Medger, K., Bennett, N.C., Ganswindt, S.B., Ganswindt, A., Hart, D.W. 2019. Changes in prolactin, cortisol and testosterone concentrations during queen succession in a colony of naked mole-rats (*Heterocephalus glaber*): a case study. *The Science of Nature* 106: 26. https://doi.org/10.1007/s00114-019-1621-1

Changes in prolactin, cortisol and testosterone concentrations during queen succession in a colony of naked mole-rats (*Heterocephalus glaber*): a case study

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Abstract

Colonies of naked mole-rats (*Heterocephalus glaber*, NMRs) are characterized by an extreme skew in lifetime reproductive success with only one female and one to three male consorts in a colony. The rest of the individuals in a colony are reproductively suppressed and much research has been focussed on elucidating that mechanism. The dopamine system and prolactin have recently been implicated in the suppression of reproduction of subordinate NMRs. To investigate the changes in prolactin during the removal of an aged reproductive female (queen) and succession of a new queen, blood samples were collected during different stages of queen removal: before queen removal, after separation, but in olfactory contact with the queen and after the total removal of the queen. Further, plasma cortisol and testosterone concentrations were determined. The colony appeared unstable prior to queen removal as indicated by high concentrations of cortisol and testosterone and lack of successful breeding. A new queen succeeded the old queen while she was still in olfactory contact. The time preceding queen succession was characterized by high levels of aggression, the death of a number of individuals, high cortisol and testosterone and low prolactin concentrations. Once the older queen was removed entirely and the new queen had given birth, prolactin concentrations increased and cortisol and testosterone concentrations decreased in subordinate NMRs. The results suggest that low prolactin levels are associated with low reproductive suppression during times of colony instability due to the removal or death of a queen.

Keywords: naked mole-rats, prolactin, cortisol, testosterone, queen succession, reproductive suppression

Introduction

Naked mole-rats (*Heterocephalus glaber*, NMR) exhibit a highly social cooperative breeding system similar to that of eusocial insects (O'Riain et al. 2000). Typically only one female (often referred to as the queen) and only a few of the largest males breed in a colony and the rest of the individuals, including both male and female subordinates, are reproductively suppressed and help with colony maintenance, foraging and rearing of the young (Jarvis 1981). Reproductive suppression happens through physiological mechanisms resulting in the suppression of ovarian cyclicity and ovulation in females and lower pituitary sensitivity to exogenous gonadotrophin releasing hormone (GnRH) in both sexes (Faulkes et al. 1990a, b, 1991). Reproductive suppression is not permanent and can be lifted when individuals are removed from the influence of the queen (Faulkes et al. 1990a; Faulkes and Abbott 1991; Margulis et al. 1995). Nevertheless, most of the subordinate individuals will never breed leading to an extreme skew in lifetime reproductive success in NMR colonies. Previous work has implicated the hypothalamic GnRH system as well as RFRP-3 and kisspeptin in the social suppression of reproduction in NMRs (Zhou et al. 2013; Peragine et al. 2017). Recent work has further suggested that the dopamine system and especially prolactin may play a role in reproductive suppression and alloparental care behaviours in NMRs (Mulugeta et al. 2017; Bennett et al. 2018).

Prolactin is well known to suppress reproduction naturally during lactation, but elevated circulating levels (hyperprolactinemia) also interfere with reproductive functions in both males and females (Kauppila et al. 1988; Egli et al. 2010; Brown et al. 2014). Furthermore, correlative studies in birds and mammals have revealed associations of prolactin with social and cooperative behaviours (Schoch et al. 1996; Carlson et al. 2006; Smiley and Adkins-regan 2016; Donhoffner et al. 2017). Male birds and mice show higher levels of prolactin if they invested in parental care (Schradin 2008a; Angelier and Chastel 2009), prolactin affects the decision to babysit in meerkat (*Suricatta suricatta*) helpers (Carlson et al. 2006) and increases social recognition in rats (Donhoffner et al. 2017). However, glucocorticoids (cortisol and corticosterone) appear to be more important than prolactin for alloparental care in cooperative breeders such as striped mice (*Rhabdomys pumilio*) and meerkats (Carlson et al. 2006; Schradin 2008b, a). In naked mole-rats, prolactin levels of subordinate individuals often exceed levels typically of hyperprolactinemia in humans, suggesting that prolactin may be important for reproductive suppression of subordinates in this species (Bennett et al. 2018). The same mechanisms may not be found in other cooperative breeding species, as the only other eusocial mammal, the Damaraland mole-rat (*Fukomys damarensis*) did not show the same prolactin pattern for subordinates (Bennett et al. 2018). Further studies are thus required to elucidate the underlying physiological mechanisms of suppression of reproduction in naked mole-rats and other cooperatively breeding mammals.

The present study monitored plasma prolactin and cortisol concentrations in a colony of NMRs during periods of queen removal and succession. Only one colony was used, in which the queen needed to be removed due to advanced age and the inability to reproduce, as the removal of the queen is often followed by severe aggression especially between the larger subordinate females and the subsequent death of a large number of individuals (Margulis et al. 1995; Clarke and Faulkes 1997). The high aggression in these large females is associated with higher testosterone levels (Clarke and Faulkes 1997) and to monitor these changes, we also measured plasma testosterone in all females. Behavioural and morphological changes, which indicate reproductive activation, were monitored throughout the study. We predicted that prolactin levels would be highest, approaching hyperprolactinemia, in the subordinate individuals before the removal of the queen and lowest when the influence of the queen is entirely removed; however, prolactin concentrations may not be as high in subordinates as was found by Bennett et al. (2018) because of the condition of the old queen. Both cortisol and testosterone concentrations are likely to increase upon removal of the queen, due to increases in aggression and associated stress levels.

Materials & Methods

Animals

NMRs were kept in tunnel systems with several plastic chambers serving as food storage, toilet and sleeping areas and connected by acrylic glass tunnels. They were fed a variety of chopped vegetables and no additional free water was provided. Nesting material consisted of wood shavings. The room temperature ranged between 29 and 30°C. The queen in one colony was very old and emaciated (body weight: 28.32 g) and unable to breed. She needed to be removed from the colony to obtain a reproductively viable colony. The queen was removed by first physically separating her from the colony, but still allowing olfactory contact through a mesh screen and after 72 days, she was removed from the colony entirely. Before the queen was removed, the colony comprised of ten female and 14 male subordinates and three putative breeding males, besides the queen (total of 26 individuals). Queens could be identified by the presence of prominent axillary and inguinal teats and a perforate vagina. We were unable to confidently identify the breeding males and we, therefore, did not distinguish between breeding and subordinate males (together referred to as subordinate individuals).

Blood collection

Blood was collected at three different stages during the removal of the queen (Figure 1). Initially, blood was collected when the colony was still intact (first bleed) after which the old queen was placed into a separate container connected to the colonies' tunnel system but only allowing olfactory contact. After 73 days of olfactory contact with the old queen, blood was taken again (second bleed) and then the old queen was removed entirely. After a further 38 days (111 days after initial separation of the old queen), all individuals were bled for the last time (third bleed). Blood was collected during the day at approximately the same time (between 10:00-14:00) for each of the three blood collection events. A venous blood sample was collected from the hind foot using heparinized micro-haematocrit tubes. It was then placed in an Eppendorf tube and immediately centrifuged at 3000× rpm. The blood plasma was removed and frozen at -80°C until hormone analysis. Animals were weighed before blood collection and care was taken that the blood volume collected was within the range of 1% of an individual's body mass. Before the physical removal of the old queen (first bleed), blood was collected from all subordinates and the breeding males. , For the second bleed, blood was only collected from a subset of individuals (for sample size see figures 2), because the colony was restructuring considerably during that time and we wanted to keep any additional stress as minimal as possible. During the third bleed, blood was collected from all individuals (including the new queen), except for one subordinate male, for which we could not obtain a sample. Any blood collection from the original queen was prevented because of her small body mass. Animals could not be marked individually preventing targeted resampling of specific individuals. In addition to the colony, blood was also collected from a separate breeding pair of naked mole-rats. This separate breeding pair successfully bred and reared young before and after the blood collection making them part of a stable and growing colony.

Hormone analysis

All plasma samples were analysed for concentrations of prolactin and cortisol. Plasma testosterone concentrations were only measured in the females (subordinates, new queen and queen from separate breeding pair). Prolactin was analysed using an enzyme immunoassay (EIA) as described by Bennett et al. (2018). Detailed assay characteristics, including full descriptions of the assay components and cross reactivity's have been provided for the cortisol EIA by Palme and Möstl (1997) and for the testosterone EIA by Palme and Möstl (1993). Sample dilutions ranged from 1/20 to 1/100 for the prolactin EIA, from 1/200 to 1/2000 for the Cortisol EIA and from 1/50 to 1/1000 for the testosterone EIA. The sensitivity of the assays was 190 pg/ml for the prolactin EIA, 20 pg/ml for the cortisol EIA and 80 pg/ml for the testosterone EIA. The intra- and inter-assay coefficient of variance, determined by repeated measurements of high- and low-value controls, was 5.67% and 6.90% and 12.06% and 14.08%, respectively, for the Cortisol EIA, as well as 4.67% and 6.21%, and 8.91% and

12.65%, respectively, for the Testosterone EIA. For the prolactin EIA, both the intra-assay precision and repeatability are < 10% according to the manufacturer and the displacement curve of serial dilutions of spiked naked mole-rat plasma samples was parallel to the standard curve with a relative variation of < 2%.

Behavioural monitoring

The colony was frequently monitored during the different stages of queen removal and any behaviours and changes in reproductive development were noted. Behavioural observations included aggression between individuals as well as observations of mating. Females were further monitored for any signs of reproductive activation such as enlarged teats, perforate vagina and pregnancy. All deaths were recorded and the body mass of these individuals was determined. Only descriptions of the behavioural observations are included as the nature of these results prevents further statistical analysis. Specific behaviours were associated with the different stages of queen succession and colony stability and as such were used to inform and discuss hormonal results at the three blood collection stages.

Statistical analysis

All statistical analyses were performed on the subordinate individuals only as the small sample sizes for the breeding individuals precluded a statistical evaluation. Comparisons with breeding individuals are done using descriptive statistics and are visualized in figures provided. Only plasma cortisol concentration was normally distributed (Shapiro-Wilk test: $p > 0.05$). Because of the small sample size, all hormone concentrations were analysed using Generalized linear models (GZLM) fitted with a linear distribution for cortisol and gamma distributions with log-link functions for prolactin and testosterone concentrations. Factors included in all analyses were the three stages of blood collection, sex and the interaction of stage and sex. Body mass was included as a covariate. Post-hoc comparisons were done using least-significant difference (LSD) pairwise comparisons. A Spearman rank correlation was used to investigate a relationship between prolactin and cortisol concentrations. IBM SPSS 25 (IBM Corp., 2017) was used for all statistical analyses and significance was assumed at $p \leq 0.05$.

Results

Observations

About one week following the separation of the old queen, but while still in olfactory contact, the female subordinates of the colony started to become aggressive towards each other. Severe fighting caused the deaths of the largest subordinate female at 18 days after queen separation and the second largest female at 26 days after queen separation (Fig. 1). These two large females had enlarged teats at the time of their deaths. After another four days, two more subordinate females and one subordinate male were killed (Fig. 1). These individuals were the smallest animals found in the colony and all were found partly eaten, which precluded any exact body mass measurements. At 34 days after removal of the old queen, one of the larger subordinate females was seen mating and she subsequently conceived as swollen, enlarged teats, a perforate vagina and an increase in the girth of the body could be seen during subsequent weeks. Just prior to the second blood collection, another large female with enlarged teats was killed (Fig. 1). No more fighting and deaths were recorded after the second bleed and total removal of the old queen. The new queen gave birth 70 days after she was seen mating with the breeding male, but all pups died four days later and three days before the last blood collection (Fig. 1).

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Hormones

Subordinate naked mole-rats showed no overall difference in plasma prolactin concentrations between females and males (Wald $\chi^2 = 0.29$, $df = 1$, $p = 0.59$) and this was consistent throughout the three blood collection times (time × sex: Wald $\chi^2 = 0.13$, $df = 2$, $p = 0.94$). Similarly, plasma cortisol concentrations were comparable between female and male subordinates (Wald $\chi^2 = 0.01$, $df = 1$, $p = 0.91$) with this also being consistent throughout the blood collection times (time × sex: Wald $\chi^2 = 2.75$, $df = 2$, $p = 0.25$).

Prolactin concentrations in subordinates were significantly different between the blood collection times (Wald $\chi^2 = 6.94$, $df = 2$, $p = 0.03$). The lowest prolactin concentrations were measured when the old queen was separated (second bleed), which was significantly lower compared to the third and last blood collections (LSD: $p = 0.02$; Fig. 2a). Prolactin concentrations were higher when the colony was intact (first bleed) than when the queen was separated (second bleed), although this was not significant (LSD: $p = 0.23$; Fig. 2a). Body mass did not affect prolactin concentrations of subordinates (Wald $\chi^2 = 0.92$, $df = 1$, $p = 0.34$). After removal of the old queen, the new, now pregnant queen showed the highest prolactin concentration measured throughout the study, which was more than nine-times higher than the overall average (Fig. 2a). There was no correlation between prolactin and cortisol in the subordinate individuals ($\rho = -0.09$, $n = 54$, $p = 0.53$).

Plasma cortisol concentrations were very high in the intact colony and during olfactory contact with the old queen (first and second bleed) and they decreased significantly during the third blood collection (LSD: $p \leq 0.01$; Wald $\chi^2 = 8.88$, $df = 2$, $p = 0.01$; Fig. 2b). A similar decrease of cortisol concentrations was also observed for the new queen from very high concentrations when the old queen was still in olfactory contact (second bleed) to lower concentrations when the old queen was removed (third bleed, Fig. 2b). Cortisol concentrations during the third blood collection were similar in the new queen, her subordinates as well as the separate breeding pair (Fig. 2b). Cortisol concentrations of subordinates were positively correlated with body mass ($R^2 = 0.04$, Wald $\chi^2 = 4.43$, $df = 1$, $p = 0.04$).

Plasma testosterone concentrations of subordinate females decreased progressively throughout the different blood collection stages and were significantly lower after the removal of the old queen (third bleed) than during the first and second blood collection (LSD: $p \leq 0.005$; Wald $\chi^2 = 19.31$, $df = 2$, $p < 0.001$; Fig. 3). Similarly, the new queen showed a decrease in testosterone concentration from second to the third blood collection (Fig. 3). Interestingly, the highest testosterone concentration was measured in the queen of the separate breeding pair (Fig. 3). Furthermore, larger female subordinates also exhibited higher testosterone concentrations (Wald $\chi^2 = 13.25$, $df = 1$, $p < 0.001$; $R^2 = 0.44$).

Discussion

Two recent studies have provided the first indications for the involvement of dopaminergic pathways and prolactin in the maintenance of eusociality and reproductive suppression of subordinates in NMRs (Mulugeta et al. 2017; Bennett et al. 2018). Non-breeding subordinate NMRs had much lower dopamine expression profiles than the breeding female and higher prolactin suggesting that the suppressing effects of dopamine on prolactin are missing in these animals (Mulugeta et al. 2017). Prolactin concentrations in subordinates often reached levels considered to be hyperprolactinemia in humans and these elevated levels may inhibit the reproductive axis by reducing GnRH release (Mulugeta et al. 2017). Similarly, Bennett et al. (2018) found very high prolactin levels in subordinate NMRs of both sexes; however, they did not observe the same patterns in the other eusocial mole-rat species, the Damaraland mole-rat, suggesting that prolactin may not play a role in reproductive suppression in this latter species. Prolactin levels vary naturally with reproductive cycles in the breeding females of both species and are very high during lactation (Bennett et al. 2018)

Although we did not observe values indicating hyperprolactinemia as observed by Mulugeta et al. (2017) and Bennett et al. (2018), we found a 180% increase of prolactin concentrations from the second bleed to the third and last bleed, which may be indicative of the changes in reproductive suppression occurring during queen removal and succession. The lowest prolactin levels were measured in subordinates at the end of a period of high colony instability indicated by high levels of aggression and the death of a number of subordinates, whereas the highest levels occurred when a new queen was firmly established and had given birth (third blood collection). Although the levels were still low and did not reach levels indicative of hyperprolactinemia in any of the individuals, possibly because of the early stage in queen succession, the levels were higher than when the old queen was still present (100% increase). The highest prolactin concentrations were measured in the new queen during the last blood collection, shortly after she had given birth and were most likely associated with lactation.

Prolactin concentrations in subordinates were unexpectedly low when the old queen was still present, but the high cortisol and testosterone concentrations at this stage may indicate that the colony was already unstable. The old queen was very emaciated and weak and was possibly not able to maintain high levels of suppressive behaviours in the form of pushing causing an increase in stress and possibly aggression by subordinate animals indicated by high cortisol and testosterone concentrations. Upon removal of the queen and placing her in olfactory contact, aggressions between female subordinates peaked. These involved primarily the large females and the fights were followed by the successive death of the largest females, which all showed signs of reproductive activation. A new queen was seen mating after removal of the old queen and after the death of two of the largest females and some smaller individuals. This period of social instability was also marked by increased concentrations of cortisol in the subordinates and in the new queen. This supports the findings by Clarke and Faulkes (1997), who also observed an increase in cortisol levels during this time of colony instability as well as recorded the death of several individuals. Furthermore, the animals involved in the agonistic encounters were the largest and oldest females (Clarke and Faulkes 1997), whereas aggression between males was low and similar to the present study, did not result in the death of males (Clarke and Faulkes 1998). It is, however, not clear why some smaller subordinates died in the present study as this has not been reported previously. We could speculate that the already unstable colony structure before the removal of the old queen resulted in the high number of deaths found in the present study; however, this requires further investigation.

The lowest concentrations of cortisol and testosterone in both nonbreeding and breeding individuals were found during the last blood collection and after the new queen had given birth. This further supports the notion that the colony was much more stable at this point and high levels of aggression had ceased. At that stage, cortisol concentrations were also comparable to those of the separate breeding pair and may possibly indicate normal cortisol levels in functioning NMR colonies.

Queen succession with the concomitant increase in aggression followed by several deaths was happening while the old queen was still in olfactory contact with the colony. Transfer of bedding from an intact colony to separated subordinate NMRs is not enough to halt reproductive activation suggesting that pheromones do not play a role in reproductive suppression (Faulkes and Abbott 1993). This suggests that the physical presence of the queen is necessary for reproductive suppression and just the olfactory and possibly pheromonal contact is not sufficient. The queen may suppress reproduction through agonistic behaviours such as shoving of subordinates of either sex.

The present study further supports the notion that prolactin plays a role in reproductive suppression of subordinate NMRs. Prolactin concentrations decrease in subordinates during times when a new queen needs to be established and the colony is at its most unstable, whereas they increase upon the recruitment of a new queen. Although the results are interesting and appear to show a relationship of prolactin with the release and reestablishment of social suppression with queen succession in NMRs, further studies are needed to confirm and extend these results.

Ethical approval

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution at which the study was conducted (animal ethics committee of the University of Pretoria EC084-15).

Acknowledgements

We would like to thank Ms Abongile Ndzungu for expert help in laboratory techniques.

Funding information

This study was funded by a South African Research Chair of Mammalian Behavioural Ecology and Physiology awarded to Nigel C. Bennett by the Department of Science and Technology and the National Research Foundation, South Africa. K. Medger acknowledges funding by the University of Pretoria.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Figures

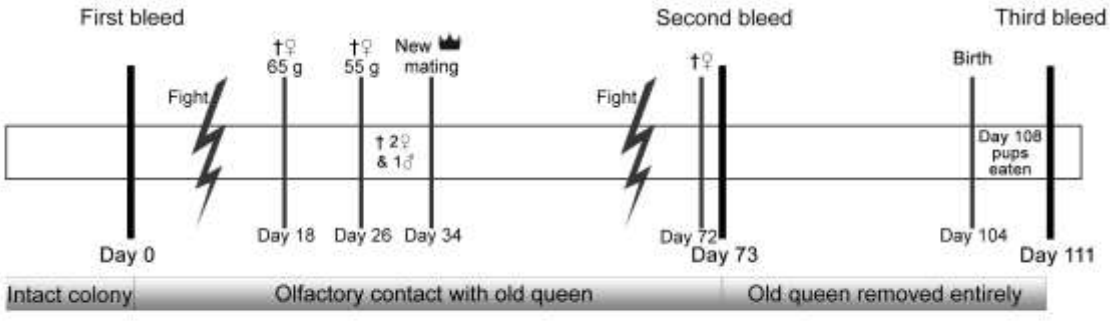

Fig. 1. A timeline showing different blood collection stages (first, second and third bleed) associated with different stages of queen removal (intact, olfactory contact and removed entirely) from a colony of naked mole-rats. Initially, the naked mole-rat colony was intact consisting of one queen, breeding males and subordinate males (♂) and females (♀). After the removal of the queen and the first bleed at day 0, fighting was recorded, several individuals died (†) and a new queen (♛) mated and subsequently, gave birth. Times for important events are given in days counted from the day of the first blood collection and the removal of the old queen and placing her in olfactory contact (Day 0).

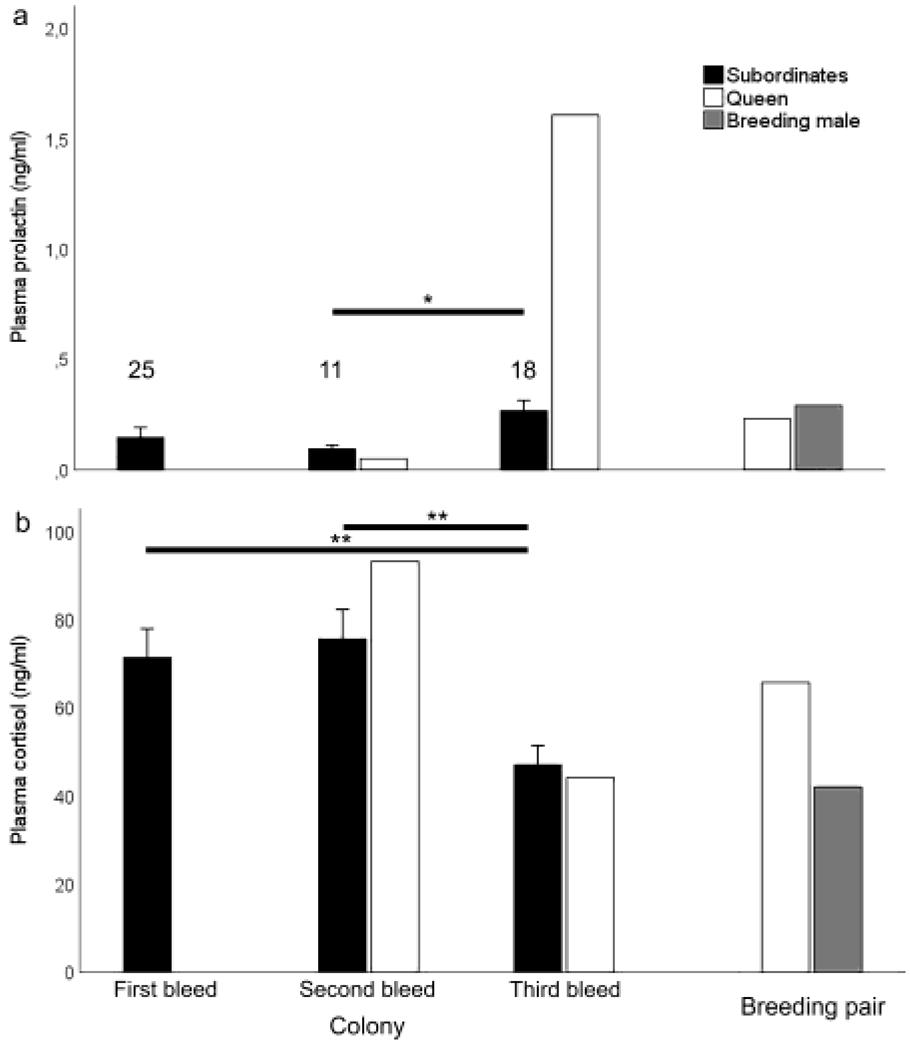

Fig. 2. Plasma prolactin (ng/ml; a) and cortisol (ng/ml; b) concentrations in male and female subordinates and a new queen of a colony of naked mole-rats (*Heterocephalus glaber*) as well as a separate breeding pair. Concentrations were measured before removal of the queen (first bleed), when queen was separated, but still in olfactory contact with the rest of the colony (second bleed) and after she was removed entirely (third bleed). A new female became queen during the separation of the old queen and is indicated as new queen. Data is shown as mean ± standard error and sample sizes are indicated for subordinates. *$p \leq 0.05$, **$p \leq 0.01$.

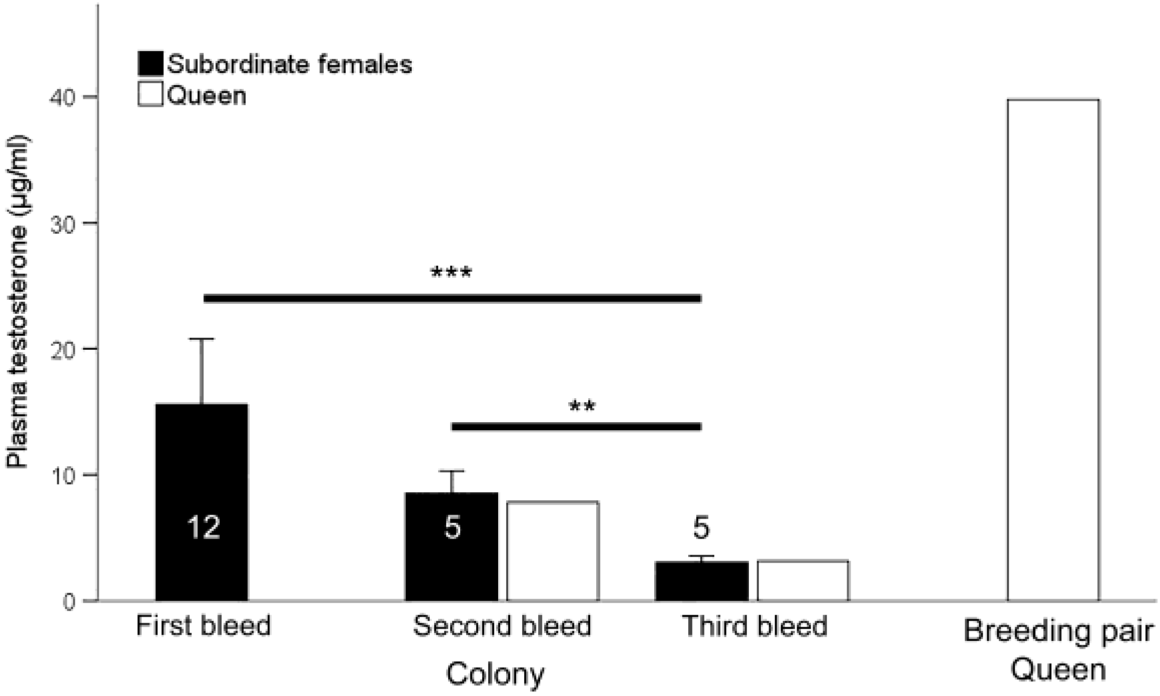

Fig. 3. Plasma testosterone concentrations (mean ± standard error; µg/ml) of subordinate and breeding female naked mole-rats (*Heterocephalus glaber*). Concentrations were measured before removal of the queen (first bleed), after the queen was separated, but still in olfactory contact with the rest of the colony (second bleed) and after she was removed entirely (third bleed). The testosterone concentration for a queen from another colony is also shown and for the new queen in the test colony. Sample sizes are included for subordinate females and asterisks are $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$.