Evidence for contrasting roles for prolactin in eusocial naked mole-rats, *Heterocephalus glaber* and Damaraland mole-rats, *Fukomys damarensis*

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Supplementary Material

Methods

Animal housing

Animals were briefly housed in their natal colonies in large plastic crates (1m x 0.5m x 0.5m) prior to sampling in the case of DMRs collected from Winton, before being taken to the University of Pretoria (UP). DMRs from the Kalahari Research Centre (KRC) and NMRs at UP and Queen Mary University of London (QMUL) were kept long-term in interconnecting tubing with communal nest, food and toilet chambers - see Tables S1 and S2. Wood shavings, sand and paper towelling served as nesting material. The room temperature ranged between 20 and 28ºC (DMRs) and 29 and 30ºC (NMRs) with relative humidity around 50-60% (Bennett & Jarvis, 1988; Faulkes *et al*., 1990, Zöttl *et al*., 2016). Animal rooms in Pretoria were maintained on a 12L:12D photoperiod, while the KRC and QMUL colonies were kept in the dark, with lights-on while daily husbandry and sampling was conducted. Photoperiod has not been shown to affect DMR and NMR behaviour (C.G. Faulkes and N.C. Bennett, unpublished data; Oosthuizen *et al*., 2003). Potential effects of the differing photoperiod regimes were tested for in this study, and ruled it out as a significant variable (see results below; F= 0.560; d.f.= 1, 124; p= 0.4558). Animals were fed on a variety of chopped vegetables and drank no free water. For both species, reproductive females were identified by the presence of prominent axillary and inguinal teats, well developed external genitalia with a perforate vagina, and/or pregnancy-related changes in girth/body size. Reproductive males were identified on the basis of observations of copulation with the reproductive female (NMRs), while in DMRs a dark stain around the periphery of the mouth and bulging testes which project from abdominal pockets discern dominant breeders. At the KRC records of dyadic behavioural interactions were used to identify socially dominant males.

Blood sampling

For NMRs, blood samples were collected between 08h00 and 15h00 as follows: The animals were hand held and venous blood samples collected from the hind foot. Approximately 300-500ul of blood was collected into heparinised micro-haematocrit tubes (University of Pretoria samples) or into a heparinised 1 ml syringe from trunk blood following euthanasia (QMUL) prior to tissue collection for other studies. Similarly, in DMRs, blood was either collected from the foot or trunk blood. All blood samples collected at the University of Pretoria and the KRC were obtained with University of Pretoria ethics committee clearance and at QMUL followed institutional guidelines.

Prolactin assay and validation

In brief, 100 μl of reference standard and diluted samples (1/2 to 1/50 in sample diluent) were transferred into coated wells of a 96-well micro-ELISA plate, respectively, and incubated for 90 min at 37°C. Subsequently, all supernatant was removed, and the plate patted dry. Immediately, 100 μl of biotinylated detection antibody was added, and incubated for 60 min at 37°C. The plate was washed 3 times, patted dry, and 100 μl of horse radish peroxide (HRP) conjugate were added and incubated for 30 min at 37°C. Subsequently, the plate was washed 5 times with wash-buffer, and patted dry. 90 μl of substrate reagent were added, and incubated for 15 min at 37°C. To terminate the enzymatic reaction, 50 μl of stop solution were added. Optical density was determined at 450 nm and results calculated using a best fit curve. The sensitivity of the assay was 0.1 ng/ml, the detection range 0.16-10 ng/ml, and coefficient of variation for repeatability was < 10%. Serial dilutions of spiked plasma pools for DMR and NMR gave displacement curves that were parallel to the respective standard curve of the PRL assay, validating the assay for use with both species (relative variation (%) of the slope of respective trend lines <2%; figure S1).

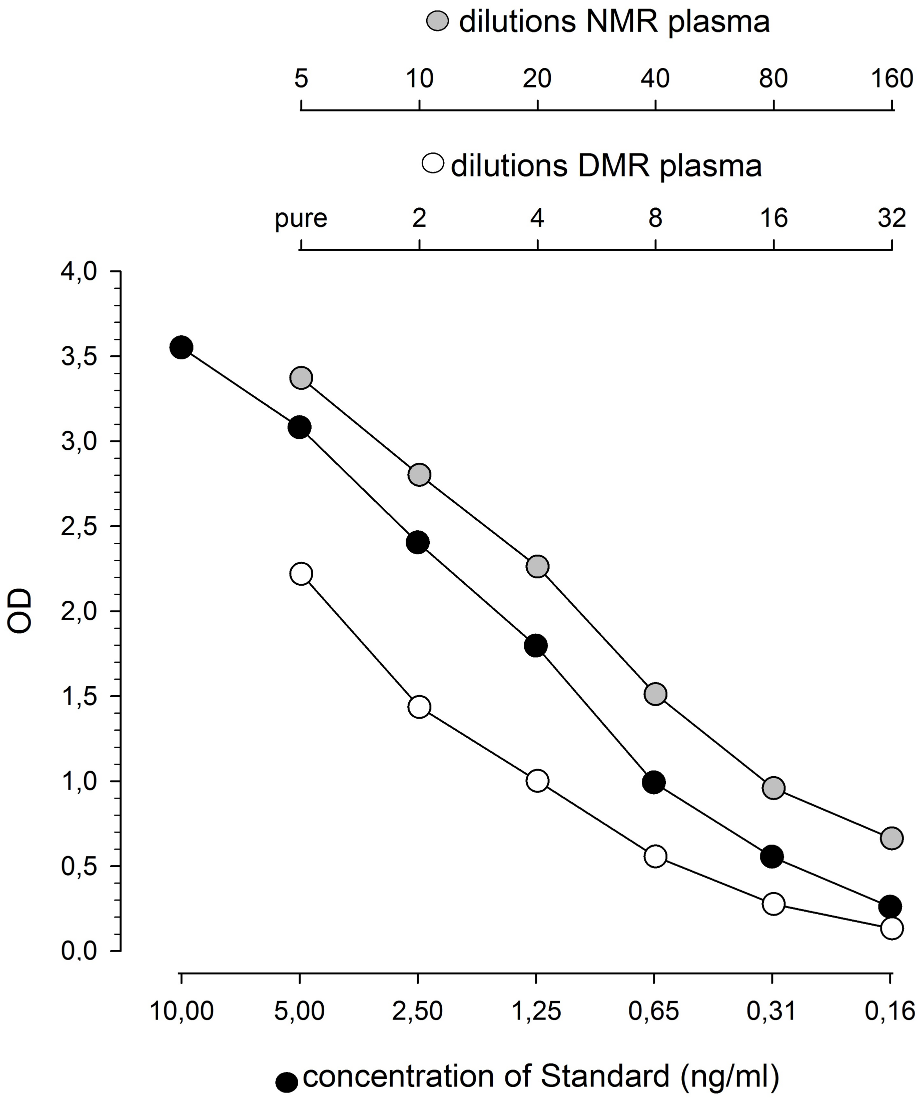

Figure S1: Assay validation revealed by parallelism of serial dilutions of spiked plasma pools for DMR and NMR, with standard for the PRL ELISA assay. Relative variation (%) of the slope of respective trendlines were <2% for DMR and NMR plasma, respectively. OD is the optical density measures the ELISA colour change, which is proportional to the concentration of PRL.

Because this is the first time PRL has been measured in mole-rats, we do not know if measures of hyperprolactinaemia in humans correspond to measures of hyperprolactinaemia in mole rats. We have chosen to include the clinical values in humans as a relevant mammalian benchmark. Normal levels of PRL are by and large similar in other mammals, and less than the benchmark values for hyperprolactinaemia in humans of 25 ng/ml that we use for context. This includes rodents, e.g. rats < 10 ng/ml (controls in Fig 3B in Egli *et al.* 2010), Chinese hamsters < 10 ng/ml, Syrian hamsters < 14 ng/ml (Parkening *et al.*, 1980) and meerkats <12 ng/ml (Carlson *et al.* 2006).

Statistical analysis

Because of the large number of animals in which prolactin levels were undetectable, and the resulting zeroes in the dataset, we used a Tweedie distribution (Tweedie, 1984) to describe the response distribution within a generalised linear model framework with prolactin levels as the response and species, sex and reproductive status (breeding or non-breeding) as predictors, with all interactions included. In separate analyses, PRL concentrations were compared in lactating and non-lactating queens for both species, and within DMRs for the effects of 12L:12D cycle versus dark/intermittent light (see methods above). The model was implemented using the R packages statmod (Giner and Smith, 2016) and tweedie (Dunn and Smyth, 2008).

Results

Summary of anova statistics from between species GLM, with prolactin levels as the response and species, sex and reproductive status (breeding or non-breeding) as predictors (spec, species; d.f., degrees of freedom):

	d.f.	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
spec	**1**	**56799**	**56799**	**50.325**	**1.51e-11 *****
sex	**1**	**139**	**139**	**0.123**	**0.726**
status	**1**	**52**	**52**	**0.046**	**0.831**
spec:sex	**1**	**2**	**2**	**0.001**	**0.970**
spec:status	**1**	**762**	**762**	**0.675**	**0.412**
sex:status	**1**	**965**	**965**	**0.855**	**0.356**
spec:sex:status	**1**	**417**	**417**	**0.370**	**0.544**
Residuals	**236**	**266362**	**1129**		

Summary of anova statistics from GLM, with prolactin levels as the response and lactating/non-lactating queens as predictors.

	d.f.	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
queens$spec	**1**	**6290**	**6290**	**12.83**	**0.00112 ****
queens$lactating	**1**	**5501**	**5501**	**11.22**	**0.00209 ****
queens$spec:queens$lactating	**1**	**3595**	**3595**	**7.33**	**0.01079 ***
Residuals	**32**	**15692**	**490**		

Summary of anova statistics from within DMR GLM, with prolactin levels as the response and Light:Dark cycle (cycle), sex and reproductive status (breeding or non-breeding) as predictors:

	d.f.	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
cycle	**1**	**16**	**16.24**	**0.560**	**0.4558**
sex	**1**	**94**	**93.93**	**3.238**	**0.0744**
status	**1**	**90**	**90.28**	**3.112**	**0.0802**
cycle:sex	**1**	**65**	**64.88**	**2.236**	**0.1373**
cycle:status	**1**	**97**	**97.11**	**3.347**	**0.0697**
sex:status	**1**	**131**	**131.43**	**4.530**	**0.0353 ***
cycle:sex:status	**1**	**40**	**39.63**	**1.366**	**0.2447**
Residuals	**124**	**3598**	**29.01**		

Signif. codes: ‘*’ 0.001 ‘**’ 0.01 ‘*’ 0.05**

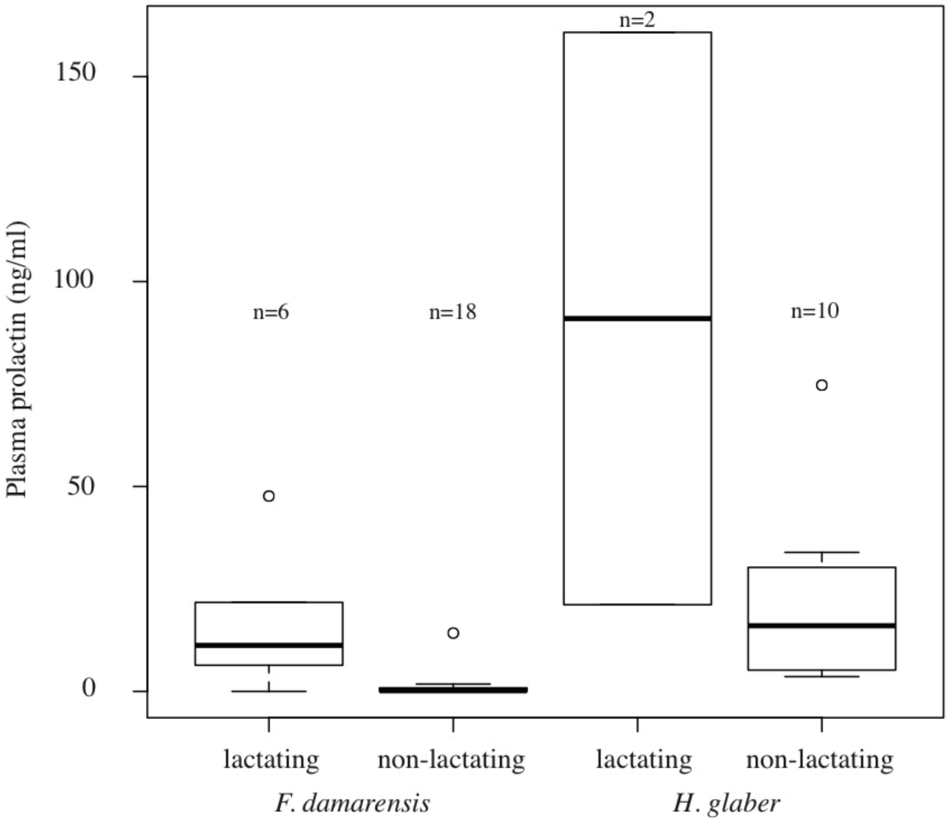

Figure S2: Plasma prolactin (PRL) concentrations for lactating and non-lactating Damaraland and naked mole-rats respectively. The box-plot midline is the median, with the upper and lower limits of the box being the third and first quartile (75th and 25th percentile) respectively. Whiskers extend up to 1.5 times the interquartile range from the top or bottom of the box to the furthest data point within that distance (beyond that distance, they are represented individually as points/'outliers').

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Supplementary Table S1 Sample details and respective prolactin values (ng/mg plasma) for naked mole-rats. NBF: non-breeding females, NBM: non-breeding males, BM: breeding males.

Animal/ sample No.	*Sex/status*	*Body mass (g)*	*Prolactin (ng/ml)*	*Colony*	*Notes*
1	NBM	45	19.50	1	
2	NBM	49	10.62	1	
3	NBF	72	19.34	1	
4	NBF	60	17.50	1	
5	NBF	40	6.50	1	
6	NBM	54	8.36	1	
7	NBF	53	11.78	1	
8	NBF	67	121.00	1	
9	NBF	40	93.56	1	
10	NBF	34	19.24	1	
11	NBM	55	38.30	1	
12	NBM	43	22.32	1	
13	NBM	40	47.09	1	
14	NBM	53	12.68	1	
15	NBF	47	88.26	1	
16	NBM	52	9.42	1	
17	NBM	38	330.30	1	
18	NBM	38	176.10	1	
19	NBM	37	209.50	1	
20	NBF	75	173.60	1	
21	NBF	63	163.10	1	
22	NBF	67	44.10	1	
23	NBM	66	20.48	1	
24	NBM	59	28.62	1	
25	NBM	46	32.34	1	
26	NBF	41	46.72	1	
27	NBF	44	39.72	1	
28	NBF	36	39.34	1	
29	NBF	49	32.72	1	
30	NBM	40	29.06	1	
31	NBF	44	11.44	2	
32	NBF	57	31.48	2	
33	Queen	73	9.02	2	44/72 days pregnant
34	NBM	56	4.74	2	
35	NBF	45	13.70	2	
36	NBF	46	7.06	2	
37	NBF	43	12.74	2	
38	NBF	47	6.02	2	
39	NBM	52	79.54	2	
40	NBM	49	5.96	2	
41	NBM	46	13.07	2	
42	NBF	40	9.46	2	

Animal/ sample No.	*Sex/status*	*Body mass (g)*	*Prolactin (ng/ml)*	*Colony*	*Notes*
1	Queen	55	21.14	3	23 days post-partum (lactating)
2	NBF	76.86	44.2	3	
3	BM	65.31	7.24	3	
4	NBF	73.3	31.36	3	
5	NBF	70.9	10.4	3	
6	NBF	49.1	21.64	3	
7	NBM	62.3	7.36	3	
8	NBM	45.75	40.68	3	
9	NBF	45.5	0	3	
10	NBM	43.3	0	3	
11	NBM	46.4	6.48	3	
12	NBF	43.3	25.12	3	
13	NBM	36.1	87.04	3	
14	NBF	36.9	50.78	3	
15	NBF	41	0	3	
16	NBM	36.3	4.34	3	
17	NBM	36	6.94	3	
18	NBM	35.6	5.84	3	
19	NBF	37.1	0	3	
20	NBM	37.4	0	3	
21	NBM	40.9	0	3	
22	NBM	28.9	0	3	
23	NBM	35.3	4.04	3	
24	NBF	35.2	0	3	
25	NBM	32.7	5.6	3	
26	NBM	28.2	0	3	
1	BM	64	6.44	3a	
2	Queen	60	5.2	3a	45/72 days pregnant
3	NBM	55.2	6.04	3a	
4	NBF	37	9.36	3a	
5	NBM	58	15.62	3a	
6	NBM	49	9.3	3a	
7	NBF	37	8.54	3a	
8	NBM	43	0	3a	
9	NBF	37	18.76	3a	
10	NBF	45	7.36	3a	
11	NBF	33	5.26	3a	
12	NBM	42	18.34	3a	
1	BM	65.9	47.92	3a	
2	Queen	55.2	160.8	3a	lactating
3	NBM	64.7	16.14	3a	
4	NBF	51	27.58	3a	

Animal/ sample No.	*Sex/status*	*Body mass (g)*	*Prolactin (ng/ml)*	*Colony*	*Notes*
5	NBM	44.4	25.5	3a	
6	NBM	52.2	61.84	3a	
7	NBF	40.3	68.58	3a	
8	NBF	38.1	16.14	3a	
9	NBM	52.4	26.2	3a	
10	NBM	52.2	21.4	3a	
11	NBF	38.1	6.52	3a	
12	NBM	37.3	53.28	3a	
13	NBM	40.6	10.36	3a	
14	NBF	40.3	9.52	3a	
15	NBM	41.4	8.98	3a	
16	NBM	39.3	7.78	3a	
17	NBM	38.2	15.12	3a	
18	NBM	30.2	10.4	3a	
19	NBM	29.3	45.12	3a	
5.01	Queen	61.7	23.02	5	pregnant
5.02	BM	48.9	29.1	5	
628	Queen	45.5	4.66	11a	Not pregnant
827	Queen	57.2	6.72	Omega	Not pregnant
527	Queen	44.8	30.26	11c	Not pregnant
881	Queen	57.5	33.92	B	Not pregnant
297	Queen	59.1	23.20	G	Not pregnant
52	Queen	52	74.72	11b	Not pregnant
555	BM	36.1	9.74	11b	
578	BM	46.6	3.92	Omega	
107	Queen	32	3.60	17a	Not pregnant
118	BM	31	7.04	17a	
33	NBM	56	90.94	B	

Supplementary Table S2 Sample details and respective prolactin values (ng/mg plasma) for Damaraland mole-rats. NBF: non-breeding females, NBM: non-breeding males, BM: breeding males. Colony names in green text signify animals housed on a 12L:12D photoperiod, while those in blue were from the KRC where colonies were kept in the dark, with lights-on while daily husbandry and sampling was conducted.

Animal/ sample No.	***Sex/status***	***Body mass (g)***	***Prolactin (ng/ml)***	***Colony***	***Notes***
1.1	Queen	119	***0.856***	Winton1	non pregnant
1.2	BM	194	0.389	Winton1	
1.3	NBF	59	0	Winton1	
2.1	Queen	116	0	Winton2	non pregnant
2.2	NBF	84	0.878	Winton2	
2.4	BM	133	1.188	Winton2	
2.5	NBF	102	0	Winton2	
2.6	NBF	120	0	Winton2	
2.7	NBM	106	0	Winton2	
3.1	BM	143	0	Winton3	
3.2	Queen	95	0.569	Winton3	non pregnant
3.3	NBF	80	0	Winton3	
3.4	NBM	88	0.59	Winton3	
5.1	NBM	98	0	Winton5	
5.2	NBM	70	0	Winton5	
5.3	BM	190	0	Winton5	
5.4	Queen	136	0	Winton5	non pregnant
5.5	NBF	65	0	Winton5	
6.1	Queen	129	0	Winton6	non pregnant
6.2	BM	149	0	Winton6	
7.1	BM	186	0	Winton7	
7.2	Queen	145	0.483	Winton7	non pregnant
8.1	NBF	120	0	Winton8	
8.2	NBM	128	0.474	Winton8	
8.3	NBM	90	0	Winton8	
8.4	BM	154	0	Winton8	
8.5	BM	188	0	Winton8	
8.6	NBF	81	0	Winton8	
8.7	Queen	112	0.332	Winton8	non pregnant
8.8	NBM	118	0	Winton8	
9.1	NBM	138	0	Winton9	
9.2	NBF		0	Winton9	
9.3	NBM	81	0.336	Winton9	
9.4	NBF	119	0	Winton9	
9.5	NBM	61	0	Winton9	
9.6	NBM	47	0.378	Winton9	
9.7	NBM	144	0	Winton9	
9.8	BM	172	0	Winton9	
9.9	NBM	80	0.396	Winton9	
9.11	NBF	84	0	Winton9	

Animal/ sample No.	***Sex/status***	***Body mass (g)***	***Prolactin (ng/ml)***	***Colony***	***Notes***
9.12	Queen	126	0	Winton9	non pregnant
9.13	NBM	106	0	Winton9	
11.1	NBM	96	0	Winton11	
11.2	NBF	47	0	Winton11	
11.3	BM	169	0	Winton11	
11.4	NBM	155	0	Winton11	
11.5	Queen	121	1.43	Winton11	non pregnant
11.6	NBM	67	0	Winton11	
11.7	NBF	114	0	Winton11	
11.8	NBM	106	0	Winton11	
11.9	NBM	118	0.512	Winton11	
11.10	NBM	77	0	Winton11	
11.11	NBF	74	0	Winton11	
11.12	NBM	70	0	Winton11	
11.13	NBF	49	0	Winton11	
5	Queen	130	8.55	Tswalu1	5 days lactating
8	Queen	142	21.71	Tswalu2	lactating
9	Queen	138	13.89	Tswalu3	9 days lactating
10	Queen	119	6.375	Tswalu4	lactating
15	Queen	157	47.65	Tswalu5	7 days lactating
G3F009	Queen	170	14.23	Crick	No Lact
NOM003	NBM	85	0	Nowak	
NOM004	NBM	91	0	Nowak	
NOF002	NBF	82	0	Nowak	
G13F012	NBF	74	0	G13	
G5F004	Queen	90	0	Grunenthal	No Lact
JA5M002	BM	91	0	Grunenthal	
SAF001	NBF	93	23.875	Sappolsky	
SAM002	NBM	132	0	Sapplosky	
SAF004	NBF	53	0	Sappolsky	
L6M009	NBM	142	0	L6	
JRM001	NBM	154	0	Jarvis	
L7F007	Queen	163	0	Aristotle	early pregnant
G10M017	BM	173	0	Aristotle	
L7F009	NBF	135	0	Aristotle	
JA1M001	BM	140	0	Lorenz	
G1F002	Queen	126	0.835	Lorenz	non pregnant?
G1F012	Queen	151	0	Burda	non pregnant?
G7M008	BM	160	0	Burda	
KR7F010	Queen	119	1.785	Hermes	non pregnant?
BO50M005	BM	116	0	Hermes	
G10M003	NBM	163	20.225	G10	
G10M002	NBM	170	0	G10	
L2M002	NBM	214	0	L2	

Animal/ sample No.	***Sex/status***	***Body mass (g)***	***Prolactin (ng/ml)***	***Colony***	***Notes***
L2M001	NBM	156	0	L2	
L3M001	NBM	189	0	L2	
FAF001	NBF	63	0	Faulkes	
G4F010	Queen	109	0.88	Faulkes	
Z3M007	NBM	144	11.995	Faulkes	
F2F002	Queen	95	0	West	non pregnant?
JA1M003	BM	115	0.885	West	
L2F017	NBF	42	0	L2	
L2M016	NBM	54	0	L2	
G10F026	NBF	63	1.035	G10	
BUF001	NBF	74	0	Burda	
G4F019	NBF	55	0	G4	
G4M017	NBM	52	0	G4	
G4F020	NBF	58	0	G4	
G4M018	NBM	64	0	G4	
G4F016	NBF	89	0	G4	
CRF004	NBF	76	0	Crick	
CRF005	NBF	81	0	Crick	
G11F011	NBF	132	0	G11	
G11F010	NBF	117	0	G11	
G11M004	NBM	170	0	G11	
G11F015	NBF	109	0	G11	
G11F003	NBF	107	0	G11	
G11F017	NBF	77	0.84	G11	
G11M018	NBM	75	0	G11	
G11M012	NBM	130	0	G11	
G11M016	NBM	144	0	G11	
G11M008	NBM	165	1.08	G11	
G11F007	Queen	147	0	G11	lactating
L2F004	NBF	154	0	L2	
L3M001	NBM	207	0	L2	
L2M001	BM	165	0	L2	
L2F017	NBF	56	0	L2	
L2M002	BM	212	0	L2	
L3F0003	NBF	153	0	L2	
L2M016	NBM	65	0	L2	
L2M012	NBM	133	0	L2	
L2M003	NBM	186	0	L2	
G1M025	NBM	197	0	G1	
G1F010	Queen	156	0	G1	
G1F022	NBF	42	0	G1	
G1M008	BM	191	0	G1	

Animal/ sample No.	***Sex/status***	***Body mass (g)***	***Prolactin (ng/ml)***	***Colony***	***Notes***
G1F021	NBF	98	0	G1	
G1F014	NBF	150	0	G1	
G1M004	BM	181	0	G1	
G1F025	NBF	51	0	G1	
G1M017	NBM	168	0	G1	
G1M026	NBM	53	0	G1	