members (Fig. 3). While the fecal estradiol levels in the focal treatment animals never achieved the maximum levels seen in pregnant queen feces, they were within the pregnancy range. These estradiol concentrations in the feces of the focal animals are also comparable to the concentration of the pellets Watarai et al. experimentally fed to animals to elevate their estradiol levels (1 g pellets with 18 ng/g estradiol). It is also unlikely that colony size prevented the transmission of hormones among individuals, because the experimental colonies were equally sized or smaller than the colonies used in the pregnancy study. Nonetheless, there are some caveats to our study that prevent rejection of the Watarai et al. hypothesis as the mechanism of elevating subordinate estradiol levels during queen pregnancy. The first would be if subordinates specifically eat queen feces, not feces from other colony members. To date, there is no evidence that this is the case (Jarvis 1991, Braude et al. 2021) but we cannot completely rule it out. This could be tested by treating queens with radiolabeled estradiol and determining if it is present in the blood or excreta of other colony members. A second caveat is that a NMR queen's pregnancy is longer than our 1 week estradiol treatment period. One week may be sufficient to induce an effect, because in Watarai et al. (2018), subordinates were fed with estradiol pellets for 4 days and an increase was detected in their feces by the second day of treatment. However, those subordinates were observed consuming the estradiol-laced pellets, whereas in our study we did not quantify coprophagy. A longer treatment period would give subordinates more opportunities to consume the high-estradiol feces. We also do not discount the findings in Watarai et al. that increased subordinate estradiol may prime

alloparental behavior, even if this increase in subordinate estradiol comes from another mechanism aside from coprophagy. It is possible that subordinates begin to reproductively activate during the queen's late pregnancy and that this early activation has adaptive value in sex hormone-driven increases in maternal care.

Our results uphold prior evidence that queen aggression is lower during pregnancy (Reeve and Sherman 1991), and generally support the hypothesis that this change may relax reproductive suppression of subordinates and allow them to begin sexually maturing (Jarvis 1991). Unfortunately, our study cannot directly tease apart the causality between queen aggression levels and subordinate estradiol levels, only the association between both of these factors with queen reproductive state. Comparison with other cooperative breeders may help elucidate this relationship. NMRs have been described as a "dominant control" model of reproductive suppression, where reproductive suppression of subordinates is imposed by the breeders. In contrast, another cooperatively breeding African mole-rat which shows cooperative alloparental care, the Damaraland mole-rat (DMRs; *Fukomys damarensis*), has been proposed to be a "self-restraint" model of reproductive suppression, where subordinates do not reproduce in the group to avoid inbreeding (Clarke et al. 2001). When DMR females are separated from their colony and housed singly for 30 days, they do not become reproductive (as determined by progesterone levels). Yet, when these singly-housed females are exposed to unrelated males, progesterone levels increase after 1–2 weeks (Clarke et al. 2001). Recent evidence indicates that DMRs are induced ovulators (i.e., contact with a male is needed to trigger ovulation; Voigt et al. 2021). Given this difference between NMRs and DMRs, if the increase in colony estradiol levels in NMRs during queen pregnancy is due to the queen's lack of control, then we predict that the DMR subordinates should not show any changes in sex hormone levels during queen pregnancy. If DMRs do show hormonal changes, then this would imply this phenomenon is unrelated to dominant control of reproductive suppression, because DMRs females should not reproductively activate without a novel male.

Our findings can also be compared to how other cooperatively breeding species manage reproductive suppression of subordinates. The changes in NMR queen behavior during pregnancy are opposite to that seen in another model of dominant control, the meerkat (*Suricata suricatta*). Dominant, breeding female meerkats become more aggressive during pregnancy and will attack and even temporarily evict subordinate females from the group. This causes an elevation in subordinate glucocorticoid levels, reduces their conception rates to zero, and causes them to have spontaneous abortions (Young et al. 2006). Though NMR queens also use some aspect of direct contact to enforce reproductive suppression (Reeve and Sherman 1991, Faulkes and Abbott 1997, Smith et al. 1997), subordinate NMRs are not obviously chronically stressed. Their glucocorticoid levels are no higher than the breeders (Faulkes and Abbott 1997; Clarke and Faulkes 1998; Edwards et al. 2020) and there is no consistent relationship between position in the group hierarchy and glucocorticoid levels (Clarke and Faulkes 1997; Edwards et al. 2020). Furthermore, prior work has found no association between levels of queen aggression and subordinate cortisol levels (Clarke and Faulkes 2001; Edwards et al. 2020). The reason for these differences between cooperative breeders is likely because NMR reproductive suppression is more absolute than that in meerkats. Subordinate NMR females do not ovulate and need 1–2 weeks of separation from the queen to become reproductive. In contrast, subordinate female meerkats ovulate and may become pregnant, though at lower rates than dominants, referred to as a "limited control" model of cooperative breeding (Clutton-Brock et al. 2001). Thus, NMR queens may not need to maintain aggression during late pregnancy because, unlike meerkats, there is low risk of subordinates conceiving. Furthermore, because of the total reproductive suppression and low social mobility in NMRs, subjecting subordinates to chronic stress or such severe levels of aggression likely has no adaptive value.

We found that when NMR queens are pregnant, estradiol levels increase colony-wide, in accordance with prior observations of physical changes in subordinates during this time (Jarvis 1991). This change is specific to queen reproductive state, because treating single individuals within the colonies with estradiol does not elevate estradiol levels in other colony members. Though it is suggestive that the decreases in queen aggression during pregnancy play a role in this hormonal change, we cannot rule out additional cues from the queen that induce this change in colony subordinates and future work, either experimental or comparative, could further tease apart the causality of aggression and other cues. However, it

is clear that the queen's reproductive state influences the physiology of the colony as a whole.

Funding

This research was supported by a University of Toronto Mississauga Post-doctoral Fellowship Award to P.D.E. and Natural Sciences and Engineering Research Council of Canada (NSERC) grants RGPIN 2018-04780 and RGPAS 2018-522465 to M.M.H.

Conflict of interest

The authors declare no conflict of interest.

Data availability statement

All data associated with this publication are available on Figshare: https://doi.org/10.6084/m9.figshare.14130488.v2.

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