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The elusive role of prolactin in the sociality of the naked mole-rat

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ABSTRACT

Despite decades of research into the evolutionary drivers of sociality, we know relatively little about the underlying proximate mechanisms. Here we investigate the potential role of prolactin in the highly social naked mole-rat. Naked mole-rats live in large social groups but, only a small number of individuals reproduce. The remaining non-breeders are reproductively suppressed and contribute to burrow maintenance, foraging, and allo-parental care. Prolactin has well-documented links with reproductive timing and parental behaviour, and the discovery that non-breeding naked mole-rats have unusually high prolactin levels has led to the suggestion that prolactin may help maintain naked mole-rat sociality. To test this idea, we investigated whether urinary prolactin was correlated with cooperative behaviour and aggression. We then administered the prolactin-suppressing drug Cabergoline to eight female non-breeders for eight weeks and assessed the physiology and behaviour of the animals relative to controls.

Contrary to the mammalian norm, and supporting previous findings for plasma, we found non-breeders had elevated urinary prolactin concentrations that were similar to breeding females. Further, prolactin levels were higher in heavier, socially dominant non-breeders. Urinary prolactin concentrations did not explain variation in working behaviour or patterns of aggression. Furthermore, females receiving Cabergoline did not show any behavioural or hormonal (progesterone) differences, and urinary prolactin did not appear to be suppressed in individuals receiving Cabergoline. While the results add to the relatively limited literature experimentally manipulating prolactin to investigate its role in reproduction and behaviour, they fail to explain why prolactin levels are high in non-breeding naked mole-rats, or how female non-breeding phenotypes are maintained.

1. Introduction

How and why animals evolve extreme sociality has long been a major puzzle for biologists. The most extreme form of social organisation, eusociality, is characterised by non-dispersing, overlapping generations of relatives that cooperatively care for the offspring of just a few individuals (Batra, 1966; Crespi and Yanegra, 1995; Michener, 1969; Sherman et al., 1995). The evolutionary costs and benefits of sociality have been debated for decades, with the majority of researchers agreeing that increased sociality among related individuals is favoured by some variation of kin selection (Abbot et al., 2011; Andersson, 1984; Bourke, 2011; Hamilton, 1964a, 1964b; Kapheim et al., 2015; Queller, 1992; West et al., 2007). Yet in contrast to the ultimate factors, the proximate mechanisms underpinning the emergence and maintenance of increased sociality have received relatively little attention, particularly in mammals, where it is poorly understood.

Jarvis (1981) first described mammalian eusociality in the naked mole-rat. Since then, the naked mole-rat has been well-studied for a number of its extraordinary traits (Buffenstein et al., 2021). Beside its ability to survive without oxygen for up to 18 min (Park et al., 2017) and its potential contributions to research of anti-aging (Buffenstein, 2005, 2008) and cancer resistance (Keane et al., 2014; Kim et al., 2011; Tian et al., 2013), the naked mole-rat exhibits the most extreme reproductive skew of any mammal, with up to 99% never reaching reproductive maturity (Jarvis, 1991; Sherman et al., 1992). Colonies can contain up to 300 individuals, but typically only one female and up to three males are reproductively active (Braude, 2000; Faulkes et al., 1991b; Jarvis, 1981, 1991; Lacey and Sherman, 1991; O'Riain and Faulkes, 2008). Other individuals remain in a pre-pubertal, reproductively-suppressed state that can continue throughout their entire lives, even after they reach full body size (Faulkes et al., 1990a, 1991a; Faulkes et al., 1994; Faulkes and Abbott, 1991, 1993). Non-breeders perform critical functions including foraging, nest maintenance, tending offspring and colony defence (Faulkes et al., 1991b; Jarvis, 1981; Lacey and Sherman, 1991).

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To remain reproductively suppressed, non-breeders must be in physical contact with the reproductive female (Faulkes and Abbott, 1993; Smith et al., 1997), who is responsible for most intra-colony aggression (Clarke and Faulkes, 1997, 2001; Faulkes and Abbott, 1993; Reeve, 1992). Some aspect of the reproductive female's behaviour is thought to initiate a molecular cascade including the suppression of the release of gonadotrophin-releasing hormone (GnRH) that results in the suppression of reproduction in non-breeders (Faulkes and Abbott, 1993). Indeed, in female non-breeders, the inhibition of GnRH secretion suppresses the release of pituitary gonadotrophin (luteinising hormone, LH) and, by inference, follicle stimulating hormone (FSH), preventing ovulation (Faulkes et al., 1990a, b). Male non-breeders also have suppressed circulating LH, as well as having lower testosterone levels than breeders, leading to fewer and lower-quality spermatozoa (Faulkes et al., 1991a; van der Horst et al., 2011). It is not yet known how GnRH activity is regulated to inhibit reproductive suppression in the naked mole-rat. One candidate is the peptide hormone prolactin, which, along with estradiol, plays a regulatory role in normal reproductive function (Araujo-Lopes et al., 2014; Brown et al., 2014, 2019; Smith et al., 2006).

Reports from diverse vertebrates indicate that prolactin can have important roles in two traits that are also essential to eusociality: reproductive timing and inhibition (Freeman et al., 2000; Ladyman et al., 2020), and social or cooperative behaviour (birds: Angelier et al., 2016; Angelier and Chastel, 2009; Smiley, 2019, elephants: Prado et al., 2020, primates: Dixson and George, 1982; Snowdon and Ziegler, 2015; Ziegler and Snowdon, 1997, humans: Gettler et al., 2012, rodents: Donhoffner et al., 2017; Guoynes and Marler, 2020; Qin et al., 2020). In mammals, circulating prolactin levels are generally low in males and non-pregnant females, but can be many times higher in breeding females (Ladyman et al., 2020; Phillipps et al., 2020). A recent study has also shown that serum prolactin and prolactin receptor gene expression appear to be associated with reproductive status in the Damaraland mole-rat, a highly-social species that has also been classified as eusocial (Voigt and Bennett, 2018). However, the complex pleiotropic and species-specific effects of prolactin mean it is hard to make specific predictions from general patterns. Instead, prolactin levels should be described separately in each species and functional experiments done to elucidate its roles more reliably.

Bennett et al. (2018) reported that non-breeding naked mole-rats of both sexes have unusually high prolactin levels, raising the intriguing possibility that prolactin might be contributing to the behaviours that are critical in the maintenance of naked mole-rat eusociality. Medger et al. (2019) measured prolactin levels during a case study of queen succession in a colony of naked mole-rats. They found that the removal of an old queen preceded a reduction in non-breeder prolactin levels, and that the queen was succeeded by a previous non-breeder after several individuals were killed. When the new queen was established, prolactin increased, testosterone decreased, and aggression subsided. While this study contains only correlational evidence from a single colony, it supports the hypothesis that the behaviour of the queen leads to high prolactin in non-breeders and this contributes to the suppression of breeding and aggression. Evidence that elevated prolactin is responsible for stimulating cooperative behaviour and inhibiting reproduction would be a tantalising suggestion for how eusociality may have evolved in the naked mole-rat lineage, the first time a mechanism for such an evolutionary transition has been explored in mammals.

In this study, we investigated associations between urinary prolactin, cooperative behaviour, reproductive status and aggression in 155 individuals from ten colonies of captive naked mole-rats. We hypothesised that urinary prolactin would be generally higher in non-breeders than in breeders and positively correlated with cooperative behaviour, as measured by an individual's time spent digging, transporting nest material, and tending to offspring. We also hypothesised that the reproductive female would be more aggressive towards individuals with low prolactin who may be reproductive rivals. We administered a widely used prolactin-suppressing fertility treatment (Cabergoline) to eight animals and predicted that animals receiving the prolactin suppressant would a) reduce the time they spend working, b) have higher urinary progesterone concentrations as they become reproductively active, and c) receive more aggression.

2. Methods

The naked mole-rats used in this study were from ten colonies of captive animals housed at Queen Mary University of London. The animal husbandry and behavioural observation methods used in this study are described in (Gilbert et al., 2020). Briefly, the animals are descendants of progenitors caught in Kenya during the 1980s. All colonies are kept in separate networks of acrylic tunnels and boxes (range length, range area), kept in a single room maintained between 26 and 32 °C. Light and noise levels vary with human activity in the surrounding rooms and corridors. Animals are fed ad libitum every day a diet of predominantly carrot, sweet potato and butternut squash, although other fruits and vegetables are occasionally included.

2.1. Study design

2.1.1. Colony and individual selection

Individuals were observed in two sampling periods (full details in Table 1). In the first, we randomly selected 133 individuals from eight colonies. In the second, we observed 79 individuals from eight colonies, of which six colonies had been observed previously. In total, 155 individuals from ten colonies were observed in at least one observation period. The observation sequence of colonies and individuals within colonies were also generated randomly. Breeders do not regularly perform working behaviour and were excluded from analyses of working behaviour.

Table 1
Summary statistics of animals used in the study.

Colony	Total number of individuals	Number of individuals observed	Number of females observed	Proportion female in observed individuals	Age (years; mean, min, max)	Mass (g; mean, min, max)
11A	28	19	10	0.53	3.3 (0.8, 7.6)	31.3 (16.7, 57.5)
11B	24	20	11	0.55	4.8 (1.5, 8.6)	39.0 (23.5, 61.2)
11C	18	15	12	0.8	2.9 (0.6, 5.7)	31.4 (16.4, 57.4)
17A	23	19	13	0.68	1.4 (0.3, 5.2)	32.8 (12.8, 69.7)
800	10	10	9	0.9	7.9 (3, 15.4)	35.2 (18.8, 60.1)
CF05A	26	11	6	0.55	3.6 (0.9, 12.6)	39.5 (23.1, 71.7)
CF27	26	20	15	0.75	6.5 (1.6, 24.3)	31.1 (18.6, 44.2)
FK100	45	22	10	0.45	3.6 (1.2, 8.4)	29.2 (16.1, 53.2)
G	9	9	6	0.67	13.8 (13.3, 14.2)	48.6 (35.0, 60.7)
Omega	29	10	6	0.6	10.7 (4.5, 26)	36.8 (24.2, 58.4)
All Colonies	238	155	98	0.63	4.7 (0.3, 26)	34.2 (12.8, 71.7)

2.2. Experimental procedures

2.2.1. Observation protocol

To facilitate collection of urine samples, before observations began, we first emptied and cleaned each colony's toilet chamber (see below). We allowed up to 10 min for the animals to settle down from any response to our activity. Observations took place between 2019 and 2021 between 08.00 and 19.00. We recorded the behaviours we observed using BORIS (Behavioral Observation Research Interactive Software) version 7.9.7 (Friard and Gamba, 2016). Each animal was observed twice a week for ten minutes, once in the morning and once in the afternoon. Animals were weighed weekly (apart from the week after a birth within the respective colony as this can lead to death of the pups) and the mean of these body masses was used in the analyses. Each individual has a unique RFID microchip and we marked each animal on its dorsal surface weekly with a unique identifier using a black marker pen to enable rapid identification during observations.

2.3. Behavioural observations

The broad behavioural categories and specific behaviours that we recorded are described in the ethogram of naked mole-rat behaviour (Lacey et al., 1991). We considered the following to be cooperative behaviours: transport of food and nest material, digging, sweeping and offspring tending. Digging and transporting behaviours were classified together as 'working' behaviour.

Aggression was recorded based on the description of shoving in Lacey et al. (1991). We did not include other agonistic behaviours such as incisor fencing or specific vocalisations associated with resource competition as these behaviours do not reflect reproductive competition. We recorded instances of aggression when individuals approached each other face-to-face, and one or both initiated a series of sharp shoves, often forcing the other individual backwards along a tunnel. We recorded reproductive behaviour (Lacey et al., 1991) on an ad hoc basis. Reproductive behaviours include copulating, ano-genital nuzzling, pregnancies, and births.

Females were classified as breeders (n = 20 and n = 18 in Experiments 1 and 2, respectively) if they had a perforated vagina, if they were seen mating, or became pregnant during the observation period. All other females (n = 61, n = 30) were classified as non-breeders and we recorded which females developed prominent vaginal membranes that did not become fully perforated. Males were classified as breeders (n = 3, n = 1) when they were seen mating or when a female became pregnant with only one adult male present. The rest were classified as non-breeders (n = 49, n = 30). Historical records of reproductive status based on this classification were also used.

2.4. Estimating dominance rank

We established the dominance hierarchy of each colony using passing behaviour, which is used as an indicator of dominance in naked mole-rats (Clarke and Faulkes, 1997). We recorded which individuals passed over the top of other individuals during face-to-face encounters in tunnels. Interactions not thought to indicate rank include tail-to-face encounters, passing in chambers or corners of tunnels, when one individual digs throughout the encounter, and when individuals do not pass directly over the top of one another.

2.5. Urine collection and hormone concentration assays

Urine samples were collected opportunistically while observing behaviour. When an individual urinated in the colony's toilet chamber, we removed the sample with a plastic pipette, transferred it to an Eppendorf tube and cleaned the toilet chamber floor with tissue paper and water. If an individual began to urinate while being weighed, we collected the urine by placing the individual in a small plastic container until they finished urinating. Samples were immediately placed on ice before being transferred to a −80 °C freezer. Observing behaviour before hormonal analysis of urine samples was undertaken meant the observations were done blindly with respect to prolactin levels. To estimate prolactin concentrations, we assayed all of the 81 samples from the first sampling period and 40 from the second, which were randomly selected from a shortlist of observation matched samples, i.e., the behaviour preceding the sample collection was known. We estimated progesterone concentrations in 65 urine samples from animals receiving the treatment (n = 32) or controls (n = 33).

Urinary hormones are expressed in units per unit of creatinine, as creatinine is produced relatively consistently throughout the day and thus differences in urine dilution are accounted for. Creatinine concentrations were estimated using a Creatinine Microplate Assay (CR01, Oxford Biomedical Research, USA) following the manufacturer's protocol. Prolactin concentrations were estimated using an immunosorbent assay (ELISA E-EL-GP0358, Elabscience Biotechnology, USA) following the manufacturer's protocol, which has previously been validated for naked mole-rats (Bennett et al., 2018; Medger et al., 2019) and the Mahali mole-rat (*Cryptomys hottentotus mahali*) (Hart et al., 2022). The sensitivity of the kit is 0.09 ng/ml and the intra-assay coefficient of variation is <10%. Serial dilutions of neat and spiked naked mole-rat urine produced displacement curves that were parallel to the standard curve, thus validating the use of this assay for urine in naked mole-rats (Fig. SI 5, Supp File 8). Progesterone concentrations were also estimated using an ELISA immunosorbent assay (ELISA EIAP4C21, ThermoFisher Scientific, USA). The product information sheet states the kit sensitivity is 47.9 pg/ml, intra-assay coefficients of variation are 3.1–5.1%, and inter-assay coefficients of variation are 4.1–7.0%. Progesterone is elevated during the luteal phase of the ovarian cycle and pregnancy and therefore a useful marker of reproductive activation and ovarian cyclicity in naked mole-rats.

Table 2
Doses of Cabergoline that successfully reduced prolactin in previous studies.

Species	Dose (mg/kg) and number of doses	Delivery	Reference
Djungarian hamster, *Phodopus campbelli*	0.25 × 1	Subcutaneous injection	Brooks et al., 2005
Lesser rice-field rat, *Rattus losea*	0.05 × 3	Not stated	Qin et al., 2020
silver fox, *Vulpe vulpes fulva*	0.015 × 2	Oral	Lengwinat et al., 2001
Wistar-Imamichi rat	0.1 × 1	Oral	Moro et al., 2001
common marmoset, *Callithrix jacchus*	0.1 × 1	Oral	Moro et al., 1999
Dog, *Canis lupus familiaris*	0.005 × 1	Subcutaneous injection	Onclin and Verstegen, 1997
Elephant, *Loxodonta Africana*	<0.005 mg/kg twice per week for 16–46 weeks	Oral	Morfeld et al., 2014
Western lowland gorilla, *Gorilla gorilla gorilla*	0.004 mg/kg twice weekly for 6 months	Not stated	Chatfield et al., 2006

2.6. Cabergoline and vehicle control administration

Cabergoline is a long-acting dopamine agonist that inhibits prolactin in mammals (Ciccarelli et al., 1997; Ferrari et al., 1988). In this study, Cabergoline (Tocris BioScience, UK) was dissolved in a solution of ethanol (3.3%, following Anokhin et al., 2017). The volume of Cabergoline-ethanol solution required varied according to each individual's body mass so each treatment was mixed with distilled water to make up a total volume of 0.4 ml per dose. Control animals received solutions with an equivalent body mass-adjusted ratio of 3.3% ethanol and distilled water. All animals received injections of the same total

volume (0.4 ml), containing the same volume of ethanol per body mass and the same dose of Cabergoline per body mass (in the Cabergoline group).

Treatments were injected subcutaneously under the loose skin of the neck at a dose of 0.5 mg/kg body mass. Previous studies found doses as small as of 0.005 mg/kg body mass could reduce prolactin in other mammals (Onclin and Verstegen, 1997, Table 2). We felt 0.5 mg/kg body mass would be enough to achieve the scientific aims of the study without an unnecessary risk of potential side effects. Treatment and control injections were administered weekly on Mondays between 9:00 am and 12:00 pm. Observations were carried out during the rest of the week.

We planned to end the study if any of the treatment animals became reproductively active, as indicated by a fully perforated vagina, mating, or pregnancy. We would have also considered extended periods of aggression or fighting as endpoints. None of these were reached. When we administered the experimental dose (0.5 mg/kg Cabergoline) to two male naked mole-rats in a pilot study to monitor any adverse side effects, we discovered signs of fighting within two weeks. We interpreted this fighting as evidence that the treatment had lowered prolactin and increased testosterone as predicted, and the animals had increased aggression as a result. We subsequently performed a separate and larger experimental validation of the Cabergoline treatment after completing the experiment by collecting urine samples longitudinally from six individuals (four female, two male) from one colony, before and after three weekly administrations of 0.5 mg/kg of Cabergoline. These results are reported in the 'Validation' results section but are not included in the dataset looking at associations between prolactin, individual characteristics, and behaviour.

2.6.1. Experimental animals

We ran a power analysis to determine the number of animals to use in the treatment and control groups. After fighting occurred in the pilot study (see above), we hypothesised most of the treatment animals would show notable changes in behaviour and physiology. On the other hand, there is a low probability that an animal receiving control injections would become reproductively active in the eight-week treatment period. We used the *power.prop.test* function in R (R Core Team, 2014) to determine the sample sizes required to detect differences in outcomes. If 0% of the control animals and 75% of the treatment animals met the predefined end point (as a binary outcome), 5.2 individuals per condition would be needed to detect this effect. We used eight animals in each group, which we thought was an appropriate compromise between power and redundancy. Our power analysis assumed 80% power and an alpha of 0.05. To select animals for the treatment and control groups, we started with a list of animals that excluded breeders, possible breeders, animals younger than four months, lighter than 20 g and individuals of unknown age. With limited time and resources available, we decided to sample one sex in this study. We chose females because there are clearer phenotypic distinctions between breeding and non-breeding females, so the study would have a more robust set of scientific end points. We randomly selected eight pairs of animals that were of similar age and body mass and from the same colony, along with up to eight others per colony to act as controls receiving neither treatment. Experimental animals were randomly allocated to the treatment or control conditions. The researcher (JDG) who recorded behavioural and physiological data was blind to whether each experimental animal had been assigned to the treatment or control condition.

2.7. Statistical analyses

2.7.1. Rank calculations

We used the Elo Rating system to calculate individual dominance ranks within colonies (R package *EloRating* (Neumann and Kulik, 2020). The Elo Rating method has several advantages over matrix-based methods such as its ability to calculate ranks within small groups and account for the loss of individuals during observations. After calculating each individual rank, we scaled each rank to account for variation in group size by dividing by the number of individuals in the respective colony. We calculated rank hierarchies for both periods; individuals that were observed in both periods received two ranks and the relevant one was used in calculations. Lower rank values (those towards zero) represent more dominant animals.

2.7.2. Multivariate analyses

We used the R package *lme4* (Bates et al., 2015) to create general and generalised mixed-effects models with the response variable as one of urinary prolactin, urinary progesterone, working behaviour or aggression. The variables used in each model are specified in the relevant results section. We included individual and colony as categorical random effects in all models to account for repeatedly sampling each individual and colony, and we included phenotypic variables reproductive status, sex, age, body mass and rank as fixed effects where appropriate. When comparing outcomes of individuals in different treatment groups, we included the interaction between the treatment group of the focal animal (treatment, control or neither in models of behaviour, treatment or control for progesterone analysis) and whether the observation took place while treatment was being administered (coded as 1 for weeks 5–12, when the treatment was being administered, and 0 for the four weeks either side).

We used the *DHARMa* R package (Hartig, 2020) to check the residuals of logistic models for uniformity, dispersion, and the presence of outliers. For the linear models, we confirmed the residuals were normally distributed and had similar variances. Where response variable distributions were semi-continuous (i.e. left-skewed with numerous zero values), we used two-part models to assess a) which variables predict whether the response variable was zero or non-zero, and b) which variables predict variation in the continuous distribution of non-zero values (Duan et al., 1983). The set of models with working behaviour duration as a response variable did not produce normally distributed residuals using a gamma distribution (with log or identity link functions), which is appropriate given the data are positive, continuous and non-normally distributed (Zuur et al., 2009). We do not report the results from these models. Instead, we report Pearson correlation coefficients and *p*-values. We were unable to model the presence or absence of prolactin using a logistic regression because all of the samples from breeders contained prolactin (i.e. there is separation in the data, Allison, 2008). To account for the separation when modelling the presence or absence of prolactin we used Bayesian models with weak priors (R package *blme*, Dorie et al., 2021).

2.7.3. Model performance

We compared models that contained different predictor variables using the following protocol. We first constructed null models for each of the response variables including only random effects. For each response variable, we then created a set of models, each containing one or two fixed effects, and compared the performance of the models as described below. We assessed the impact of treatment group on the experimental outcomes through the interaction between treatment and time because we wanted to know whether behaviour and progesterone levels change when the treatment was being administered i.e., it is not a direct comparison between the individuals in different treatment groups.

Akaike Information Criterion (AIC) values estimate how well a model approximates an unknown reality relative to other models (Burnham et al., 2011; Burnham and Anderson, 2002); smaller AIC values indicate better models. Second-order AICs (AICcs) were generated for each model to compare the performance of different combinations of predictor variables. As well as assessments of relative model performance, we calculated the variance explained by each model. For models with numerical response variables, the conditional coefficient of determination (R package *MuMIn*, Bartoń, 2020) was used. We calculated McFadden's pseudo-R^2 (McFadden, 1973) for logistic models with

Table 3
Summary statistics for urine samples.

	All samples	Observation matched
Number of samples	118	79
Breeder	19	5
Breeding female	18	5
Breeding male	1	0
Non-breeder	99	74
Non-breeding female	44	34
Non-breeding male	55	40
Number of individuals	66	45

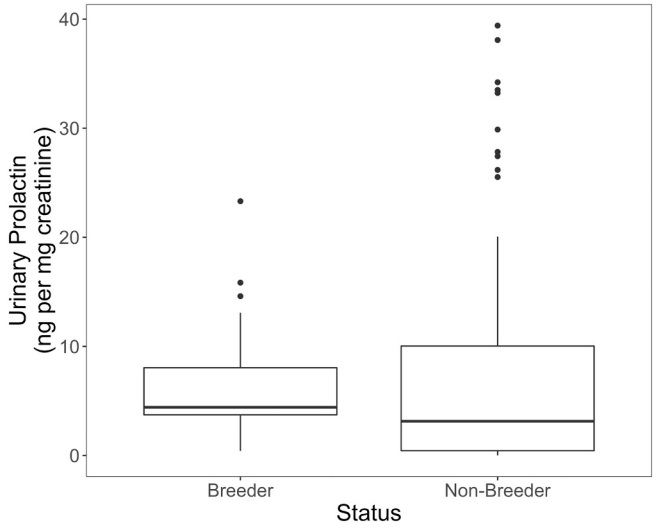

Fig. 1. Urinary prolactin levels were similar in breeders and non-breeders. Thick horizontal bars indicate medians, boxplots are 25th and 75th percentiles. Whiskers extend up to 1.5 times the interquartile range outside of the boxes.

binary outcomes apart from the Bayesian model for the presence or absence of prolactin. We do not report an estimate of explained variance for this model. In the results tables we report model coefficients and standard errors (SEs), the AICc, ΔAICc versus the null model and R^2. All statistical analyses were carried out in R Studio Version 4.1.1 (R Core Team, 2014). Figures were made using the *ggplot2* (Wickham, 2016) or *interact_plot* (Long, 2021) packages.

3. Results

3.1. General description

A total of 155 naked mole-rats were observed for between 6 and 38 (mean 21.5) ten-minute periods. We assayed 121 urine samples to estimate prolactin concentrations. Three samples were excluded because the absorbances and concentrations were outside the range of the respective standard curve, leaving 118 urine samples from 66 individuals (1–10 per individual, mean 1.8, Table 3). 99 samples were from 55 non-breeders, the remaining 19 were from 12 breeders. We wanted to test whether urinary prolactin predicted working behaviour shortly before or after the urine sample was collected. To do this, we used 74 urine samples collected during observation sessions in which the behaviour of the animal urinating was observed (referred to as observation-matched samples). Of the 74 observations that had corresponding urine samples, worker behaviour was observed in 49 (70%). The average duration of worker behaviour in these observations was 180.5 s (range 0–599 s, standard deviation 209.5 s).

Table 4
Results of linear mixed-effects models in the format:
Urinary Prolactin ~ *Colony* + *Individual* + *Fixed Effect*
where *Urinary Prolactin* is urinary prolactin concentration (continuous) and *Colony* and *Individual* are random effects. The null model contained only random effects while other models included one of sex (categorical, male or female), age (years, continuous), mass (grams, continuous) and rank (0–1, scaled, continuous) as fixed effects. β = regression coefficient. SE = standard error. Bold text indicates a *p*-value less than 0.05.

Variable	Linear regression models for prolactin by individual characteristics (82 samples from 49 non-breeders)																	
	Null			Sex			Age			Mass			Rank			Mass and rank		
	β	SE	*p*	β	SE	*p*	β	SE	*p*	β	SE	*p*	β	SE	*p*	β	SE	*p*
Intercept	2.181	0.133	**<0.001**	2.080	0.206	**<0.001**	2.327	0.203	**<0.001**	3.732	0.559	**<0.001**	1.058	0.377	**0.005**	2.648	0.723	**<0.001**
Sex (Base = F)				0.166	0.269	0.538												
Age							−0.028	0.028	0.317									
Mass										−0.050	0.017	**0.003**				−0.039	0.017	**0.023**
Rank													1.903	0.604	0.002	1.383	0.593	**0.020**
Model AICc	524.12			526.01			525.42			517.99			517.05			514.71		
AICc versus Null model	–			+1.89			+1.30			−6.13			−7.07			−9.41		
R^2	0.00			0.01			0.02			0.16			0.27			0.28		

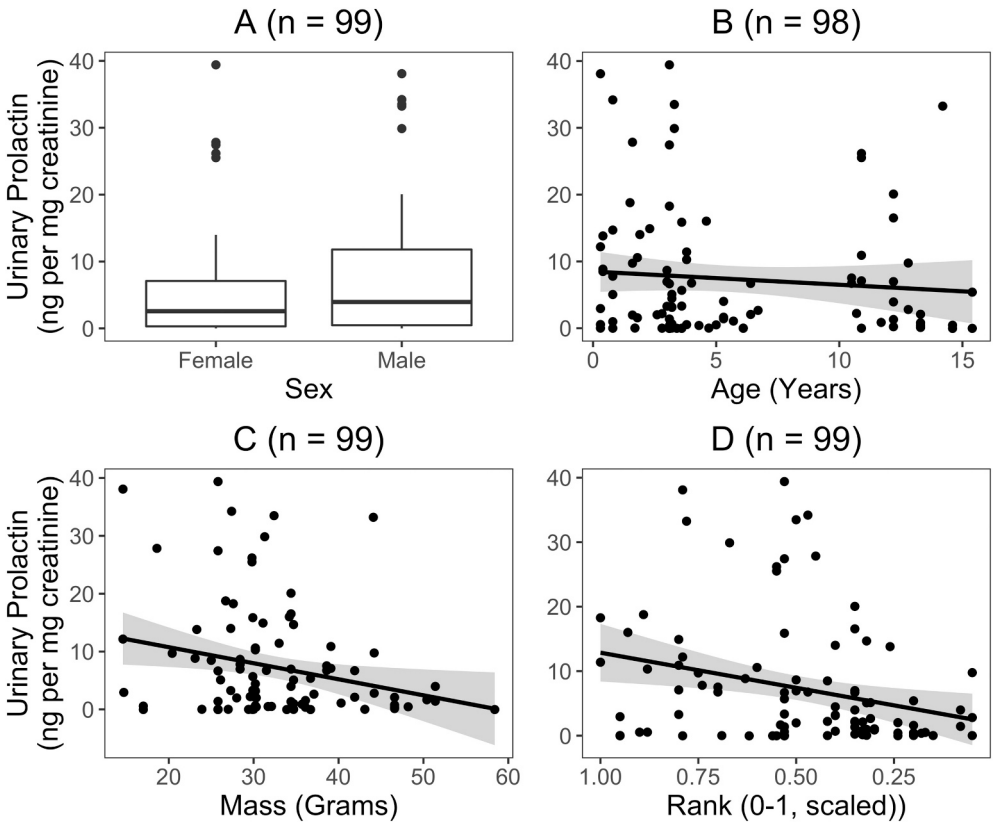

Fig. 2. Urinary prolactin did not vary with sex (A) or age (B), but was lower in heavier (C), and in high-ranking (D) non-breeders. The straight lines are general linear regressions made using *ggplot2*'s *stat_smooth* function (Wickham, 2016). Note that these are different from the models described below, which also include colony and individual as random effects. Sample sizes (n) given in brackets.

3.2. Who has the most prolactin?

We detected prolactin in 101 of 118 samples (85.6%) with a mean prolactin concentration of 7.26 ng prolactin/mg creatinine (0–39.41, standard error (SE) 0.85). The prolactin data were heavily skewed towards lower values; the median of 3.87 ng prolactin/mg creatinine was approximately half of the mean and 30% of the samples were below 1 ng prolactin/mg creatinine.

We detected prolactin in 100% of the samples from breeders and 82% of the samples from non-breeders, although the average concentration was slightly higher in the non-breeder samples (7.33 ng prolactin/mg creatinine, 0–39.41, SE 0.98) than in the samples from breeders (6.87 ng prolactin/mg creatinine, 0.42–23.3, SE 1.34; Fig. 1). Overall, urinary prolactin was similar in breeders and non-breeders. Reproductive status did not explain variation in urinary prolactin (Table SI 1).

3.3. Prolactin in non-breeders

We ran mixed-effects models to test which individual characteristics predicted the concentration of urinary prolactin. We found that sex, reproductive status, age, body mass and rank did not predict whether prolactin was detected or not, and none of the models performed well according to McFadden's pseudo-R-squared (Table SI2). Individual characteristics did not predict which individuals had detectable urinary prolactin, but models featuring mass and rank performed better than the null model when predicting the concentration of prolactin. Model outputs (Table 4) confirm the trends in Fig. 2C and D; heavier individuals and more dominant individuals had less urinary prolactin. These two variables performed better and explained considerably more variation than the null model. When included together in a single model, the AIC reduced further, indicating better performance, and both variables retained similar effect sizes.

3.4. Does prolactin predict who works or for how long?

Older, heavier individuals were less likely to be observed working (Table 5), while adding prolactin to the model did not improve the model's performance. In focal observation periods that recorded working behaviour, we found weak correlations between work duration and prolactin (positive), age and body mass (both negative) (Table 6). Individual characteristics seem to be more important for working behaviour than prolactin (Fig. 3).

3.5. Prolactin and aggression

84% of breeders were recorded initiating aggression compared with only 14% non-breeders. 58% of breeders also received aggression along with 38% of non-breeders. Aggression generally occurs between reproductive rivals, but can also be directed towards low-ranking individuals. We calculated the average prolactin concentration for each individual and looked at whether it predicted if the individual initiated or received aggression. Whether an individual initiated or received aggression at any point was not predicted by the individual's mean prolactin, mean work duration, sex, age, body mass, or rank (Table SI 3, Table SI 4, Fig. 4). Reproductively active animals were more likely to be observed initiating aggressive, but reproductive status did not affect whether an individual received aggression.

3.6. Validation

We attempted to validate the treatment by collecting 50 urine samples before and after administering the Cabergoline treatment to six

Table 5
Results of logistic mixed-effects models in the format:
Presence of working behaviour ~ Colony + Individual + Fixed Effect
where *Presence of working behaviour* is whether working behaviour was observed in the focal period (binary), and *Colony* and *Individual* are random effects. The null model contained only random effects while other models included one of sex (categorical, male or female), age (years, continuous), Mass (grams, continuous) and rank (0–1, scaled, continuous) as fixed effects. β = regression coefficient. Bold text indicates a *p*-value less than 0.05.

Variable	Logistic regression models of work by prolactin and individual characteristics (74 samples from 41 non-breeders)																	
	Null			Prolactin			Age			Mass			Rank			Age and Mass		
	β	SE	*p*	β	SE	*p*	β	SE	*p*	β	SE	*p*	β	SE	*p*	β	SE	*p*
Intercept	0.778	0.451	**0.084**	0.726	0.473	0.125	1.670	0.607	**0.006**	4.472	1.479	**0.002**	0.165	0.718	0.819	4.455	1.769	**0.010**
Sex (Base = F)				0.009	0.039	0.807												
Age							−0.177	0.073	**0.016**							−0.012	0.093	0.899
Mass										−0.111	0.041	**0.007**						
Rank													1.252	1.283	0.329	−0.113	0.061	0.065
Model AICc	98.22			100.40			94.11			91.13			99.53			91.65		
AICc versus Null model	–			+2.18			−4.11			−7.09			1.31			−6.57		
R^2	0.03			0.03			0.10			0.13			0.04			0.15		

Table 6
Pearson's correlation values between work duration and urinary prolactin, age, mass, and rank. 49 samples from 28 individuals were used in the correlations of work with prolactin, body mass and rank. One sample from one individual was excluded in the correlation with age as we do not know the exact age of that individual.

Variable	Pearson correlation with work duration	*p*-Value
Prolactin	0.16	0.28
Age	−0.16	0.28
Mass	−0.14	0.33
Rank	0.01	0.92

animals from Colony 17A. Two samples were excluded due to having creatinine concentrations that exceeded the upper limit of the standard curve leaving a total of 48 samples. Prolactin was detected in all 24 samples taken before treatment was administered and all 24 samples taken when the treatment was being administered. The means of 3.80 ng/mg creatinine before and 4.34 ng/mg creatinine during Cabergoline administration were similar (Fig. 5, Table 7) indicating that, uniquely, Cabergoline failed to suppress prolactin as expected in the naked mole-rat. During the eight-week administration of Cabergoline and the control, we found no differences on measures of working behaviour, aggression, or progesterone (Supplementary File 7).

4. Discussion

Naked mole-rats are among the most social of all vertebrates. Low dispersal rates and high reproductive skew mean siblings can remain as non-breeding helpers in their colony and cooperate for many years (Jarvis, 1981; Lacey and Sherman, 1991). The mechanisms responsible for maintaining reproductive suppression and high levels of cooperation are poorly understood, although previous studies have suggested prolactin may have an important role in naked mole-rat sociality (Bennett et al., 2018; Medger et al., 2019). In this study, prolactin did not predict variation in working behaviour or aggression. Moreover, the administration of a drug that inhibits prolactin by mimicking the effects of dopamine (a dopamine agonist) did not appear to reduce prolactin and had no detectable impact on either behaviour or reproductive function.

4.1. Prolactin and reproductive suppression

Across mammals, prolactin levels are generally low in non-pregnant, non-lactating individuals (Ladyman et al., 2020; Phillipps et al., 2020), yet we recorded prolactin in 82% of non-breeder urine samples. As reported by Bennett et al. (2018), we also found non-breeders had higher average prolactin concentrations than breeders, although the difference was small. Our results from the breeding females are consistent with the general mammalian pattern of prolactin levels with expected peaks and troughs over the reproductive cycle (Ladyman et al., 2020).

High prolactin levels are well known to interrupt reproduction and affect fertility across mammals (Freeman, 2021; Ladyman et al., 2020) and administration of prolactin can suppress gonadotrophin-releasing hormone (GnRH) levels (Calogero et al., 1996; Koike et al., 1991), suppressing reproductive function at the level of the hypothalamus. Previously it was shown that when breeders are removed, they are succeeded by large, high-ranking individuals (Clarke and Faulkes, 1997). Our finding that large, high-ranking individuals have lower urinary prolactin could help to explain how they are able to achieve rapid reproductive activation should the opportunity arise.

The levels of prolactin detected in non-breeders here and in Bennett et al. (2018) strongly suggest prolactin may be part of the mechanism that suppresses reproduction in naked mole-rats. To directly test this hypothesis, we administered a commonly used fertility drug (Cabergoline) that suppresses prolactin, predicting that when prolactin is lowered their reproduction would be “switched on” even in the presence of the

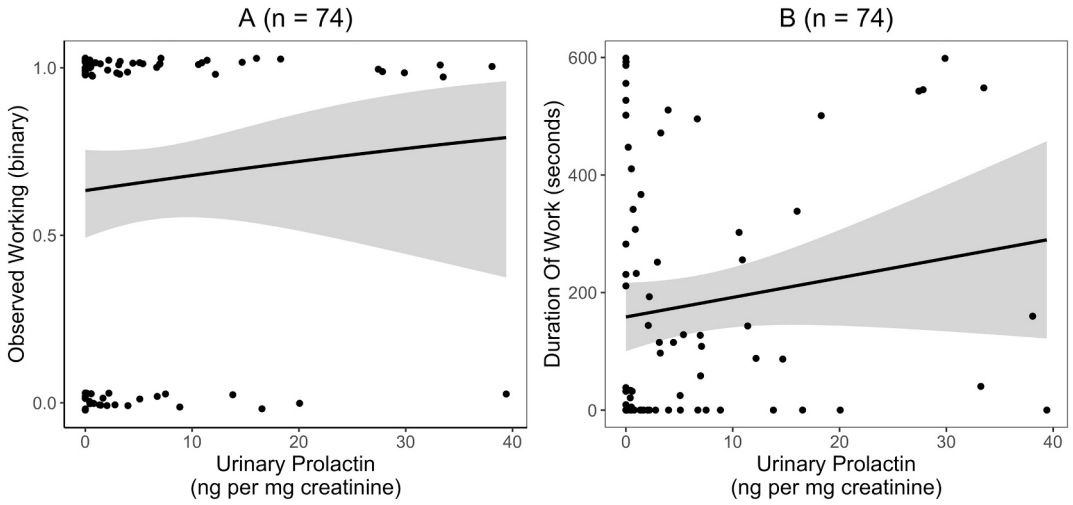

Fig. 3. Urinary prolactin did not predict whether an individual was observed working (A) or how long they worked for (B). The straight lines are general linear regressions made using *ggplot2's stat_smooth* function (Wickham, 2016). Note that these are different from the models described below, which also include colony and individual as random effects.

suppressing effects of the breeding queen. However, and unexpectedly, none of the animals that received the Cabergoline differed from control animals in their behaviour, external phenotype (prominence of genitalia) or urinary progesterone levels (as a marker of ovulation). In a subsequent validation of the treatment, animals receiving Cabergoline did not have lower urinary prolactin levels after receiving the treatment compared to before.

One explanation for the failure of Cabergoline is that the inhibition of prolactin by dopamine is not conserved in naked mole-rats, or more likely that the D2 dopamine receptor that Cabergoline interacts with does not function in the normal mammalian way. Cabergoline and the similarly structured Bromocriptine are ergot-derived alkaloids that have previously been used as a research tool to reduce prolactin in hyperprolactinaemic individuals of many mammalian and avian species, as well as a fertility drug in humans. Since being developed to treat hyperprolactinemia in humans (Ciccarelli et al., 1997; Ferrari et al., 1988), Cabergoline has also been shown to suppress prolactin in Djungarian hamsters (Brooks et al., 2005), lesser rice-field rats (Qin et al., 2020), silver foxes (Lengwinat et al., 2001), Wistar-Imamichi rats (Moro et al., 2001), common marmosets (Moro et al., 1999), dogs (Onclin and Verstegen, 1997), Western lowland gorillas (Chatfield et al., 2006) and elephants (Morfeld et al., 2014). The failure of Cabergoline to produce its normal pharmacological effect in naked mole-rats is unexpected and, as far as we know, unprecedented. Future research should focus on the neuroanatomical basis of the relationship between dopamine and prolactin in naked mole-rats, together with the molecular biology of the D2 dopamine receptors that are normally stimulated by the dopamine agonists Bromocriptine and Cabergoline (Kvernmo et al., 2006), in particular those in the lactotrophic pituitary cells where dopamine inhibits the expression of the gene responsible for producing prolactin (Fitzgerald and Dinan, 2008; Freeman et al., 2000). It is possible that D2 receptors are down-regulated in the lactotroph cells in naked mole-rats, which would explain both why prolactin levels are high in non-breeders and why Cabergoline did not suppress prolactin in this study. Another possibility that we have ruled out is that amino acid variation in the D2 receptor protein prevents the normal binding of Cabergoline to the relevant active sites – inspection of publicly available sequence data reveals that all such sites are highly conserved across mammals including NMRs and Damaraland mole-rats (CG Faulkes, unpublished observations).

The application of RFamide-related peptide-3 can prevent reproductive activation in naked mole-rats (Peragine et al., 2017), possibly mediating the stress-based glucocorticoid pathways that sometimes contributes to reproductive suppression in mammals (Freeman, 2021). Future research could administer exogenous prolactin, which might also inhibit reproductive function in animals separated from the suppressive effects of a dominant female. Full characterisation of this network will require monitoring prolactin levels through reproductive activation, investigating the distribution and activity of prolactin and RFRP-3 in more detail.

4.2. *Prolactin and cooperative behaviour*

Another critical feature of cooperative group-living is the ability to work together effectively to maximise collective output. Naked mole-rats are interesting in this regard because work is unevenly distributed among non-breeders (Gilbert et al., 2020; Jarvis, 1981; Lacey and Sherman, 1991; Mooney et al., 2015) and they exhibit an unusual decoupling of age, growth and size (O'Riain and Jarvis, 1998). The causes of variation in cooperative behaviour are still unknown (Gilbert et al., 2020; Mooney et al., 2015). Prolactin has been hypothesised as a predictor of prosocial behaviour in naked mole-rats because of evidence from other species (Bennett et al., 2018; Rosenbaum and Gettler, 2018b); it has been reported in association with many parental traits in other vertebrate species (Freeman et al., 2000; Guoynes and Marler, 2020) and has even been referred to as "the hormone of paternity" (Schradin and Anzenberger, 1999, p. 199).

A considerable amount of research has looked for associations between prolactin and prosocial behaviour, producing a large set of mixed results. In birds, correlations between prolactin and offspring care have been seen in non-breeding helpers in some cooperatively breeding birds (Schoech et al., 1996; Vleck et al., 1991), but not others (Khan et al., 2001). Studies looking at correlations between prolactin and chick survival have also produced mixed findings (Cottin et al., 2014; Ouyang et al., 2011; Thierry et al., 2013; Wang et al., 2020). Work in zebra finches found that suppressing prolactin inhibits parental behaviour, but increasing prolactin did not increase combined (female plus male) parental effort (Smiley and Adkins-Regan, 2018a, 2018b). On the other hand, Buntin et al. (1991) found that administering prolactin to non-breeding ring doves stimulated alloparental food regurgitations.

Results from mammalian studies have been just as equivocal. Some studies suggest prolactin increases in response to parental status (Ziegler et al., 1996) or parental care (Mota et al., 2006; Roberts et al., 2001a, b). Carlson et al. (2006) found higher prolactin levels in meerkats that proceeded to babysit rather than forage, while Roberts et al. (2001a, b) found suppressing prolactin in common marmosets made them less

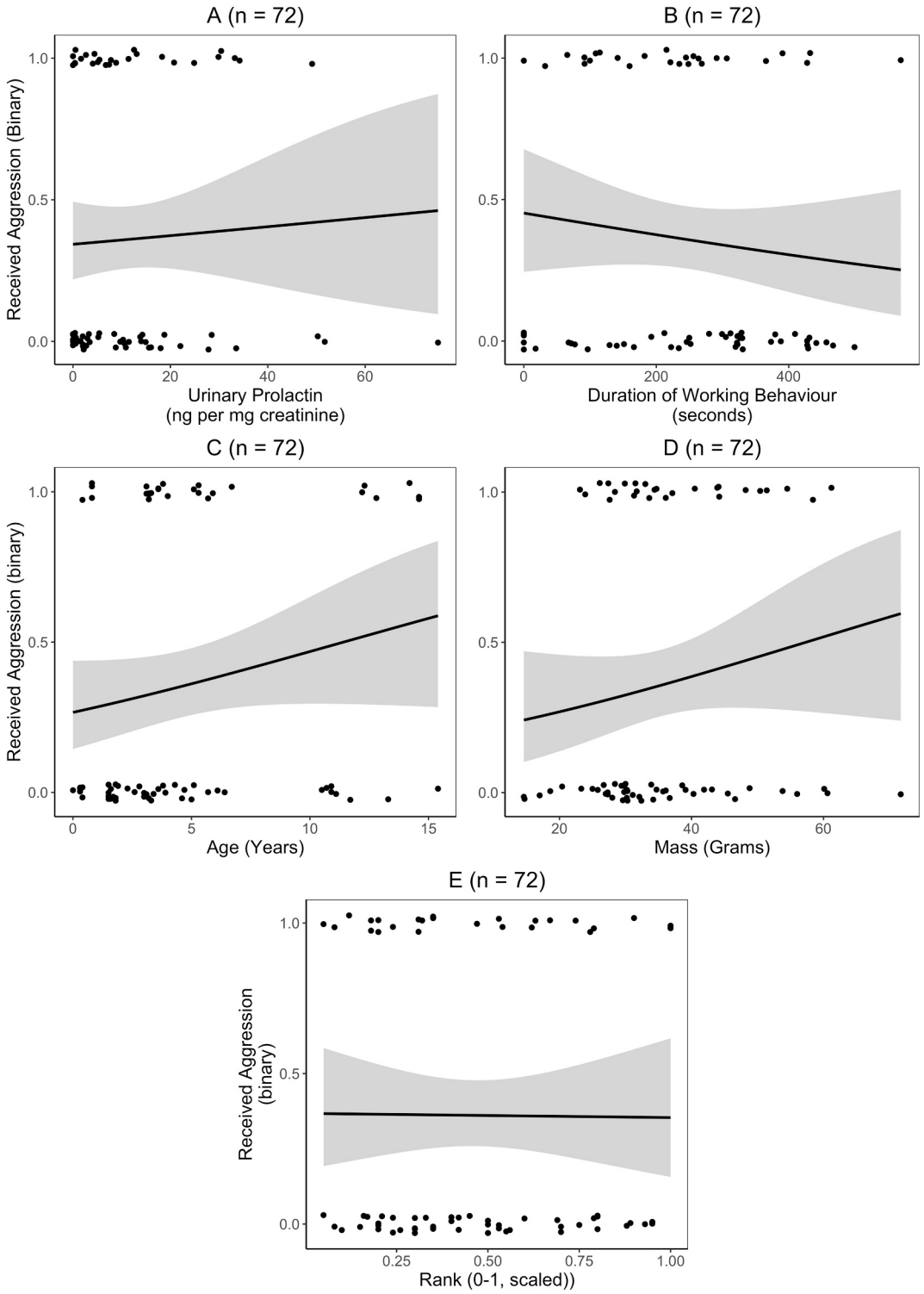

Fig. 4. Whether an individual received aggression was not associated with urinary prolactin (A), duration of working behaviour (B), or rank (E). Older (C), heavier (D) animals were more likely to receive aggression, but the relationships were weak. Aggression is whether the individual received aggression at any point during observations (i.e. is binary). Prolactin and working behaviour are the means for each individual. Lines are logistic regressions made with R's *stat_smooth* function. Points are "jittered" using *geom_point* to display overlapping points.

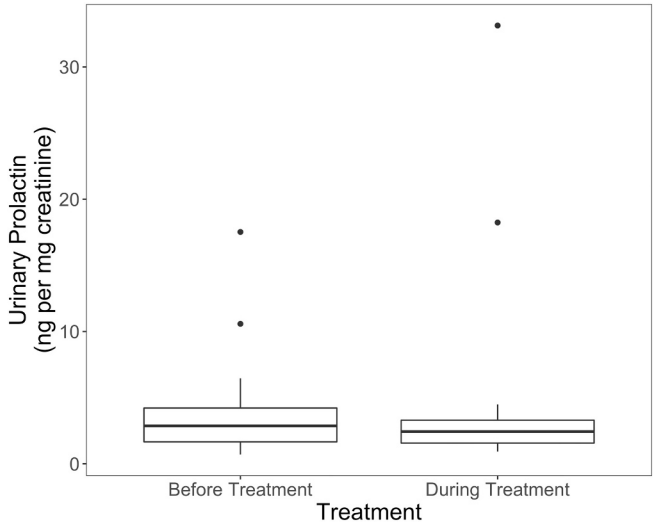

Fig. 5. Urinary prolactin was similar before and after the administration of weekly Cabergoline doses (0.50 mg/kg body mass). *Thick horizontal bars indicate medians, boxplots are 25th and 75th percentiles*. Whiskers extend up to 1.5 times the interquartile range outside of the boxes.

Table 7
Results of mixed-effects models in the format:
Urinary Prolactin ~ *Individual* + *Fixed effect*
where *Urinary Prolactin* is the natural logarithm of the concentration of urinary prolactin and *Individual* is a random effect. The null model contained just the random effect and other models included *Cabergoline Treatment* as a categorical fixed effect with two levels. β = regression coefficient. SE = standard error. Bold text indicates a *p*-value less than 0.05.

Variable	Linear model of prolactin by Cabergoline Treatment (48 samples from 6 individuals)					
	Null (intercept only)			Cabergoline Treatment		
	β	SE	*p*	β	SE	*p*
Intercept	0.311	0.102	**0.002**	0.295	0.120	**0.014**
Cabergoline Treatment				0.031	0.121	0.798
Model AICc	93.86			96.18		
AICc versus Null model	–			+2.32		
R^2	0.06			0.06		

likely or slower to retrieve infants; strong support for a causal role of prolactin in parental behaviour. Other studies have produced results that do not support this hypothesis. For example, Ziegler et al. (2009) and Almond et al. (2006) found no evidence that suppressing prolactin reduces parental care in common marmosets, and Brooks et al. (2005) also found no effect of prolactin suppression on paternal care in Campbell's dwarf hamsters. This mixed and complicated set of results highlights the need to document species-specific patterns before we can look for commonalities across taxa (Bales and Saltzman, 2016; Hashemian et al., 2016; Storey and Ziegler, 2016).

Intriguing research in captive elephants indicates that hyperprolactinemia caused by social stress often leads to anovulation (Brown et al., 2016; Prado et al., 2019; Prado-Oviedo et al., 2013). Interestingly, estimates of the elephants' temperaments suggest that those with high prolactin appear to be more "caring" (Prado et al., 2020). We hypothesised something similar; social stress elevates prolactin, which suppresses reproduction and stimulates prosocial behaviour. However, we found no evidence that prolactin is associated with working behaviour in naked mole-rats. Urinary prolactin did not explain whether an individual was observed working, or the amount of time the individual spent working. There were also no differences in working behaviour between animals receiving Cabergoline and animals receiving a control.

4.3. Prolactin and aggression

Aggression is an important behaviour in naked mole-rat colonies; it is thought to be responsible for a significant proportion of deaths (Delaney et al., 2013, 2021) and is likely to form part of the mechanism maintaining reproductive suppression in non-breeders. Reproductive females are responsible for the majority of aggression within naked mole-rat colonies and aggression is often targeted towards particular individuals (Clarke and Faulkes, 1997, 2001; Margulis et al., 1995). Reeve (1992) argued that aggression would be targeted at two groups of individuals: reproductive rivals and "lazy" workers. Previously, research has failed to find evidence that aggression is targeted based on working behaviour (Jacobs and Jarvis, 1996), but some data suggest it is generally aimed at those most likely to escape reproductive suppression (Clarke and Faulkes, 2001; Margulis et al., 1995) — aggression is often used to suppress reproduction in other taxa (Kapheim et al., 2015; Saltzman et al., 2008).

In this study, we found no evidence that aggression was targeted based on the recipient's age, body mass, rank, mean prolactin or mean work duration. Dominant female meerkats are aggressive towards females that are most likely to provide reproductive competition (Young et al., 2006) and it is possible that the reproductive female targets her rivals using a component of reproductive status that we did not assess. For example, they may target individuals with rising progesterone (females) or testosterone (males) as these are the first signs that an individual is becoming reproductively active (Faykoo-Martinez et al., 2021; Margulis et al., 1995). Aggression may also be used in response to the behaviour of senior non-breeders, who might adjust their behaviour in anticipation of a change in reproductive status, as has been shown in a cooperatively breeding fish (Zöttl et al., 2013).

Medger et al. (2019) found plasma prolactin was associated with colony stability in naked mole-rats, with higher levels of aggression coinciding with reduced prolactin. The absence of evidence that urinary prolactin predicts individual working behaviour or patterns of aggression only highlights our poor understanding of the role of prolactin in this species. Social behaviour is controlled by a network of hormones and neurotransmitters (Holmes and Goldman, 2021; Vullioud et al., 2021), which will all likely contribute to naked mole-rat behaviour. For now, prolactin's role within this system remains unexplained.

5. Conclusions

Increasing social complexity gives organisms the ability to exploit new niches and life histories (Maynard Smith and Szathmáry, 1999). While the evolutionary forces that favour cooperative breeding and increased sociality have received plenty of attention (Emlen, 1982; Kokko and Jennions, 2008; Lukas and Clutton-Brock, 2012; Rosenbaum and Gettler, 2018a; West et al., 2011), proximate mechanisms are poorly understood. Here, we confirm that naked mole-rat prolactin levels are comparable in breeders and non-breeders, but we found no evidence that prolactin underpins cooperative working behaviour in naked mole-rats. We also report that the dopamine-agonist Cabergoline failed to reduce prolactin in a validation study, a result that creates more questions than it answers. Further research is needed to identify the molecular pathways responsible for maintaining sociality in the naked mole-rat.

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Research data

All raw data and scripts are available in the supplementary data, including raw behavioural data (Supp 1, Supp 2), data relating to individuals used in the study (Supp 3), results of hormone assays (Supp 4), and scripts used to analyse the data (Supp 5, Supp 6). The supplementary files are available at doi:10.17632/rfnjyx9htg.1.

Ethical approval

The research was carried out in accordance with institutional guidelines, Home Office Project Licence P0A56C73B and Animal Ethics Committee University of Pretoria reference NAS252/2021.

Declaration of competing interest

The authors declare no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yhbeh.2022.105196.

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