Anovulation in non-reproductive female Damaraland mole-rats 
\textit{(Cryptomys damarensis)}

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Within colonies of Damaraland mole-rats \textit{(Cryptomys damarensis)}, anovulation in non-reproductive females is thought to play an important role in maintaining reproductive skew. Pituitary sensitivity and ovarian structure were examined in three groups of females that differed with respect to their social environment and breeding status to determine whether anovulation is due to inhibitory social cues or is merely the result of a lack of copulatory stimulation. The contribution of gonadal steroid negative feedback to neuroendocrine differences in the reproductive systems of the respective groups was also investigated. LH secretion after a 0.5 \(\mu\)g GnRH challenge in females that had been removed from the presence of the breeding individuals for at least 6 months (removed non-reproductive females) was significantly higher than in non-reproductive females in the colony, but significantly lower than in reproductive females. In both removed non-reproductive females and reproductive females, corpora lutea were observed in ovaries of seven of eight females, indicating that ovulation occurs spontaneously in subordinate females on removal from the breeding pair. Circulating progesterone concentrations in removed non-reproductive females were significantly higher than in non-reproductive females, indicating that circulating progesterone is not responsible for infertility in non-reproductive females. Indeed, after hystero-ovariectomy, reproductive females continued to show significantly greater GnRH-stimulated LH secretion than non-reproductive females. Thus, differential inhibition of gonadotrophin secretion in breeding and non-breeding females occurs independently of gonadal steroids. It is concluded that female Damaraland mole-rats are spontaneous ovulators and that anovulation results from inhibitory social cues within the colony, not a lack of copulatory stimulation. Since non-reproductive females are infertile, inhibition of the hypothalamo–pituitary–gonadal axis has the potential to play a causal role in maintaining reproductive skew in colonies of \textit{C. damarensis}.

Introduction

In co-operatively breeding species, helping is thought to be important for the breeding success of reproductive individuals and, thus, potential helpers should not compete by breeding independently (Emlen, 1991). At the proximate level, dominant females may suppress reproduction in subordinate females directly through interruption of sexual behaviour (for example wolf, \textit{Canis lupus}; Packard et al., 1985) or by causing the premature death of the offspring of subordinate females (wild dog, \textit{Lycaon pictus}; Malcolm and Marten, 1982). Alternatively, harassment of subordinates (yellow baboons, \textit{Papio cynacephalus}; Wasser and Starling, 1988) or exclusion of subordinates from food resources crucial for reproduction (red deer, \textit{Cervus elephas}; Clutton-Brock et al., 1986) by the dominant female, may lead to increased stress and subsequent impairment of fertility in subordinates. The physiological response to physical and emotional stress has a negative impact on fertility and reproductive function (Johnson \textit{et al.}, 1992). Proximate physiological mechanisms of rank-related reproductive inhibition may also occur independently of the generalized stress response, via specialized neuroendocrine pathways, in co-operatively breeding callitrichid monkeys (Snowdon, 1996). Rather than being suppressed by the dominant female, subordinates may restrain themselves from breeding, in response to inhibitory social cues, because it is in their best interests to do so (Snowdon, 1996). Removal of the dominant breeding female results in the initiation of ovarian cyclicity and ovulation in subordinate females (Abbott, 1988).

Wild colonies of co-operatively breeding Damaraland mole-rats show an extreme reproductive skew characterized by a single breeding pair and as many as 41 non-breeding individuals (Bennett and Jarvis, 1988; Jarvis and Bennett, 1993). Non-breeding individuals are typically highly related and an incest avoidance mechanism, which does not depend on the proposed socially induced infertility, prevents
incestuous breeding (Bennett et al., 1996). Field studies indicate that over 90% of the non-breeding individuals do not breed during their lifetime (Jarvis and Bennett, 1993). Histological and endocrine differences between the reproductive axes of breeding and non-breeding female Damaraland mole-rats (Cryptomys damaarensis) have been interpreted as evidence of socially induced infertility in non-breeding females (Bennett et al., 1993, 1994; Bennett, 1994). Furthermore, Bennett et al. (1994) proposed that prostegesterone produced by luteinized unruptured follicles results in an endocrine state similar to that of pseudo-pregnancy, maintaining the anovulatory state in non-breeding females. If non-breeding females are infertile while resident in the colony, anovulation may contribute to the proximate mechanism maintaining reproductive skew. However, Burda (1995), working on the less social common mole-rat from Zambia, Cryptomys anselli, proposed that subordinate females of the genus Cryptomys are in fact not infertile, but that their pseudopregnant-like state can be interrupted, possibly by frequent multiple copulations with an unrelated male, leading to ovulation. Burda (1995) proposed that the reproductive skew is maintained solely through behavioural incest avoidance and anovulation is the result of a lack of stimulation rather than reproductive inhibition. Although a later study by Bennett et al. (1996) found that pituitary sensitivity to exogenous GnRH increased in non-breeding females housed together in the absence of the breeding female, indicating that the social environment does affect the reproductive axis of non-breeding females, the initiation of ovulation was not investigated.

Thus, the aims of the present study were: (i) to determine whether anovulation observed in non-breeding females is attributable to socially induced infertility or to a lack of copulatory stimulation by unfamiliar conspecifics; and (ii) to determine whether gonadal steroids are responsible for maintaining a state of anovulation in non-breeding females through increased negative feedback effects.

Materials and Methods

Animals

Damaraland mole-rats were collected at Dordabis, Namibia (22° 58′ S; 17° 41′ E), and at Hotazel, Northern Cape Province, South Africa (27° 17′; 22° 58′ E). Breeding individuals were captured in the field and additional breeding pairs were formed in the laboratory by pairing unrelated males and females. Females were classified as reproductive (RF) once they had given birth to at least one litter. Two groups of non-reproductive females were used in the study. One group of females originated from functionally complete colonies in which the breeding individuals were present (NRF), whereas in the second group, the reproductive individuals were removed and the colonies were reproductively quiescent (rNRF). Since the reproductive male is typically the heaviest individual in the colony (Jacobs et al., 1991), large males were removed, whereas smaller male siblings were left in the colony. A strong incest avoidance prevents mating between siblings and their parents in the colony (Bennett et al., 1996).

Colonies, ranging in size from reproductive pairs to groups of ten individuals, were housed in plastic crates (1.0 m × 0.5 m × 0.5 m) with nesting boxes. Animals were provided with wood shavings and shredded paper towelling for nesting material. The animals were maintained in a constant temperature room at 25°C in continuous dark and were fed and cleaned under red light. Animals were provided with freshly chopped vegetables each day.

Experiment 1

LH was measured in blood samples taken immediately before and 20 min after a low dose of exogenous GnRH to compare the pituitary responsiveness in RF, NRF and rNRF (n = 10 in each group). A dose of 0.5 μg GnRH was used to determine whether differences in LH response were comparable with those obtained in previous studies in which a 2.0 μg GnRH dose was used (Bennett et al., 1993, 1996). Approximately 300 μl whole blood was collected from veins in the feet of hand-restrained animals. Blood was collected in heparinized microhaematocrit capillary tubes after venepuncture using a sterile hypodermic needle. Blood samples were centrifuged at 500 g for 20 min and the plasma was stored at −40°C until required for LH bioassay. The GnRH (kindly supplied by R. P. Millar, Department Chemical Pathology, UCT) was synthesized using solid phase methodology and had a purity of greater than 98% homogeneity (Millar et al., 1989). GnRH was administered in a 200 μl physiological saline vehicle as a single s.c. injection. Control animals were injected s.c. with 200 μl sterile physiological saline (0.9% (w/v) NaCl).

Experiment 2

Ovaries were obtained from RF (n = 8), NRF (n = 10) and rNRF (n = 8) to determine whether differences in pituitary sensitivity are correlated with differences in ovarian function. Reproductive females were killed irrespective of the stage of the oestrous cycle or pregnancy. Groups containing the rNRF were maintained without the breeding individuals for at least 10 months before the experiment. A further two NRF were removed from their colony and housed singly for approximately 6 months. After i.m. injection of an excess dose (50 μg) of ketamine hydrochloride (Ketalar, Warner-Lambert Research Laboratories, Johannesburg), individuals were weighed and blood samples were obtained by cardiac puncture for progesterone determination from the RF (n = 8), NRF (n = 8) and rNRF (n = 6). Ovaries were removed immediately, fixed in Bouin’s solution for 14–16 h and stored in 70% ethanol until sectioning. Serial sections of 5 μm were cut on the longitudinal axis through the equatorial region of each ovary after routine dehydration and embedding in paraffin wax. Every seventh section was mounted on a glass slide and counter-stained using haematoxylin and eosin. Ovarian size, degree of follicular development and the presence of corpora...
lutea were recorded. The maximum ovarian length and breadth was measured using an ocular graticule at \( \times 25 \) magnification or digital callipers. Ovarian volume was calculated using the formula for the volume of an ellipsoid: 
\[ V = \frac{4}{3} \pi ab^2, \]
where \( a \) = \( \frac{1}{2} \) maximum length and \( b \) = \( \frac{1}{2} \) maximum breadth (Woodall and Skinner, 1989).

**Experiment 3**

Ten reproductive (ovxRF) and ten non-reproductive females (ovxNRF) were hystero-ovariectomized to determine the role of ovarian steroid negative feedback in the maintenance of socially induced infertility. After hystero-ovariectomy, females were allowed to recover for 2 months before the experiment. Blood samples were obtained immediately before and 20 min after the administration of a bolus injection of 2.0 \( \mu \)g GnRH for LH determination. The results were compared with those obtained from groups of intact RF (\( n = 10 \)) and NRF (\( n = 10 \)) injected with 2.0 \( \mu \)g GnRH. Control animals for each of the four groups (\( n = 6 \) in each group) received physiological saline instead of GnRH.

**Hormone determinations**

**Luteinizing hormone bioassay.** Concentrations of plasma and pituitary LH were determined using the LH bioassay, which is based on the production of testosterone by dispersed mouse Leydig cells (Van Damme et al., 1974). Details of the assay have been published by Harlow et al. (1984), Hodges et al. (1987) and Faulkes et al. (1990). This assay method has an advantage over conventional LH radioimmunoassay since it measures only biologically active LH. Plasma samples were assayed at dilutions of either 1:20 or 1:40. LH standard (2nd International Standard 1988, Code 80/552, NIBSC, UK; Storring and Gaines Das, 1993) was used over the range 0.0625–2.0 miu ml\(^{-1}\). The amount of testosterone produced during the incubation was determined by radioimmunoassay (see Hodges et al., 1987).

Serial doubling dilutions of mole-rat plasma, obtained from a reproductive female after a 2.0 \( \mu \)g GnRH challenge, were assayed to validate the bioassay for use in the Damaraland mole-rat. After logit-log transformation of the data (Chard, 1987), the slope of the curve was compared with that of the LH standard curve using the Statistica computer package (Statsoft, Tulsa). The slopes of the two lines were not significantly different (ANCOVA, \( F_{1.4} = 0.02, P = 0.90 \)). The sensitivity of the assay (determined at 90% binding) was 0.1 miu per tube. Intra- and inter-assay coefficients of variation were both 10% (\( n = 18 \) and \( n = 4 \), respectively).

**Progesterone radioimmunoassay.** Progesterone assays were performed using a coat-a-count progesterone kit (Diagnostic Products Corporation, Johannesburg) as described by Bennett et al. (1994). The antiserum is highly specific for progesterone with a crossreactivity to all naturally occurring steroids of <0.5%, with the exception of 17α-dihydroprogesterone (3.4%), 11-deoxycorticosterone (2.4%), 5β-pregnan-3,20-dione (3.2%) and 5α-pregnan-3,20-dione (9.0%). Standard concentrations ranged from 0.3 to 127.2 nmol l\(^{-1}\).

The assay was validated for C. damarensis plasma by testing the slope of the curve produced using serial doubling dilutions of unextracted mole-rat plasma obtained from a pregnant female (over the range 1:1 to 1:64) against that of the standard curve. After logit-log transformation of the data (Chard, 1987), the slopes of the lines were compared using the Statistica computer package and were found not to differ significantly (ANCOVA, \( F_{1.4} = 0.1, P = 0.75 \)). In addition, mole-rat plasma spiked with cold progesterone at three concentrations yielded a recovery estimate of (109.3 ± 5.8%). The minimum detection limit of the assay was 0.36 nmol l\(^{-1}\). The intra-assay coefficient of variation for the single assay was 4.4% (\( n = 20 \)).

**Results**

**Pituitary sensitivity**

Plasma LH secretion was significantly greater after the administration of 0.5 \( \mu \)g exogenous GnRH compared with basal concentrations in RF (Student’s \( t \) test, \( t_{6} = –16.8, P < 0.0001 \); Fig. 1), NFR (\( P < 0.0001, t_{6} = –8.9 \)) and rNRF (\( P < 0.0001, t_{6} = –10.4 \)). Mann–Whitney \( U \) tests revealed that there were no significant differences in control animals in any of the three groups. The mean plasma LH concentration after GnRH stimulation in NRF was significantly lower than that obtained in both rNRF (ANOVA, \( F_{1.27} = 30.4, P < 0.0001 \); LSD test after ANOVA, \( P < 0.05 \)) and RF (\( P < 0.0001 \)). However, there was also a significant difference between the mean GnRH stimulated plasma LH concentrations in rNRF and RF (\( P < 0.0001 \)). There was no significant difference in LH secretion between 0.5 \( \mu \)g and 2.0 \( \mu \)g GnRH challenges in NRF, rNRF or RF.

**Ovarian structure**

Mean body weight (± SEM) did not differ significantly between NRF (121.7 ± 11.3 g), NFR (108.9 ± 9.5 g) and RF (123.3 ± 5.4 g). A significant difference was observed in

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![Fig. 1. Bioactive plasma LH concentrations (mean ± SEM) immediately before (□) and 20 min after (■) a single challenge (s.c.) of 0.5 μg GnRH (n = 10 in each group) and a saline control (○) (n = 6 in each group) in female Damaraland mole-rats. NRF: non-reproductive females; rNRF: non-reproductive females housed in the absence of the breeding pair; RF: reproductive females. P < 0.0001 for post-GnRH values versus pre-GnRH values (Student’s t test). a: P < 0.05 versus NRF and P < 0.0001 versus RF (LSD test after ANOVA).](image)
ovarian volume between at least two of the three groups (Kruskal–Wallis, $H_2 = 8.8, P < 0.05$, Fig. 2). The ovaries of RF could be clearly distinguished from those of the NRF housed in the colony, both macro- and microscopically. The mean ovarian volume in the reproductive group was significantly greater than that of NRF (Mann–Whitney U test, $U = 14, P < 0.05$). Large corpora lutea, which filled much of the stroma of seven of eight RF (a corpus albicans was observed in the other RF), resulted in a macroscopic granular appearance compared with the smooth contour of the ovaries of NRF. In contrast, although follicular development was observed in ovaries of all NRF, only two of ten in the group contained a single corpus luteum. High densities of luteinized unruptured follicles resulted in the ‘paved-like’ appearance of the stroma (see Bennett et al., 1994). In contrast, far fewer luteinized unruptured follicles were present in the ovaries of RF; most of the stroma was occupied by the corpora lutea and developing follicles. The stroma of rNRF resembled that of NRF, with the exception of the corpora lutea that were observed in the ovaries of seven of eight females. Despite the presence of corpora lutea, there was no difference in mean ovarian volume between NRF and rNRF. The large corpora lutea in RF resulted in the significantly greater ovarian volume than that of the rNRF ($U = 6, P < 0.01$). None of the animals in the group housed in the absence of the breeding pair (rNRF) were pregnant and no placental scars were observed in their uteri.

**Plasma progesterone concentrations**

Mean plasma progesterone concentrations are presented (Fig. 3). Kruskal–Wallis analysis ($H_1 = 14.3, P < 0.05$) revealed significant differences in circulating progesterone concentrations between the three groups of females. Concentrations in NRF were significantly lower than both rNRF (Mann–Whitney U test, $U = 11, P < 0.05$) and RF ($U = 0, P < 0.05$). Progesterone concentrations in these two groups were characterized by high degrees of variation. Progesterone concentrations from the three pregnant RF were above the upper detection limit of the assay and were assigned values of 63 nmol l$^{-1}$, corresponding to approximately 15% binding on the assay standard curve.

**Effect of hystero-ovariectomy on pituitary sensitivity**

Mean basal and 2.0 μg GnRH-stimulated plasma LH concentrations in intact RF and NRF, and ovxRF and ovxNRF are presented (Fig. 4). The response to an exogenous 2.0 μg GnRH challenge was significantly higher than basal plasma LH concentrations in both ovxRF (Student’s $t$ test, $t_{18} = 11.0, P < 0.0001$) and ovxNRF ($t_{18} = 7.4, P < 0.0001$). Mann–Whitney U tests showed that there was no response to a saline injection in either of the groups. Hystero-ovariectomy resulted in an increase in pituitary sensitivity to GnRH. GnRH-stimulated LH secretion was significantly greater in both ovxRF ($t_{18} = -5.7, P < 0.0001$) and ovxNRF ($t_{18} = -3.7, P < 0.002$), compared with intact RF and NRF, respectively. Although the basal concentrations of plasma LH were insufficient volumes prohibited the re-assay of these samples at a more appropriate dilution. In contrast to NRF, in which progesterone concentrations did not exceed 5.0 nmol l$^{-1}$, only three of eight RF showed progesterone concentrations below 10.0 nmol l$^{-1}$.
Damaraland mole-rats may ovulating within 7 days after removal (Faulkes et al., 1991) and a review of their reproductive characteristics revealed that most species are spontaneous ovulators (Jarvis and Bennett, 1991). In the present study, comparison of the GnRH-stimulated LH concentrations in the two non-reproductive females possessing a corpus luteum with those non-reproductive females that did not revealed that they were among the highest in the group and resembled LH concentrations found in non-reproductive females housed in the absence of the breeding pair. Bennett et al. (1994) examined 12 non-reproductive females from two colonies and found no corpora lutea. Thus, only two of 22 non-reproductive females examined to date have shown evidence of ovulation. This indicates that ovulation is unusual in non-reproductive females, and in the present study may have been related to specific events within a single colony.

Thus, non-reproductive females are typically anovulatory while they remain in the colony but start ovulating spontaneously in the absence of the breeders. The Bathyergidae have strong hystricomorph affinities (Jarvis and Bennett, 1991) and a review of their reproductive characteristics revealed that most species are spontaneous ovulators (Weir, 1974). In the eusocial naked mole-rat (Heterocephalus glaber), progesterone profiles indicate that subordinate females removed from the colony can start ovulating within 7 days after removal (Faulkes et al., 1990). Spontaneous ovulation in female Damaraland mole-rats may have important implications in terms of the proximate mechanisms responsible for maintaining reproductive skew. Willingstorfer et al. (1998) proposed that a lack of ovulation in non-reproductive females in the colony can be interpreted either as evidence of suppression of reproduction in a spontaneously ovulating species or as a lack of copulatory-induced ovulation in an induced ovulating species. On the basis of evidence obtained in the present study and other studies on C. damarensis (Bennett et al., 1996), it appears that non-reproductive females are infertile while the breeders are present, indicating that anovulation reflects a socially induced infertility. The fact that non-reproductive females are infertile while in the colony supports the hypothesis that inhibition of reproductive function in non-reproductive females contributes to the proximate mechanisms involved in the maintenance of reproductive skew. Although incest avoidance mechanisms (which may include infertility) may be sufficient to prevent breeding between non-reproductive members of the colony, unrelated males do enter the colony (N. C. Bennett, personal observation) and represent potential breeding opportunities for subordinate females. The fact that multiple reproductive females have not been found in more than 100 wild-caught colonies (N. C. Bennett and A. J. Molteno, personal observation) indicates that additional mechanisms operate to maintain reproductive skew. This proposal is in contrast to the hypothesis that anovulation in non-reproductive females is merely due to a lack of stimulation by an unrelated male and, consequently, that reproductive skew in colonies of Cryptomys is maintained solely through incest avoidance (Burd, 1995; Willingstorfer et al., 1998). The fact that these authors did not work on C. damarensis indicates that there are species specific differences within the genus Cryptomys with respect to reproductive function and, consequently, the mechanisms responsible for maintaining reproductive skew.

Infertility may function to prevent breeding through one or both of two mechanisms. If unrelated males mate with non-breeding females (whether females solicit the males or not), inactivity of the reproductive axis may function to prevent fertilization and pregnancy directly as a result of the lack of fertilizable ova. Alternatively, breeding among subordinates may be prevented through an absence of mating with unrelated males due to low concentrations of ovarian hormones. Ovarian hormones play a critical role in promoting the expression of reproductive behaviour in many species (Johnson and Everitt, 1995). Reproductive female Damaraland mole-rats typically solicit copulations from males during the mating sequence (Bennett, 1990) and inadequate gonadal steroid concentrations may result in the absence of proceptive (and receptive) sexual behaviour. In the dwarf mongoose (Helogale parvula), low oestrogen concentrations lead to low mating rates that cannot be explained by direct aggression by dominant females (Creel et al., 1992). Thus, the effect on non-reproductive females, both behavioural and physiological, of an unrelated male entering the colony requires investigation to determine how reproductive inhibition functions in maintaining reproductive skew.

Given that ovulation occurs spontaneously in non-reproductive females housed in the absence of the breeding pair, the difference in pituitary sensitivity between these
females and reproductive females is interesting. This difference is mirrored by a greater ovarian volume in reproductive females compared with non-reproductive females housed in the absence of the breeding pair, whereas there is no difference between the latter group and non-reproductive females. It can be hypothesized that smaller corpora lutea in non-reproductive females housed in the absence of the breeding pair are non-functional due to inadequate LH stimulation. Some rodents require the act of coitus to produce a fully functional corpus luteum (Short, 1984). However, in the present study, pregnant reproductive females showed the highest progesterone concentrations and possessed the largest ovaries due to large corpora lutea that occupied the entire stroma in these females. An increase in the size of the corpus luteum and steroid output is associated with pregnancy in rats (Heap and Flint, 1984). Thus, differences in the reproductive axis between reproductive females and cyclic non-breeding females are probably effects of pregnancy, rather than prerequisites for breeding.

In contrast to a previous study in which urinary progesterone was measured (Bennett et al., 1996), non-reproductive females housed in the absence of the breeding pair showed significantly higher circulating progesterone concentrations than non-reproductive females. Increased progesterone secretion would be expected in females housed in the absence of the breeding pair since corpora lutea were present in their ovaries, indicating that urinary progesterone is not a suitable metabolite as an indicator of ovarian function in this species.

In both social and seasonal reproductive inhibition, a gonadal independent mechanism of gonadotrophin suppression is apparent (Legan et al., 1977; Martin et al., 1983; Karsch et al., 1984; Abbott, 1988). In C. damarensis, although there was a significant increase in pituitary sensitivity to GnRH in both reproductive and non-reproductive hysterectomy-removed females compared with intact females, a significant difference in GnRH-stimulated LH secretion was still apparent between the two hysterectomy-removed groups. Thus, although gonadal steroids clearly affect pituitary sensitivity and play an important role in determining the plasma LH concentrations in intact females via their negative feedback effects, the absence of negative feedback does not abolish the relative lack of pituitary sensitivity in non-reproductive females. This finding indicates that gonadal steroid independent mechanisms are involved in the suppression of gonadotrophin secretion, possibly involving the increased activity of inhibitory neurotransmitters. A number of studies have implicated endorphins in the natural suppression of reproductive function (Sirinathsinghji and Martini, 1984; Roberts et al., 1985; Johnson et al., 1992; Aurich et al., 1994) and may play a similar role in socially induced infertility.

In summary, the present study shows that anovulation in subordinate females represents a socially induced infertility, which occurs independently of gonadal steroids, supporting the hypothesis that reproductive inhibition plays a role in maintaining reproductive skew.

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