Received: 8 April 2024 | Revised: 13 August 2024 | Accepted: 25 August 2024

DOI: 10.1111/jne.13444

ORIGINAL ARTICLE

Journal of Neuroendocrinology WILEY

The curious case of the hypothalamic–pituitary–gonadal axis dysfunction in subordinate female naked mole-rats (*Heterocephalus glaber*): No apparent role of opioids and glucocorticoids

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Funding information
NRF, Grant/Award Number: 64756; University of Pretoria; Technology Innovation Agency (South Africa); University of Cape Town

Abstract

The naked mole-rat (*Heterocephalus glaber*) is a unique model mammal in which to study socially induced inhibition of the hypothalamic–pituitary–gonadal (HPG) axis. Naked mole-rat groups exhibit a high degree of reproductive bias in which breeding is restricted to one female (the queen) and one male, with subordinate non-breeding colony members rarely, if ever, having the opportunity to reproduce due to a dysfunctional HPG axis. It is posited that aggression directed at subordinates by the queen suppresses reproduction in these subordinates, yet the underlying physiological mechanisms causing this dysfunction are unknown. This study aimed to investigate the possible factors contributing to the dysfunction of the HPG axis in subordinate female naked mole-rats with a specific focus on the role of ovarian feedback and stress-related factors such as circulating glucocorticoid and endogenous opioid peptides. The results showed that stress-related factors appear to not mediate the suppression of reproductive function in subordinate female naked mole rats. Indeed, in some cases, the activation of the stress axis may lead to reproductive activation instead of deactivation. At the same time, the role of ovarian sex steroid feedback in reproductive suppression is likely limited and not clearly delineated. This study highlights the need for detailed studies to elucidate the mechanism of reproductive suppression in this unique model mammalian species which may shed light on, and reveal novel mechanisms, in the social regulation of reproduction.

KEYWORDS
aggression, cortisol, endogenous opioid peptides, luteinising hormone, reproductive suppression

1 | INTRODUCTION

The naked mole-rat (*Heterocephalus glaber*) is a eusocial subterranean rodent that lives in large colonies and exhibits a high degree of

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reproductive skew in which breeding is restricted to a small subset of individuals, with subordinate non-breeding colony members rarely, if ever, having the opportunity to reproduce.[1–3] The colony's reproduction is usually monopolised by a single dominant female (the queen) and one dominant male.[2,4] All other females in the colony are non-breeding females (subordinate females) and show physiological suppression with a dysfunctional reproductive axis (or hypothalamic–pituitary–gonadal [HPG] axis), resulting in diminished follicular development and anovulation in these subordinate females.[2,3,5,6] Most evidence points to the dysfunction of the HPG-axis in subordinate naked mole-rat females being caused by the disrupted secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus and/or reduced sensitivity to GnRH of the anterior pituitary, leading to reduced circulating concentrations of gonadotropins (such as luteinising hormone [LH]),[3,7] yet the causes of this are currently unknown.

It has been suggested that aggression directed at subordinates by the queen suppresses reproduction in these subordinates.[8] Naked mole-rat queens are behaviourally dominant to male and female subordinates, shoving, biting and even occasionally killing a close female competitor and other colony members. The queen performs the majority of the shoving (up to 96%) within a colony[5] and typically targets a subset of the larger females.[9] Therefore, it is unsurprising that subordinates can become sexually functional through the removal from the colony and/or removal/death of the queen.[1,5,9–11] Removal of a subordinate female from their natal colony can result in the activation of ovarian cyclicity in as little as 7 days, and pregnancy may arise following the first oestrous cycle.[7,8,12,13] The removal of the queen allows for some females (primarily the larger females) to become sexually functional until a new queen is established, usually as a result of fighting and the subsequent death of competing beta females. New queens result in the re-establishment of suppression in the remaining colony members.[8,11,14]

Queen-performed aggression towards other females (particularly larger subordinate females) has been suggested to induce stress in recipients.[15] When animals are confronted with a stressor, such as aggressive behaviour directed by the queen, the hypothalamus-pituitary (HP)-adrenal (HPA) axis is activated and this, in turn, results in an increase in the secretion of glucocorticoids, cortisol in the case of African mole-rats,[16–19] by the adrenal glands.[15] In some species, glucocorticoids have previously been linked to HPG axis function as an inhibitor of GnRH secretion through a variety of pathways.[20] Furthermore, under physically stressful conditions, endogenous opioid peptides (EOPs) are produced due to their analgesic properties. However, despite the apparent benefits of opioids, it has long been known that opioids have an inhibitory effect on reproductive function, such as down-regulating the release of various pituitary hormones and impairing the function of the HPG axis.[21] Therefore, cortisol and/or EOPs may be potential candidates through which naked mole-rat queens induce inhibition of GnRH and LH secretion, consequently resulting in anovulation in female subordinate naked mole-rats.[3]

To date, the impact of the opioid system on the naked mole-rat reproductive axis has not been investigated. While differences in glucocorticoid metabolites between subordinates and queens have been investigated,[22,23] the effect of the activation of the HPA axis (through physical stress) and circulating cortisol on the functionality of the female naked mole-rat HPG axis has never been directly tested.

In the past, studies by Faulkes et al.[3] selected significantly lighter subordinate females for comparison with queens whilst assessing the degree of HPG dysfunction. Yet, it is the larger subordinate females (often referred to as soldiers[24,25]) that have been observed to exhibit a relaxed form of reproductive suppression compared with smaller and younger subordinate females (often referred to as workers[14,24,25]) even though soldier subordinate females are likely to be exposed to increased physical aggression. As such, this study matched subordinate females of similar body masses (soldiers) to queens and compared their pituitary response, through the monitoring of LH, to an exogenous GnRH challenge to queens and smaller worker subordinate females under similar experimental conditions.[3] Next, we examined the HPA response to acute or chronic physical stressors in queens and soldier subordinate females by measuring the circulating cortisol concentrations in the plasma to assess whether queens and subordinates possess a varied HPA response to stress. Furthermore, we examined the possible relationship between basal concentrations of circulating cortisol and LH to explore the role of cortisol, and ultimately stress, as an inhibitory agent of subordinate female HPG axis. Through the use of the mu-opioidergic receptor antagonist (naloxone), we aimed to examine whether EOPs could play a role in the suppression of reproduction in soldier-subordinate females naked mole-rats. If EOPs are implicated in the physiological suppression of the subordinate female naked mole-rats by acting on the gonadotrophs of the pituitary, then circulating plasma LH should be elevated significantly following a single or a series of injections of naloxone. Finally, this study aimed to eliminate the possible role of progesterone and oestrogen's negative feedback effects in the reproductive axis's dysfunction of females through the monitoring of the pituitary response to an exogenous GnRH challenge in intact and ovariectomised females.

2 | MATERIALS AND METHODS

2.1 | Study animals

A total of 24 naked mole rats (12 queens and 12 subordinate females) from 12 captive colonies were used for this study. The animals were housed in artificial Perspex® and glass burrow systems in the University of Cape Town Department of Zoology. Animals were provided with various freshly chopped vegetables and fruit daily. Mole-rats do not drink free water and obtain all their water requirements from the food resources which in nature comprise the underground storage organs of geophytes.[26] The animals were maintained in rooms at 28°C and humidity of 60% to simulate conditions in the tunnels in their natural habitat. Animal rooms were maintained on a 12-h light and 12-h dark photoperiod (06:00–18:00 light). The photoperiod has not been shown to affect mole-rat circadian biology,[27,28] so all sampling occurred during the morning (08:00–10:00) where possible. The University of Cape Town ethics committee approved all experimental procedures.

A queen was defined as a female that had produced five or more litters prior to the onset of the experiment. Subordinate females were defined as adult females that had not bred and were still housed within their natal colony.[16]

2.2 | Surgical procedure

Hystero-ovariectomies (removal of the uterus, cervix, ovaries and fallopian tubes) were performed on a subset of six queens and six subordinate females. Animals were returned to their natal colony within 2 h of their operation. A minimum period of 6 weeks was allowed for recovery before further experimentation (as reported by Molteno and Bennett[29]). The remaining subset of six queens and six subordinate females were deemed intact females as they still possessed their uterus, cervix, ovaries and fallopian tubes.

2.3 | Experimental design

Animals were divided into one of four groups. Group 1 comprised six intact queen naked mole-rats, while group 2 consisted of six intact subordinate female naked mole-rats. Groups 1 and 2 originated from six colonies, with intact subordinates and intact queens from each. Group 3 consisted of six hystero-ovariectomised queen naked mole-rats, while group 4 consisted of six hystero-ovariectomised subordinate female naked mole-rats. Groups 3 and 4 originated from six colonies, a hystero-ovariectomised queen and subordinate from each. The intact queens, hystero-ovariectomised queens, intact subordinates and hystero-ovariectomised subordinates were matched for body mass (intact queens: 58.21 ± 14.8 g; hystero-ovariectomised queens: 57.26 ± 7.43 g; intact subordinates: 42.63 ± 8.00 g; hystero-ovariectomised subordinate: 47.09 ± 9.96 g; analysis of variance (ANOVA): $F = 0.54$, $p = .66$). This differs from previous studies where smaller subordinate females were selected.[3] Age was not considered in this study as it does not determine a subordinate's rank or role in the colony (i.e., soldier or worker), which is instead influenced by body mass,[19,23,30] yet all animals were older than 2 years at the time of the experiment.

Seven separate experiments (Experiments 1–7) were conducted on each of the four groups over one calendar year to allow for sufficient recovery time between experiments (Figure S1). Due to the small body mass of naked mole-rats, multiple bleeds were required over an extended period to obtain sufficient blood sample volume (240 µL per bleeding event) for LH and cortisol measurement without endangering the animal's health. All animals remained in their natal colonies during this time.

All injections were administered subcutaneously as a bolus. Blood was obtained by piercing a superficial blood vessel in the animal's hind foot using a sterilised needle. The blood was then collected using heparinised microhematocrit tubes, transferred to Nunc^R tubes, and centrifuged (500 *g* for 6 min). After centrifuging, the plasma was then pipetted off into new Nunc^R tubes and stored at −20°C before the radioimmunoassay. All animals were returned to their colonies after injections and bleedings. In the case of the cortisol samples, blood was obtained within 3 min of handling the animal.

The GnRH was synthesised in the laboratory of R.P. Millar using solid phase methodology (the purity of GnRH was greater than 98% homogeneity).[31] Powdered naloxone was stored at −4°C and mixed with saline before administration.

2.3.1 | Experiment 1: Social class effect on pituitary sensitivity to GnRH

All intact queens and intact subordinates were administered a single, subcutaneous dose of 0.5 µg of GnRH in 200 µL physiological saline solution. Blood was taken immediately before (pre) and 20 min (post) after the GnRH administration, and plasma LH was subsequently measured. Data were extracted from Faulkes et al.,[3] along with communication with the authors, to obtain the pre- and 20 min post-circulating LH levels after the administration of 0.5 µg of GnRH in 200 µL physiological saline solutions to workers (significantly lighter than their queen) subordinate females. Pre- and post-GnRH challenge plasma LH concentrations of intact queens and intact subordinates (soldiers) were compared with those recorded by Faulkes et al.[3]

2.3.2 | Experiment 2: The effect of female steroid feedback on pituitary sensitivity to GnRH

All hystero-ovariectomised queens and hystero-ovariectomised subordinates received a single, subcutaneous dose of 0.5 µg of GnRH in 200 µL physiological saline solution. Blood was taken immediately pre- and 20 min after (post) the GnRH administration and plasma LH was subsequently measured. Pre- and post-GnRH administration plasma LH concentrations of hystero-ovariectomised queens and subordinates were compared to pre- and post-GnRH administration plasma LH concentrations of intact queens and subordinates from Experiment 1.

2.3.3 | Experiment 3: Determining the relationship between basal concentrations of circulating cortisol and LH

Intact queens and subordinates and hystero-ovariectomised queens and subordinates were subjected to a once-off bleeding event to measure baseline plasma cortisol and LH.

2.3.4 | Experiment 4: The effect of breeding status on the adrenal response to acute stress

The six intact queens and subordinates were administered a single subcutaneous dose of 200 µL physiological saline solution. Blood was

taken immediately pre-, and 20 min after (post) the saline injection and plasma cortisol was subsequently measured.

2.3.5 | Experiment 5: The effect of breeding status on the adrenal response to chronic stress

The six intact queens and subordinates received 200 µL of physiological saline injections hourly over 10 h. Blood was taken immediately pre- and 20 min after (post) the last saline injection and plasma cortisol was subsequently measured.

2.3.6 | Experiment 6: The effect of a single dose of naloxone on pituitary sensitivity to GnRH

All animals in groups 1–4 (intact queens and subordinates and hystero-ovariectomised queens and subordinates) were bled prior to any treatment (pre). All animals in groups 1–4 then received a single injection of 250 µg naloxone in 250 µL physiological saline. The dosage of 250 µg in 250 µL of physiological saline was based on the amount administered for a 40 g animal (similar dosages to that used in rats by Leposavic et al.[32]). A GnRH challenge (0.5 µg in 200 µL physiological saline) was then performed 20 min after the naloxone. Blood was collected 20 min after (post) the GnRH challenge and plasma LH titre was measured on both pre- and post-blood samples.

2.3.7 | Experiment 7: The effect of multiple doses of naloxone on pituitary sensitivity to GnRH

The effect of frequent low doses of naloxone was investigated in groups 1 through 4 (intact queens and subordinates and hystero-ovariectomised queens and subordinates). A GnRH (0.5 µg in 200 µL physiological saline) challenge was performed before the start of the naloxone injections. Animals then received hourly injections of 125 µg of naloxone in 125 µL of physiological saline for 10 h. The dosage of 125 µg in 125 µL was based on the amount administered for a 40 g animal (similar dosages to that used in rats by Leposavic et al.[32]). A second GnRH (0.5 µg in 200 µL physiological saline) challenge was performed 20 min after the last naloxone injection. Blood was taken 20 min after the first GnRH challenge (post-GnRH only) and 20 min after the second GnRH challenge (post-GnRH + multidose naloxone). Plasma LH concentration was then measured.

2.4 | Luteinising hormone assay

LH activity was measured using an in vitro bioassay based on the production of testosterone by mouse Leydig cells.[33] Plasma samples were assayed at a dilution of 1:20 LH pituitary preparation (2nd International Standard 1988, code no. 80/552, NIBSC, UK) was used over the range 1.4–200 µ.mL^{-1}. Checks for parallelism to the standard curve were carried out to validate the LH assay after GnRH administration. Following the logit-log transformation of the data,[34] the parallelism of the LH standard and the serial dilution of mole-rat plasma were tested. The curve was parallel and not significantly different from the reference preparation. The sensitivity of the assay (determined at 90% binding) was 12.7µ/tube or 2.5 µ/mL. Intra and inter-assay coefficient of variation for repeated measurement of quality control was 8% ($n = 4$) and 11% ($n = 8$), respectively. The assay has been validated for use in the naked mole-rat by Van Der Westhuizen et al.[6] All blood samples were analysed separately.

2.5 | Cortisol assay

Plasma cortisol concentrations were determined using a commercially available kit Coat-a-Count Cortisol (TRKCO2 Diagnostic Products Corporation, Los Angeles, CA), as described by Hart et al[17] The assay was able to determine cortisol concentrations of between 1.26 and 1380 nmol.L^{-1}. The cross-reactivity of the antiserum was less than 1% with all naturally occurring steroids, with the exception of 11-deoxycortisol (11.4%). Cross-reaction with prednisolone was 76% and with prednisone was 2.3%. The assay was validated for plasma by testing for parallelism using serial doubling dilutions of mole-rat plasma obtained from an individual with high cortisol concentrations (range, 1:1–1:32). The slope of the lines did not differ significantly (ANCOVA: $F_{1,6} = 4.7$, $p < .05$). The sensitivity of the assay (suppression of binding of 10%) was 6.1 ng mL^{-1}. Intra- and inter-assay coefficients of variation were 2.3% and 4.1%, respectively. All blood samples were analysed separately.

3 | DATA ANALYSIS

Statistical analyses were performed on R 2022.02.0 and GraphPad Prism 8.4.3. Statistical significance was denoted by $p \leq .05$, and data are presented as mean ± standard error (SEM). Data were checked for normality (Shapiro–Wilk test) and equal variance (Levene's test). All data were normally distributed. Post hoc comparisons were made using Tukey's honestly significant difference (HSD) tests. Only biologically relevant post hoc comparisons are reported.

3.1 | Experiment 1

A two-way ANOVA was utilised to compare the pre- and post-GnRH challenge plasma LH concentrations for six small worker intact subordinates (extracted from Faulkes et al.[3]), six large soldier intact subordinates (this study) and 12 intact queens (six from this study and six extracted from Faulkes et al.[3]).

3.2 | Experiment 2

A two-way ANOVA with repeated measures was utilised to compare the pre- and post-GnRH challenge plasma LH concentrations for six

intact queens, six intact subordinates, six hystero-ovariectomised queens and six hystero-ovariectomised subordinates.

3.3 | Experiment 3

A one-way ANOVA was utilised to compare the baseline circulating LH and cortisol concentrations for six intact queens and subordinates and six hystero-ovariectomised queens and subordinates, respectively. A linear regression line was fitted to find the relationship between baseline circulating LH and cortisol concentrations for all 24 females.

3.4 | Experiments 4 and 5

Two separate two-way ANOVA, with repeated measures, were utilised to compare the cortisol concentrations for six intact queens subordinates prior to and after an acute or chronic stressor event.

3.5 | Experiments 6 and 7

A separate two-way ANOVA, with repeated measures, was utilised to compare the pre- and post-GnRH challenge circulating plasma LH levels for six intact queens and subordinates and six hystero-ovariectomised queens and subordinates after a single administration of naloxone (Experiment 6). An additional two-way ANOVA, with repeated measures, was utilised to compare the post-GnRH challenge circulating LH levels (post-GnRH only) and the post-GnRH challenge circulating LH levels following multiple administrations of naloxone (post-GnRH + multidose naloxone) for six intact queens and subordinates, six hystero-ovariectomised queens and subordinates (Experiment 7).

4 | RESULTS

4.1 | Experiment 1

In all intact females, there was a significant rise in plasma LH between pre- and post-administration of GnRH ($F_{1,36} = 125.8$, $p < .0001$, Figure 1).

The female group (worker intact subordinates, soldier intact subordinates, intact queens from this study and intact queens from Faulkes et al.[3]) significantly contributed to the variation in circulating LH between all intact females ($F_{3,36} = 12.6$, $p < .0001$, Figure 1). When pre- and post-plasma LH concentrations were combined worker intact subordinates had significantly lower circulating LH levels compared with soldier intact subordinates (post hoc: $p = .001$) and both sets of intact queens (post hoc: $p \leq .01$ for both, Figure 1). While, soldier intact subordinates and both sets of intact queens displayed no statistically significant difference in plasma LH concentration (post hoc: $p \geq .06$, for all, Figure 1).

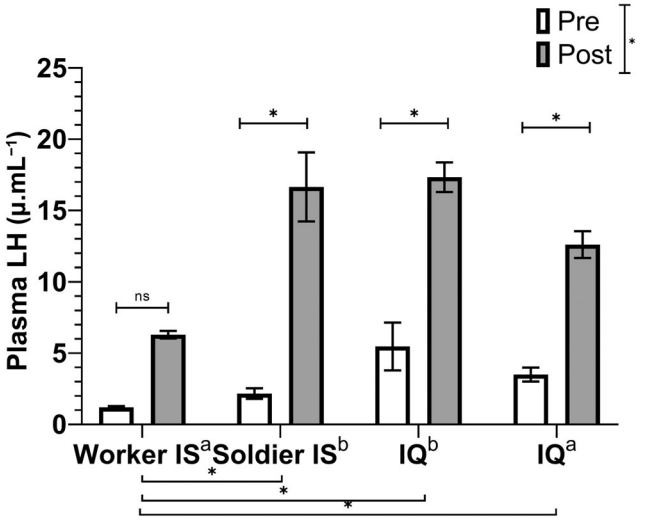

FIGURE 1 Mean (±SE) prior to a single gonadotropin-releasing hormone (GnRH) injection (0.5 mg in 200 μL saline) plasma luteinising hormone (LH, $\mu.mL^{-1}$) concentration (pre) and the post-GnRH administration plasma LH ($\mu.mL^{-1}$) concentration for worker intact subordinates (IS), soldier ISs and intact queens (IQ) ($n = 6$ for all groups) female naked mole-rats (*Heterocephalus glaber*). Worker ISs were defined as female subordinates significantly lighter than their queens. Soldiers ISs were defined as those female subordinates of similar masses to their queens. [a] denotes data extracted from Faulkes et al.,[3] while [b] denotes data from this study. Significant relationships ($p \leq .05$) are indicated with '*'.

The interaction between time (pre- and post-GnRH challenge) and female group (worker intact subordinates, soldier intact subordinates, intact queens from this study and intact queens from Faulkes et al.[3]) significantly contributed to the variation in circulating LH ($F_{1,36} = 4.90$, $p = .006$). On closer inspection, all intact female groups displayed no statistically significant difference in pre-LH concentrations to one another (post hoc: $p \geq .29$ for all, Figure 1). Similarly, worker intact subordinates' pre- and post-GnRH challenge LH concentrations displayed no statistically significant difference in plasma LH titres (post hoc: $p = .16$). In contrast, soldier intact subordinates (post hoc: $p < .0001$) and the two sets of intact queens (post hoc: $p \leq .001$, for both) displayed a significant increase in plasma LH after the administration of GnRH (Figure 1). Worker intact subordinates post-administration of GnRH LH concentrations were significantly lower than those of soldier intact subordinates (post hoc: $p < .0001$) and the two sets of intact queens (post hoc: $p \leq .04$, for both) (Figure 1). Soldier intact subordinates and the two sets of intact queens displayed no statistically significant difference in post-GnRH challenge LH concentrations (post hoc: $p \geq .18$ for all, Figure 1).

4.2 | Experiment 2

For all females (intact queens and subordinates and hystero-ovariectomised queens and subordinates combined) in this study, there was a significant effect of time (pre- and post-administration of GnRH) on the variation in circulating LH plasma LH ($F_{1,40} = 106.6$,

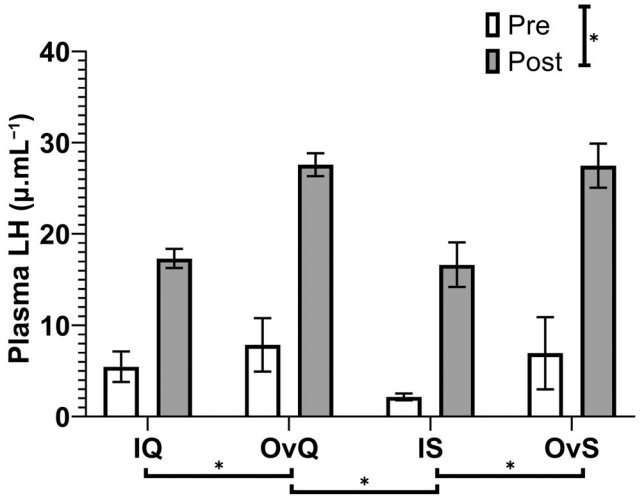

FIGURE 2 Mean (±SE) before (pre) and after plasma luteinising hormone (LH, μ.mL^{-1}) concentration following a single gonadotropin-releasing hormone (GnRH) (0.5 mg in 200 μL saline) administration for intact queens (IQ), hystero-ovariectomised queens (OvQ), intact subordinates (IS) and hystero-ovariectomised subordinates (OvS) ($n = 6$ for all groups) female naked mole-rats (*Heterocephalus glaber*). Significant relationships ($p \leq .05$) are indicated with '*'.

$p < .0001$), with a rise in plasma LH after GnRH administration (Figure 2).

Furthermore, female group (intact queens, intact subordinates, hystero-ovariectomised queens and hystero-ovariectomised subordinates) significantly contributed to the variation in circulating LH ($F_{3,40} = 6.68$, $p < .001$, Figure 2), when pre- and post-plasma LH concentrations were combined. Hystero-ovariectomised queens had significantly higher combined circulating LH levels compared with intact queens (post hoc: $p = .04$) and intact subordinates (post hoc: $p = .004$) (Figure 2). Similarly, hystero-ovariectomised subordinates possessed significantly higher combined circulating LH levels compared with intact subordinates (post hoc: $p = .01$) (Figure 2). Hystero-ovariectomised queens and hystero-ovariectomised subordinates (post hoc: $p = .99$), intact queens and intact subordinates (post hoc: $p = .82$) and intact queens and hystero-ovariectomised subordinates (post hoc: $p = .07$), respectively, displayed no statistically significant difference in circulating LH levels (Figure 2). There was no significant interaction between time (pre- and post-administration of GnRH) and the female group (intact queens, intact subordinates, hystero-ovariectomised queens and hystero-ovariectomised subordinates) ($F_{1,40} = 1.67$, $p = .19$).

4.3 | Experiment 3

Female group (intact queens, intact subordinates, hystero-ovariectomised queens and hystero-ovariectomised subordinates) significantly contributed to the variation in circulating LH of this study ($F_{3,20} = 13.4$, $p < .001$, Figure 3A). On closer examination, intact subordinates were found to have lower circulating baseline LH levels compared to intact

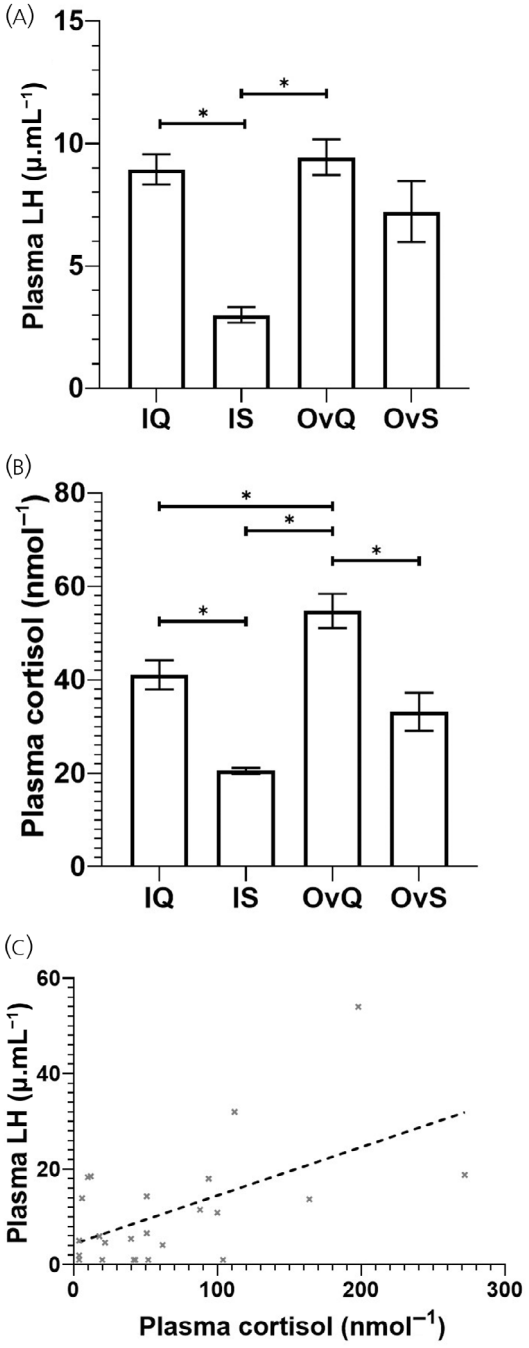

FIGURE 3 Mean (±SE) baseline plasma (A) luteinising hormone (LH, μ.mL^{-1}) and (B) cortisol (nmol.L^{-1}) for intact queens (IQ), hystero-ovariectomised queens (OvQ), intact subordinates (IS) and hystero-ovariectomised subordinates (OvS) ($n = 6$ for all groups) female naked mole-rats (*Heterocephalus glaber*). (C) The positive relationship between baseline plasma LH (μ.mL^{-1}) and cortisol (nmol.L^{-1}) for all naked mole-rat females. Significant relationships ($p \leq .05$) are indicated with '*'.

queens (post hoc: $p = .001$), hystero-ovariectomised queens (post hoc: $p = .004$) and hystero-ovariectomised subordinates (post hoc: $p = .007$, Figure 3A). Intact queens, hystero-ovariectomised queens and hystero-ovariectomised subordinates displayed no statistically significant

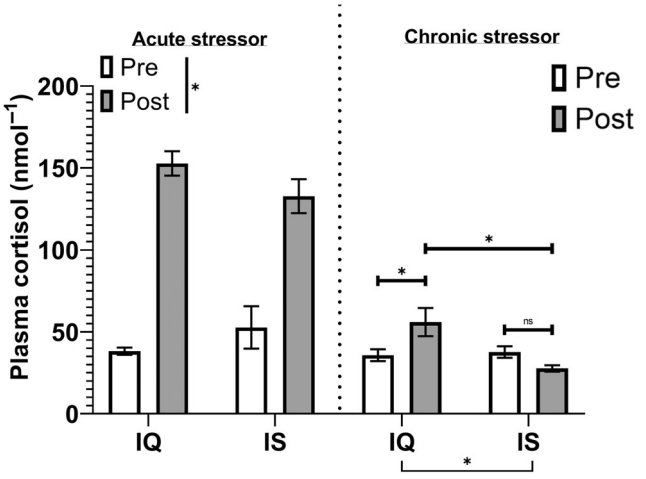

FIGURE 4 Mean (±SE) plasma cortisol (nmol.L^{-1}) prior to (pre) following (post) a single 200-μL injection of saline (acute stressor) and 10 hourly 200-μL injections of saline injections (Chronic stressor) for intact subordinates (IS) and intact queens (IQ) ($n = 6$ for all groups) female naked mole-rats (*Heterocephalus glaber*). Significant relationships ($p \leq .05$) are indicated with '*'.

difference in levels of baseline LH levels compared to one another (post hoc: $p \geq .23$, Figure 3A).

Similarly, female groups (intact queens, intact subordinates, hystero-ovariectomised queens and ovariectomised subordinates) of this study significantly contributed to the variation in circulating cortisol titres ($F_{3,20} = 20.5$, $p < .001$, Figure 3B). Intact subordinates possessed significantly lower baseline cortisol levels compared to intact queens (post hoc: $p = .001$) and hystero-ovariectomised queens (post hoc: $p < .0001$), but intact subordinates baseline cortisol levels were not significantly different than hystero-ovariectomised subordinates (post hoc: $p > .05$, Figure 3B). Similarly, hystero-ovariectomised subordinates possessed significantly lower baseline cortisol levels compared to hystero-ovariectomised queens (post hoc: $p = .001$, Figure 3B). Hystero-ovariectomised queens possessed significantly higher baseline cortisol levels than intact queens (post hoc: $p = .03$), and hystero-ovariectomised subordinates and intact queens showed no statistically significant difference in plasma cortisol levels (post hoc: $p = .32$, Figure 3B).

Baseline circulating levels of LH were found to be significantly affected by baseline plasma cortisol concentrations for all 24 females ($F_{1,22} = 10.2$, $p = .004$, $y = 0.10x + 4.33$). As baseline plasma cortisol concentrations increased, so did baseline circulating levels of LH (Figure 3C).

4.4 | Experiments 4 and 5

Overall, for all intact females from this study (intact queens and intact subordinates), there was a significant effect of time on plasma cortisol following an acute stressor (pre vs. post; $F_{1,20} = 112.4$, $p < .0001$, Figure 4). In contrast, a chronic stressor (pre vs. post) showed no statistically significant effect on plasma cortisol in all intact females from this study ($F_{1,20} = 1.01$, $p = .37$, Figure 4). When pre- and post-plasma cortisol titre was combined the female group (intact queens and intact subordinates) did not differ significantly from one another under an acute stressor ($F_{1,20} = 0.09$, $p = .77$), but did under a chronic stressor ($F_{1,20} = 6.72$, $p = .02$) with intact subordinates possessing lower plasma cortisol levels under a chronic stressor (Figure 4). Similarly, there was no significant interaction between time (pre- and post-stressor) and the female group (intact queens and intact subordinates) under an acute stressor ($F_{1,20} = 3.54$, $p = .07$). Yet, this interaction did significantly contribute to the variation in female cortisol levels under chronic stress ($F_{1,20} = 8.83$, $p = .008$, Figure 4). On closer investigation, intact queen's cortisol concentrations following chronic stress were significantly higher than their pre-stress trial concentrations (post hoc: $p = .05$, Figure 4). While intact subordinates' cortisol concentrations after chronic stress were lower than their pre-stress concentrations; however, this was not significant (post hoc: $p = .52$, Figure 4). However, post-stressor plasma cortisol concentrations for intact queens were significantly higher than intact subordinate cortisol concentrations after a chronic stress event (post hoc: $p = .004$, Figure 4). All remaining comparisons showed no statistically significant difference (post hoc: $p \geq .08$).

4.5 | Experiment 6

Overall, for all females (intact queens, intact subordinates, hystero-ovariectomised queens and hystero-ovariectomised subordinates) from this study, time (pre- and post-administration of a single administration of naloxone and GnRH) significantly contributed to the variation in circulating LH, with a significant rise in plasma LH following the administration ($F_{1,40} = 78.6$, $p < .0001$, Figure 5A). Furthermore, the female group (intact queens, intact subordinates, hystero-ovariectomised queen and hystero-ovariectomised subordinates) significantly contributed to the variation in circulating LH ($F_{3,40} = 14.1$, $p < .0001$, Figure 5A), when pre- and post-plasma LH concentrations were combined. Intact subordinates possessed significantly lower plasma LH titres compared with intact queens (post hoc: $p = .02$), hystero-ovariectomised queens (post hoc: $p < .0001$) and hystero-ovariectomised subordinates (post hoc: $p = .003$, Figure 5A). Similarly, hystero-ovariectomised queens possessed significantly higher plasma LH titres compared with intact queens (post hoc: $p = .01$) and ovariectomised subordinates (post hoc: $p = .05$, Figure 5A). Hystero-ovariectomised subordinates possessed no significant difference in plasma LH titres compared to intact queens (post hoc: $p = .89$).

The interaction between time (pre- and post-single administration of naloxone and GnRH) and the female group (intact queens, intact subordinates, hystero-ovariectomised queens and hystero-ovariectomised subordinates) significantly contributed to the variation in plasma LH ($F_{3,40} = 3.12$, $p = .04$). On closer inspection, all-female groups possessed no significant difference in pre-single administration of naloxone and GnRH LH concentrations to one another (post hoc: $p \geq .06$ for all,

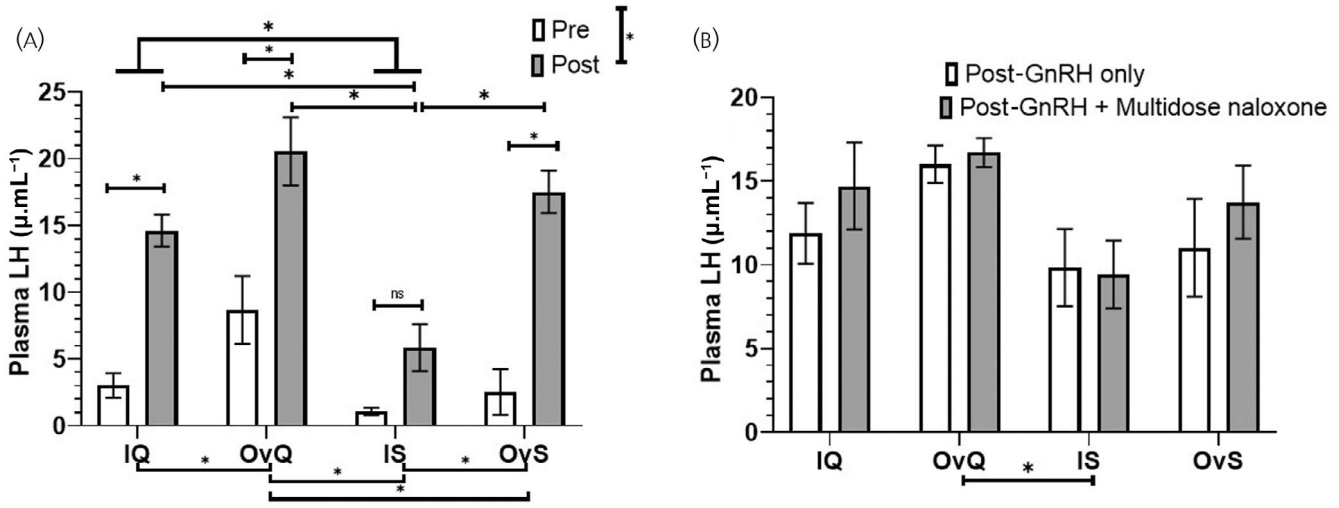

FIGURE 5 (A) Mean (±SE) before (pre) and after (post) plasma luteinising hormone (LH, $\mu.mL^{-1}$) concentrations following a single gonadotropin-releasing hormone (GnRH) (0.5 mg in 200 μL saline) and naloxone (250 μg in 250 μL saline) administration. (B) Stimulated plasma LH response following a single GnRH challenge (post-GnRH only) and a single GnRH challenge after eight 1 hourly naloxone injections (125 μg in 125 μL saline) (post-GnRH + multidose naloxone) for intact queens (IQ), hystero-ovariectomised queens (OvQ), intact subordinates (IS) and hystero-ovariectomised subordinates (OvS) female naked mole-rats (*Heterocephalus glaber*) ($n = 6$ for all groups). Significant relationships ($p \leq .05$) are indicated with '*'.

Figure 5A). A significant rise in plasma LH titre after GnRH-naloxone administration was detected in intact queens (post hoc: $p = .001$), hystero-ovariectomised queens (post hoc: $p = .004$) and hystero-ovariectomised subordinate (post hoc: $p < .0001$, Figure 5A). In contrast, no statistically significant difference in plasma LH titre after a single naloxone and GnRH administration was detected in intact subordinates (post hoc: $p = .52$, Figure 5A). Intact subordinates possessed significantly lower post-single administration of naloxone and GnRH LH concentrations compared to intact queens (post hoc: $p = .02$), hystero-ovariectomised queens (post hoc: $p < .0001$) and hystero-ovariectomised subordinates (post hoc: $p = .001$, Figure 5A). Meanwhile, intact queens, hystero-ovariectomised subordinates and hystero-ovariectomised queens had no statistically significant difference in post-single administration of naloxone and GnRH LH concentrations (post hoc: $p \geq .25$ for all, Figure 5A).

4.6 | Experiment 7

There was no significant difference in plasma LH post-GnRH only and post-GnRH + Multidose naloxone from females in this study (intact queens, intact subordinates, hystero-ovariectomised queens and hystero-ovariectomised subordinates) ($F_{1,40} = 0.98$, $p = .33$). Similarly, the interaction between time (post-GnRH only and post-GnRH + multidose naloxone) and the female group (intact queens, intact subordinates, hystero-ovariectomised queens and hystero-ovariectomised subordinates) did not significantly contribute to the variation in plasma LH ($F_{3,40} = 0.29$, $p = .83$). However, the female group (intact queens, intact subordinates, hystero-ovariectomised queens and hystero-ovariectomised subordinates) significantly contributed to the variation in circulating LH ($F_{13,40} = 3.55$, $p = .02$, Figure 5B) when post-GnRH only and post-GnRH + Multidose naloxone plasma LH concentrations were combined. Intact subordinates possessed significantly lower plasma LH titres compared with hystero-ovariectomised queens (post hoc: $p < .0001$, Figure 5B). All other female groups' LH concentrations showed no statistically significant differences (post hoc: $p = .24$, Figure 5B).

5 | DISCUSSION

This study aimed to investigate the putative factors contributing to the dysfunction of the HPG axis in subordinate female naked mole-rats. Specifically, we investigated the following:

a. whether there was a higher tone of ovarian negative feedback in subordinates which could be overcome by hystero-ovariectomising subordinates.
b. whether there was a higher tone of EOPs in subordinates induced by the queen which could be overcome by administering the opioid antagonist, naloxone, to subordinates.
c. whether there were higher levels of cortisol induced by the queen in subordinates which led to suppression of the HPG axis in subordinates.

However, our findings indicate that none of these suggested mechanisms is solely involved in the dysfunction of subordinate females' HPG axis as hystero-ovariectomy, antagonism of EOPs and the presence of low plasma cortisol concentrations did not relieve the suppression in subordinate females.

Hystero-ovariectomised queens and subordinates had higher basal plasma LH levels compared to intact queens and subordinates. This finding is expected since hystero-ovariectomy relieves the HP axis from the negative feedback effects of sex steroids such as oestrogen and progesterone, and the peptides inhibin and follistatin on LH and follicle-stimulating hormone secretion,[35] thus resulting in higher LH levels, which has been found in other social species of mole-rats.[29,36] This indicates that the subordinate females used in this study (those that are of similar mass to queens) may possess ovarian negative feedback; however, it is unlikely that this ovarian feedback loop is the primary cause of reproductive suppression due to their similar pituitary response to GnRH in both hystero-ovariectomized breeding and non-breeding female naked mole-rats. Clearly further research is needed to robustly negate the ovarian negative feedback as a mechanism of reproductive suppression.

Second, it has been well documented that beta females, those females that are the largest subordinate females of the colony (soldiers), are frequently the object of an increased frequency of directed aggression by the dominant queen compared with smaller subordinate females, workers.[8,9] Such aggression could activate the various stress axes, including the HPA and EOP axes of these soldier females when compared to workers, which would likely result in a greater dysfunction of the HPG axis. However, this study, along with that of Faulkes et al.,[3] is in agreement with van der Westhuizen et al.,[14] who found that the largest subordinate females of the colony, the soldiers, possessed a functional HPG axis equal to that of the reproductive queen. This implies that either a physical stressor does not result in the activation of the stress axis, or the various stress axes do not play a role in the dysfunction of the HPG axis.

Edwards et al.[22] showed that subordinate naked mole-rats possessed similar glucocorticoid concentrations (through the measurements of faecal glucocorticoid metabolites) to their breeding counterparts. In contrast, the current study observed that subordinate naked mole-rats possessed lower cortisol levels than their breeding counterparts. This may be due to the fact that this current study only used soldier subordinate females, whereas Edwards et al.[22] utilised subordinate females of all body masses.

In the case of the naked mole-rat, it would appear that much like dwarf mongooses (*Helogale parvula*),[37] wolves (*Canis lupus*)[38] and female common marmosets (*Callithrix jacchus*),[39–41] dominants have higher baseline stress levels than subordinates. Creel[37] suggested that it is not surprising that reproductive function is rarely mediated by glucocorticoids given the range of pathologies (e.g., decreased immune function and loss of muscle mass) associated with raised glucocorticoid levels. Dominant individuals may thus carry a higher fitness cost, which may explain why subordinates appear to accept their inferior status so readily.[37,42] However, the HPA axis may be more sensitive in dominants, such as queens, resulting in raised levels of cortisol.

Intact queens showed a significant increase in plasma cortisol following an acute and chronic physical stressor. In contrast, intact subordinates only showed a significant increase in plasma cortisol following an acute but not a chronic physical stressor. When examining the response to an acute versus a chronic stressor, it would appear that the latter results in the down-regulation of cortisol, particularly in intact subordinates. As an animal habituates to a stressor and consequently no longer perceives it as a threat; the stress response is then reduced.[43] In the subordinate naked mole-rat, cortisol depression was evident after only 10 h following the onset of multiple injections. This would suggest that naked mole-rats have a marked predisposition towards down-regulation under chronic stress conditions. This may well represent an adaptation by subordinates to living in close proximity to a dominant and aggressive queen who routinely patrols the natal burrow system, shoving and biting colony members that she encounters en route.[1,5,9,10]

Contrary to our initial predictions, it is unlikely that the opioid system is directly involved in the suppression of gonadotropins in female subordinate naked mole-rats. After administering a single naloxone dose prior to the GnRH challenge, three of the four groups (intact queens, hystero-ovariectomised queens and hystero-ovariectomised subordinates) showed a significant increase in LH concentrations similar to other group-living mole-rat species, Damaraland (*Fukomys damarensis*) and highveld (*Cryptomys hottentotus pretoriae*).[29,44] Surprisingly, intact subordinates showed a loss of a significant rise in LH upon a single naloxone dose prior to the GnRH challenge, which is the converse to what would be expected if EOPs were operational in maintaining reproductive suppression in subordinates. Previous studies have observed differences between the naked mole-rat's and other mammal's opioid systems, including functional differences in the mu-receptors.[45] Usually, in mammals, the behavioural response to mu-receptor agonists, such as morphine, includes analgesia, sedation and decreased aggression.[46] However naked mole-rats display morphine-induced hyperactivity, motor dysfunction and extreme aggression, which are reversible by naloxone.[45,47,48] This suggests that the functional difference in the mu-receptors of naked mole-rats may cause naloxone to have a suppressive effect on gonadotropin secretion rather than a stimulatory effect. As such, like with cortisol, the production of EOPs in response to a stressor may result in a rise in LH and the female's sexual functionality. Abbott[49] suggested that a short-term blockade (e.g., a single injection of naloxone) of opioid receptors is insufficient to stimulate an increase in plasma LH. Yet in our study, multiple naloxone injections (over an 10-h period) resulted in similar plasma LH levels in all four groups.

This study supports the notion that the suppression of reproduction in subordinate female naked mole-rats is likely not mediated by higher sensitivity to ovarian feedback, or stress-related axis activation and highlights the need for exploration of other factors involved in the reproductive suppression in this unique model mammalian species. An attractive candidate is prolactin[11,18,50] which when administered to mice inhibits neurokinin B and kisspeptin which are stimulators of GnRH.[51–53] Moreover, cyclicity can be restored when administering daily kisspeptin concomitantly with prolactin in these mice and also in hyperprolactinemic women.[54]

AUTHOR CONTRIBUTIONS

Daniel W. Hart: Formal analysis; visualization; writing – original draft. **E. Roberts:** Data curation; investigation; methodology;

writing – original draft. **M. J. O'Riain:** Conceptualization; data curation; funding acquisition; investigation; methodology; project administration; supervision; writing – original draft. **R. P. Millar:** Conceptualization; writing – original draft. **N. C. Bennett:** Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; supervision; writing – original draft.

ACKNOWLEDGEMENTS

We thank Professor Jennifer Jarvis for the use of her naked mole-rats and insights.

FUNDING INFORMATION

This study was funded by University of Pretoria to N. C. Bennett (grant no. 64756), Technology Innovation Agency (South Africa) and NRF grants to R. P. Millar and the University of Cape Town.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because rights to these raw data are held by the institute and further permission is needed to release such data. Requests to access the datasets should be directed to DWH, u10022725@tuks.co.za.

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SUPPORTING INFORMATION

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How to cite this article: Hart DW, Roberts E, O'Riain MJ, Millar RP, Bennett NC. The curious case of the hypothalamic–pituitary–gonadal axis dysfunction in subordinate female naked mole-rats (*Heterocephalus glaber*): No apparent role of opioids and glucocorticoids. *J Neuroendocrinol*. 2024;36(10):e13444. doi:10.1111/jne.13444