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The pattern of reproduction in the mole-rat *Heliophobius* from Tanzania: do not refrain during the long rains!

M.K. Ngalameno, A.D.S. Bastos, G. Mgode, and N.C. Bennett

Abstract: The genus *Heliophobius* Peters, 1846 comprises at least six cryptic, topotypical species in the *Heliophobius argenteocinereus* Peters, 1846 species complex. The current study investigated the breeding patterns of a wild-caught population from Tanzania where the putative species *Heliophobius argenteocinereus emini* Noack, 1894 resides. Individuals were collected on a monthly basis for an entire calendar year. Assessment of fœtus presence, gonadal histology, reproductive-tract morphometrics in combination with gonadal steroid (plasma progesterone and œstradiol-17β in females and testosterone in males) measurements and field observations revealed that rainfall is important for the onset of breeding. The results further confirmed that breeding is limited to a single, yearly reproductive event synchronised to the long rainfall pattern. The distinct breeding peak in July is associated with an elevation in gonadal mass, increase in concentrations of reproductive hormones, and presence of Graafian follicles and corpora lutea in the ovaries of females. These reproductive parameters coincided with the end of the long rainfall period, whereas presence of young in the maternal burrow system corresponded with the start of the short rainfall of East Africa. These findings confirm *Heliophobius* has a single breeding opportunity each year, and this species is therefore vulnerable to any changes that may impact their climatically attuned breeding patterns.

Key words: *Heliophobius*, solitary, hormones, histology, precipitation, radioimmunoassay, reproduction, cyt *b*.

Résumé : Le genre *Heliophobius* Peters, 1846 comprend au moins six espèces topotypiques cryptiques dans le complexe d'espèces *Heliophobius argenteocinereus* Peters, 1846. L'étude s'est penchée sur les motifs de reproduction d'individus capturés à l'état sauvage d'une population de Tanzanie où réside l'espèce putative *Heliophobius argenteocinereus emini* Noack, 1894. Des individus ont été prélevés mensuellement pendant une toute une année. L'évaluation de la présence de fœtus, l'histologie des gonades, la morphométrie du système reproducteur combinée à des mesures de stéroïdes gonadiques (progestérone plasmatique et œstradiol-17β chez les femelles et testostérone chez les mâles) et des observations de terrain révèlent que la pluie est importante en ce qui concerne le début de la reproduction. Les résultats confirment en outre que la reproduction est limitée à un seul évènement annuel synchronisé avec la longue saison des pluies. Un pic de reproduction net en juillet est associé à des augmentations de la masse des gonades et des concentrations d'hormones de reproduction et à la présence de follicules de De Graaf et de corps jaunes dans les ovaires des femelles. Ces paramètres de reproduction coïncident avec la fin de la longue saison des pluies, alors que la présence de jeunes dans le réseau de tunnels maternel correspond au début de la courte saison des pluies en Afrique de l'Est. Ces constatations confirment qu'*Heliophobius* n'a qu'une seule occasion de reproduction par année, cette espèce étant donc vulnérable à tout changement pouvant avoir une incidence sur ses motifs de reproduction synchronisés avec le climat. [Traduit par la Rédaction]

Mots-clés : *Heliophobius*, solitaire, hormones, histologie, précipitation, dosage radio-immunologique, reproduction, cyt *b*.

Introduction

In subterranean mole-rats, breeding is constrained by the ecological conditions and the restrictive nature of the burrow system (Bennett et al. 2000). The subterranean niche prevents the use of many environmental cues used by surface-occurring species; for example, change in day length (photoperiod) is unlikely to act as a proximate cue because of the slight annual difference in day length. Rainfall on the other hand is increasingly implicated as a cue for triggering breeding in several solitary subterranean mammals (Dennis and Marsh 1997; Bennett and Faulkes 2000; Šumbera et al. 2003; Herbst et al. 2004; Hart et al. 2006; Katandukila et al. 2013), but a consistent trend is yet to emerge as the importance of this cue varies geographically and across different levels of sociality.

Temperature is another important cue that subterranean mammals may utilize for their activity patterns (Lovegrove 1989; Šklíba et al. 2007, 2014; Lovy et al. 2013; Oosthuizen and Bennett 2015). The temperatures within the burrow systems of African mole-rats are generally more muted compared with those experienced above ground, but diurnal and seasonal fluctuations in temperature do occur albeit at lower amplitudes compared with those of aboveground conditions (Bennett et al. 1988; Roper et al. 2001; Burda et al. 2007).

In South Africa, the mole-rats occurring in winter rainfall regions mate and conceive only during the winter rainfall months,

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with the young born in the spring when food is widely available and the soil is workable enabling animals to burrow to search for food in the form of underground storage organs of geophytes. Furthermore, the moist soil allows dispersal of the young and the establishment of independent burrow systems in three solitary species: the Cape mole-rat (*Georychus capensis* (Pallas, 1778)), the Namaqua mole-rat (*Bathyergus janetta* Thomas and Schwann, 1904), and the Cape dune mole-rat (*Bathyergus suillus* (Schreber, 1782)) (Bennett and Jarvis 1988; Herbst et al. 2004; Hart et al. 2006). In eastern Africa, these reproductive events have been found to co-incide with the long and short rainfall periods in the solitary East African root rat (*Tachyoryctes splendens* (Rüppell, 1835)), an allopatric and phylogenetically diverse subterranean rodent mole (Katandukila et al. 2013). The solitary silvery mole-rat (*Heliophobius argenteocinereus* Peters, 1846) is reported to breed seasonally in Zambia, Kenya, and Malawi (Copley 1950; Scharff et al. 2001; Šumbera et al. 2003). In Malawi, breeding has been reported at the end (or) the second half of a rainy season (Šumbera et al. 2003), rather than at the beginning of the rainy season.

All solitary South African species of bathyergid mole-rats studied to date have been reported to be seasonal breeders with their reproduction linked to rainfall (Bennett and Jarvis 1988; Herbst et al. 2004; Hart et al. 2006). In contrast, the majority of social mole-rats breed throughout the year (Bennett et al. 1988; Sichilima et al. 2008, 2011; Oosthuizen and Bennett 2009). There are, however, two social species that breed seasonally with rainfall: the common mole-rat (*Cryptomys hottentotus hottentotus* (Lesson, 1826)), which occurs sympatrically with the solitary mole-rat genera *Georychus* Illiger, 1811 and *Bathyergus* Illiger, 1811 (Spinks et al. 1997, 1999), and the highveld mole-rat (*Cryptomys hottentotus pretoriae* (Roberts, 1913) = *Cryptomys hottentotus natalensis* Roberts, 1913) (van Rensburg et al. 2002; van der Walt et al. 2001).

The *Heliophobius argenteocinereus* cryptic species complex represents an ideal study group for teasing apart variation in environmental drivers of reproduction in subterranean mammals. Members of this taxon are widely distributed throughout eastern, central, and southern Africa, and were until recently believed to be monotypic. A phylogeographical study confirming the presence of multiple topotypical species within the complex allows for a more thorough investigation of reproductive cues. As all species are solitary and share common ancestry, differences in the timing of reproduction will be strongly linked to locality-specific environmental factors. The Emin's mole-rat (*Heliophobius argenteocinereus emini* Noack, 1894) was selected for this study because it occurs in an area that is geographically and climatically distinct from the single *Heliophobius* species for which data are available, viz. *H. argenteocinereus* from Malawi (Šumbera et al. 2003), but allopatric with another solitary subterranean species, i.e., *T. splendens*, for which there is extensive reproductive data (Katandukila et al. 2013).

In the absence of nuclear data corroborating the topotypically discrete mitochondrial lineages (Faulkes et al. 2011), we take a conservative taxonomic approach and refer to the taxon under study as *Heliophobius*. However, it is pertinent to mention that available chromosome data, although limited, are suggestive that the species identified by Noack (1894) as *Heliophobius argenteocinereus emini* has a $2n = 52$, whereas specimens from eastern Zambia have a $2n = 62$ and those from Kenya have a $2n = 60$ (George 1979; Scharff et al. 2001; Deuve et al. 2008; Faulkes et al. 2011).

By investigating the reproductive biology of *Heliophobius* from postmortem examination of gonads and plasma steroids of animals sampled on a monthly basis for a year in Tanzania, as has been done for other subterranean mammals (e.g., van Rensburg et al. 2002; Herbst et al. 2004; Schoeman et al. 2004; Hart et al. 2006; de Bruin et al. 2012; Katandukila et al. 2013), and by recording pregnancies, we can determine whether reproductive activity coincides with the rainfall patterns that are characteristic in eastern Africa; namely, the long wet season from February to early June and the shorter dry season in September, October, and No-

vember. The results have important implications for the species in light of changes in climate of equatorial Africa, where rainfall periods shift dramatically interannually in concert with El Niño Southern Oscillations and where temperature and rainfall have increased and decreased by a mean of 0.23 °C and 3.3%, respectively, per decade since 1960 (McSweeney et al. 2010a, 2010b).

Materials and methods
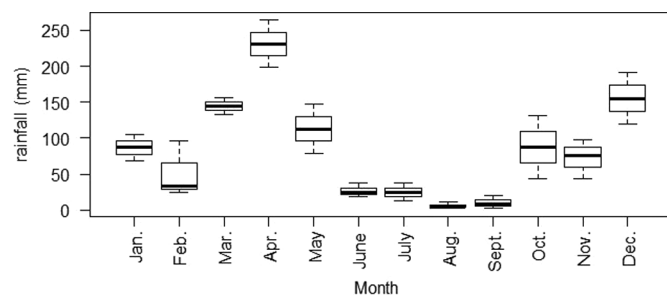
Study site and sampling

Heliophobius were captured from four main sites in the Mvomero District, Tanzania: 6.82036°S, 37.67056°E; 6.97858°S, 37.55001°E; 6.98003°S, 37.54636°E; 6.98005°S, 37.54635°E. The study was conducted over 12 consecutive months, from January to December 2013. All sites are classified as agri-ecological zones with highly fertile soils supporting a variety of food and cash crops (Morogoro Regional Socio Economic Profile (MRSP)). The study area has two rainy seasons with a mean annual rainfall of 600 mm; the longer period of rainfall (i.e., heavy rainfall) is from March to May with a mean of 470 mm, whereas a shorter period of rainfall of 130 mm is from October to December. Dry months are June, July, August, and September (MRSP). June and July months are the transition months between heavy rains and the dry season. Annual mean temperature ranges from 18 to 30 °C with high humidity during March, April, and May, as well as October and November (MRSP) (Fig. 1).

Animals were collected from areas subject to widespread crop destruction, where the mole-rat is considered an agricultural pest. Animals were collected using Hickman live traps or by excavation of burrow systems (Hart et al. 2006; Sichilima et al. 2008). Five adult female and five adult male mole-rats were captured monthly for one calendar year, resulting in a total of 120 animals (i.e., 60 adult males and 60 adult females). Burrows for capturing animals were selected randomly by opening a distinct burrow system at least 100 m from one another. The animals were euthanized with an overdose of chloroform and the body mass subsequently recorded. Burrow entrances were left open to check for subsequent blocking, and if arising, traps were inserted (multiple occupancy usually resulted when there was a mother and offspring).

The presence or absence of elongated teats and (or) a perforate or imperforate vagina were recorded in females. Blood was drawn from the heart of the sacrificed animal using heparin-coated syringes and centrifuged at 500*g* (3000 rev/min) for 10 min. Following centrifugation, plasma was removed using a pipette, transferred to a new tube, and stored at –20 °C. Gonads (testes or ovaries) were removed and fixed in Bouin's fixative for a period of 18 h prior to being stored in 70% ethanol. The reproductive status of females was further assessed by recording the presence or absence of embryos or fœtuses following the opening of the abdominal cavity. Lactating females could be identified by the presence of prominent teats that released milk on squeezing and (or) the occupancy of young, either juvenile(s) or infant(s), in their burrows. Juveniles and subadults of both sexes co-habiting their mother's burrow were recorded on capture to assess recruitment to the population of *Heliophobius* from reproductive animals collected.

The mass (g) of the gonads was measured using a Sartorius scale (Sartorius AG, Goettingen, Germany). Following weighing, the gonads were stored in picric acid for 24 h and subsequently stored in 70% ethanol until being used for histological preparation. The gonads were then sequentially dehydrated by placing them in containers of increasing alcohol concentration and subsequently embedded in a block of paraffin wax before sectioning at a thickness of 7 μm using a rotary microtome (820 Spencer; American Optical, Scientific Instrument Division, Buffalo, New York, USA). Sections were mounted on microscopic slides after being dipped in water at 45 °C mixed with gelatine as an adhesive. The mounted sections were subsequently dried in an oven at 36 °C for a period of 72 h and then stained with Ehrlich's haematoxylin and counter-

Fig. 1. Mean (±SD) monthly rainfall (mm) of Mvomero District, Morogoro, Tanzania, for January until December 2014. Data from Tanzania Meterology Agency.

stained with eosin (Drury and Wallington 1967). The stained sections were covered by slide cover slips secured with resin solution (Entellan® glue; Merck KGaA, Darmstadt, Germany) and dried in an oven at 36 °C for 48 h.

Testicular histology

Thirty randomly selected sections taken from the mid-region of the testes were used to estimate the mean diameter of seminiferous tubules with a light microscope (Diaplan; Ernst Leitz Wetzlar GmbH, Wetzlar, Germany). Seminiferous tubules were photographed at 10× magnification using a digital camera attached to a microscope (Moticam 1000, 1.3 megapixel, USB 2.0; Motic China Group, Ltd., Xiamen, People's Republic of China). The diameters (μm) of 1800 seminiferous tubules were measured using Motic Images Plus 2.0ML (Motic China Group, Ltd., Xiamen, People's Republic of China). The larger diameter of the seminiferous tubules implies active testes with greater sperm production.

Ovarian histology

The sectioned ovaries were examined under a light microscope for the numbers of primordial, primary, secondary, tertiary, and Graafian follicles, as well as corpora lutea, following Bloom and Fawcett (1962), van Rensburg et al. (2002), and Hart et al. (2006). Magnifications of 100×, 200×, and 400× were used consecutively (van Rensburg et al. 2002; Hart et al. 2006). The follicles of each category for each section were counted. Counting error was taken into account using the method of Borgeest et al. (2004). The sections were photographed using a digital camera attached to a light microscope (Moticam 1000, 1.3 megapixel, USB 2.0; Motic China Group, Ltd., Xiamen, People's Republic of China).

Radioimmunoassay

Progesterone

Plasma progesterone concentrations were measured using a commercially available kit (Coat-a-Count MG12171; IBL International GmbH, Hamburg, Germany). The assay enabled the determination of testosterone concentrations over the range 6–1200 ng/dL. The cross-reactivity of the antibody with other naturally occurring steroids was <0.1% except for 5α-pregnan-3,20-dione (3.4%), 17α-hydroxyprogesterone (1.5%), 20-β-dihydroprogesterone (3.2%), and 11-deoxycorticosterone (0.3%). A plasma sample with a high concentration of progesterone was double diluted using the assay buffer as a matrix 1:1 to 1:8 then assayed. The slopes of serial double dilution and standard curve were compared with check for parallelism (ANCOVA, $F_{[1,2]} = 5.26$, $P = 0.07$, $n = 4$) following a log–logit transformation of the data (Chard 1987). The intra-assay coefficient of variation for the plasma pool was 6.7% and sensitivity of the assay was 1.35 nmol/L.

Œstradiol-17β

Œstradiol-17β assays were performed as described by Herbst et al. (2004) using the Coat-a-Count MG12101 œstradiol kit (IBL International GmbH, Hamburg, Germany). The cross-reactivity of the antibody to all

naturally occurring steroids was 10% with œstrone, <5% with œstriol, œstrone-β-ᴅ-glucoronide, œstone-3-sulphate, ᴅ-equilenin, 17β-œstradiol-3-monosulphate, testosterone, and androsterone. The assay was validated for plasma of *H. a. emini* by testing the slope of the curve produced using serial dilutions of unextracted mole-rat plasma obtained from a female with high œstradiol concentrations (over the range 1:1 to 1:32) against the standard curve. After log–logit transformation of the data (Chard 1987), slopes of the lines were compared and found not to differ significantly from the reference preparation (ANCOVA, $F_{[1,4]} = 4.4$, $P = 0.09$, $n = 4$). The intra-assay coefficient of variation for repeated determinations of quality control was 8.3% and sensitivity of the assay was 10 pg/mL.

Testosterone assays

The plasma testosterone concentrations were measured using the commercially available Coat-a-Count MG12191 testosterone kit (IBL International GmBH, Hamburg, Germany). The assay was able to determine testosterone concentrations of 6–1200 ng/dL. Cross-reactivity of the Coat-a-Count testosterone antibody was 1.8% with 19-nortestosterone, 0.31% with dihydrotestosterone, and <0.01% with other steroids tested. We validated the assay by testing for parallelism between the serial dilutions of *H. argenteocinereus* plasma (obtained from an individual with high testosterone concentrations) and the standard curve (Chard 1987). The curves were parallel and not significantly different from the reference preparation (ANCOVA, $F_{[1,2]} = 1.8$, $P = 0.2$, $n = 6$). The intra-assay coefficient of variation was 7.6%. The sensitivity of the assay (90% binding) was 5 ng/dL or 0.175 nmol/L.

Descriptive and statistical analyses were performed using R i386 3.2.2 and Excel 2007. The relationship between variables such as gonadal mass, volumes, and rainfall were evaluated using correlation statistical analyses. The normality of the data within each month was tested using the Shapiro–Wilk normality test (Lilliefors 1967); consequently, parametric tests were used in subsequent analyses. Annual variation in hormone concentrations and gonadal metrics was investigated using ANOVA with Tukey's honest significant difference (HSD) test to evaluate significant differences. All descriptive results in the figures are presented as mean ± SD.

Species designation

Clade and species assignments were determined by cyt *b* gene amplification and by nucleotide sequencing of 10 randomly selected individuals (5 males and 5 females), using previously described primers and thermal cycling conditions (Bastos et al. 2011). Full-length gene sequences submitted to GenBank under accession numbers KX060579–KX060580 were complemented with homologous data from two prior studies (Faulkes et al. 2004, 2011) and the best-fit model of sequence evolution identified under Akaike's information criterion corrected for small sample size in Mega5 (Tamura et al. 2011) was subsequently used for maximum-likelihood inferences.
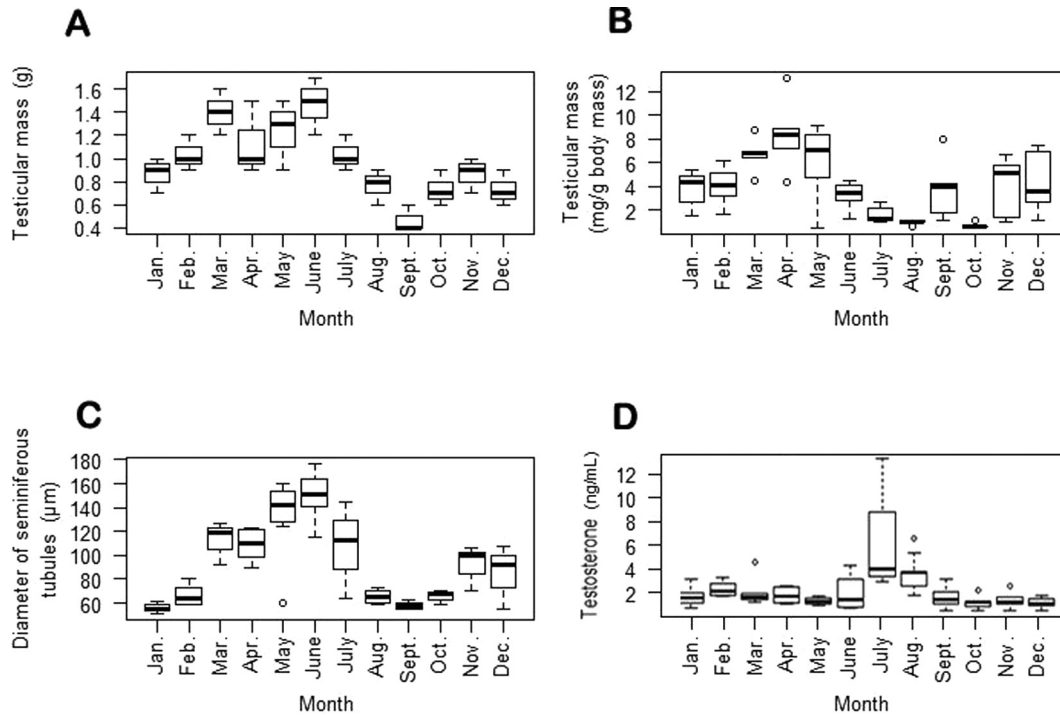
Two haplotypes were recovered from the 10 individuals selected at random for genetic characterisation. One of these had 100% nucleotide sequence identity to AY425935 and AY425938, which correspond to clade 3 *Heliophobius* museum voucher QM1026 and QM1023, respectively, and the other represents a new haplotype that differs from the aforementioned reference strains at nucleotide position 604 (G/A). This first base position mutation results in a nonsynonymous amino acid substitution at position 202 (Ala/Thr). The two haplotype sequences generated in this study cluster within the *H. a. emini* clade defined by Faulkes et al. (2011), with 100% bootstrap support (not shown).

Results

Testicular metrics and histology

The testicular mass increased over the period February to March and decreased from July to August and remained reduced until January (Fig. 2A). The mass of the testes was significantly different

Fig. 2. Monthly fluctuations (mean ± SD) for raw testicular mass (A), testicular mass milligrams per gram body mass (B), diameter of seminiferous tubule (C), and plasma testosterone concentration (D) in Emin's mole-rat (*Heliophobius argenteocinereus emini*) from January until December 2014.

between months ($F_{[11,48]} = 4.811$, $P < 0.0001$, $n = 60$), with the months of the long wet season having males with heavier testes per gram mass of animal than those of the dry months and the short rains of October and November ($F_{[1,58]} = 15.58$, $P < 0.0002$, $n = 60$) (Fig. 2B). Significant differences were clearly observed between June and August ($P = 0.012$), June and December ($P = 0.005$), March and December ($P = 0.02$), May and December ($P = 0.033$), October and June ($P = 0.008$), and October and May ($P = 0.04$). Testicular mass of the breeding and nonbreeding animals was not influenced by body mass ($F_{[3,56]} = 2.033$, $P = 0.16$, $n = 60$).

The mean diameter of the seminiferous tubules was greatest in May, June, and July, whereas the lowest mean diameter was in August, September, and October and again in January (Fig. 2C). Variation in the diameter of seminiferous tubules between months was significant ($F_{[11,1788]} = 115.9$, $P < 0.0001$, $n = 1800$), with breeding months (the long rains) showing males with increased diameter of seminiferous tubules than nonbreeding months (dry months and short rains) ($F_{[1,1328]} = 390.9$, $P < 0.0001$, $n = 1330$). Significant differences were observed between September and April, October and April, June and August, March and August, May and August, June and December, March and December, May and December, as well as May and February ($P < 0.0001$). The width of the seminiferous tubules was positively correlated with the peak of rainfall (Pearson's correlation, $r = 0.123$, $P < 0.0001$, $n = 1799$).

Ovarian metrics and histology

The mass of the ovaries of females were greatest in March, April, and May, but remained relatively small for the rest of the year (Fig. 3A). Ovarian mass varied between breeding and nonbreeding months ($F_{[1,58]} = 82.91$, $P < 0.0001$, $n = 60$) (Fig. 3B). Significant differences were observed between August and April, December and April, November and April, March and December, May and January, and May and July ($P < 0.0001$).

The follicular development varied over the year, and as expected, primordial follicles were uniform and showed no clear pattern, but the highest numbers were recorded in March through to July

(Table 1). Primary follicles increased from June to March, increasing dramatically in April and then decreased, reaching the lowest number in October (Table 1). The numbers increased almost three-fold in November and then decreased to almost by half the amount in December (Table 1). The number of secondary follicles increased in January, peaked in June, then decreased gradually through to September. The numbers increased again in October and November and finally dropped off again in December (Table 1). Tertiary follicles increased sharply from January to April, slightly decreased in May, and gradually decreased in November after which they increased sharply (Table 1). Graafian follicles increased in number from January and reached a peak in April. The number decreased gradually from May to October, increased in November, and decreased again in December (Table 1). The number of corpora lutea increased gradually from February to May, followed by a sharp increase in June and July. The numbers decreased to zero in September, then increased marginally from October to December (Fig. 3C).

Hormonal profiles

The concentration of testosterone in the males was generally low throughout the year, but increased in April and peaked in June, after which the concentrations dropped through to March (Fig. 2D). The concentration of testosterone varied between months ($F_{[11,46]} = 3.212$, $P = 0.00261$, $n = 58$).

The concentration of testosterone in male mole-rats increased during the long rains and was lowest during the dry season with the maxima coinciding with peaks in precipitation (see Figs. 1 and 2D).

Levels of œstradiol increased from April, peaked in July, after which there was a gradual decline through to October, followed by a slight increase in November and December (Fig. 3D). Progesterone was high in June, July, and August, and then declined from September onwards, remaining very low for all other months (Fig. 3E). There was a significant positive correlation between progesterone concentration and number of corpora lutea (Pearson's correlation, $r = 0.42$, $P < 0.0002$, $n = 75$).

Fig. 3. Monthly fluctuations (mean ± SD) for raw ovarian mass (A), ovarian mass milligrams per gram body mass (B), number of corpora lutea (C), plasma œstradiol-17β concentration (D), and plasma progesterone concentration (E) in Emin's mole-rat (*Heliophobius argenteocinereus emini*) from January until December 2014.
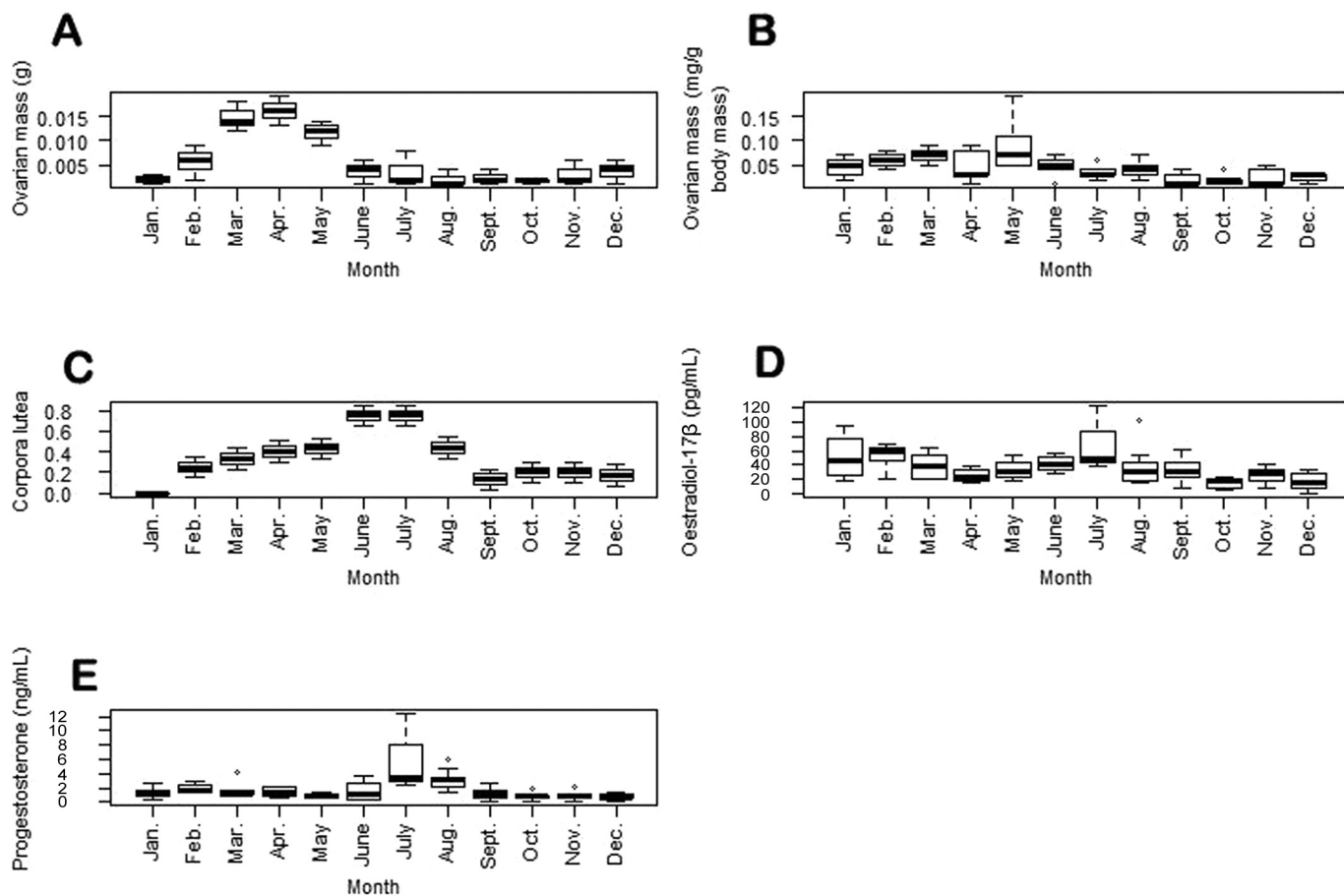

Table 1. Number (mean ± SD) of follicular cell types found in the ovary of Emin's mole-rat (*Heliophobius argenteocinereus emini*) over a single calendar year.

Month	Follicular cells				
	Primordial	Primary	Secondary	Tertiary	Graafian
January	19±2.14	0.38±0.0	0.23±0.43	0.15±0.37	0.08±2.7
February	18.75±2.06	0.25±0.5	0.25±0.5	0.5±0.57	0.25±0.5
March	20±3.29	0.67±0.54	0.33±0.51	0.5±0.54	0.5±0.54
April	18.4±2.7	0.8±0.48	0.4±0.54	0.6±0.54	0.8±0.44
May	19.4±2.44	0.57±0.53	0.43±0.53	0.57±0.53	0.57±0.53
June	19.4±2.38	0.25±0.5	0.5±0.5	0.25±0.5	0.5±0.57
July	19±1.63	0.25±0.5	0.25±0.5	0.25±0.5	0.25±0.5
August	15±4.47	0.33±0.5	0.22±0.44	0.11±0.33	0.11±0.33
September	18.5±2.73	0.25±0.46	0.25±0.46	0.13±0.35	0.13±0.35
October	14.6±3.78	0.2±0.44	0.4±0.54	0.2±0.44	0.2±0.44
November	18.2±2.59	0.6±0.54	0.4±0.54	0.6±0.54	0.4±0.54
December	12.67±2.5	0.33±0.49	0.17±0.4	0.17±0.4	0.33±0.51

Pregnancies and lactation

A total of 120 *H. a. emini* were sampled (60 males and 60 females). Out of 60 sampled female animals, 12 (20%) were pregnant and 13 (21.7%) were lactating. Pregnancies were observed initially in June when two of five females (40%) were pregnant. The percentage increased in July when all five female animals sampled (100%) were pregnant. Pregnancies decreased in the following months, whereby three females (60%) and two females (40%) were found to be pregnant in August and September, respectively. No pregnancies were observed after September or before June. This is reflected in the number of corpora lutea, which increased from March to May (these are probably corpora lutea of ovulation). In June and July, there was a peak in the numbers of corpora lutea, the corpora lutea of pregnancy, which dropped gradually in August and reached the lowest number from September to February (Fig. 3C).

A total of 50% of pregnant females were found to have five embryos (three on the left uterine horn and two on the right uterine horn), 35% had six embryos (three on either uterine horn), and 15% were found to have five embryos (four on the left uterine horn and one on the right uterine horn). Based on the number of embryos, the mean litter size of *Heliophobius* from Tanzania was

5.3 ± 0.5. However, the number of pups found in maternal burrows was lower with a mean litter size of 4.4 ± 0.5. Lactating animals were first observed in September, of which four of the five females were lactating (80%). All female animals sampled in October and November were lactating and this decreased dramatically in December, where only one out of five sampled females were found to be lactating (20%).

Presence of juveniles

A total of 47 juveniles (20 male and 27 female) were sampled in maternal burrow systems. Juveniles were captured in the months of September, October, and November. The presence of juveniles coincided with the short rainfall. We predict that in the months of December to February, these individuals would disperse to establish their own independent burrow systems, dispersing off from the maternal burrow system.

Discussion

A field-based study based on postmortem examination of animals using a number of reproductive indices in *Heliophobius* from Tanzania showed a single clear breeding period. The reproductive-tract morphometrics, gonadal histology, and measurement of circulating gonadal steroids revealed one single distinct breeding period that coincided with the precipitation pattern of the long rains in Tanzania. Œstradiol-17β and the presence of Graafian follicles in April and May imply that the females ovulate during the height of the long wet season. Likewise, male *Heliophobius* also showed peaks in testosterone around April and June, had larger seminiferous tubules, and heavier testicular mass around April to June. These findings suggest that the adult males and females prepare for mating during the period of April to June. The mating period may reflect a time when the sexes are more tolerant of one another to promote copulation and ensure maximal reproductive success, but it is also a period when increased territoriality between same-sex individuals may arise, as has been found in other subterranean mammals during the breeding season (Bennett and Jarvis 1988; Bennett et al. 1991). It is unknown if the sexes use seismic communication as has been reported for other solitary bathyergids (Bennett and Jarvis 1988; Narins et al. 1992; Herbst et al. 2004; Hart et al. 2006) and spalacids (Heth et al. 1987; Rado et al. 1987; Hrouzkova et al. 2013; Katandukila et al. 2013).

The sexes synchronise the maturation of their reproductive organs and the sperm and ova to maximise conception before the end of the wet months. The rainfall results in the soil becoming more workable so that excavation and digging can arise and hence the sexes pair up; however, we currently have little information of how this arises in the field. Patzenhauerova et al. (2010) using microsatellites report *H. argenteocinereus* to exhibit polygyny. The population that they studied was found to have a female-biased sex ratio. Because of the large distances between burrow systems, it is speculated that males might seek females above ground.

The presence of numerous corpora lutea of pregnancy, higher circulating progesterone concentrations, and presence of embryos suggest that maximum pregnancies occur in June and July, falling off in August, following the long rains. These pregnancies probably reflect the optimal period when mating could arise and when constraints on burrowing are minimal as has been suggested for other subterranean mammals (Emlen and Oring 1977; Isaac and Johnson 2003). Lactation was first reported in September, but was frequent in October and November, which coincides with the short rainfall period. As a consequence, the soils would be soft and workable during this time and would allow lactating females with young to access the geophytes and grass rhizomes and thus enable these mothers to care for the juveniles over this period. The span of first pregnancy to first lactation and maximal pregnancy to maximal lactation is around 3 months, which fits with the estimated gestation of 87 days reported by Jarvis (1969)

and Šumbera et al. (2003) for *Heliophobius* from Kenya and Malawi, respectively. By breeding seasonally, animals can time their reproduction such that the pups are born at a point that maximises offspring survival in the environment (Ims 1990).

Many organisms use environmental proximate cues to trigger the onset of reproductive activity. In this study, it would appear that the onset of the long rains triggered reproductive activity. In contrast to *T. splendens*, mating did not take place early in the rains, but moreover during the latter part of the long rains. The role of photoperiod in subterranean rodents appears to be relatively unimportant. In the spalacid, the photoperiod of the Middle East blind mole-rat (*Spalax ehrenbergi* Nehring, 1898) has a role in thermoregulation (Haim et al. 1983); similarly, several species of bathyergid are able to entrain their locomotory activity patterns to different lighting schedules (Oosthuizen et al. 2003; Hart et al. 2004; De Vries et al. 2008). However, rainfall is undoubtedly the most important environmental cue acting on subterranean mammals in that it can be detected underground because it softens soil and also brings about plant growth and flushes of vegetation (Dennis and Marsh 1997). Indeed, all solitary South African bathyergid mole-rats studied to date gauge their breeding events with extended periods of rainfall (Bennett and Jarvis 1988; Herbst et al. 2004; Hart et al. 2006). The results of our study are consistent with those for *Heliophobius* from Malawi (Šumbera et al. 2003) in that reproduction arises in the latter part of the long rains.

The finding of a single litter born per annum per adult female corresponds well with the findings of Copley (1950) and Šumbera et al. (2003), who found a single litter to be produced during the long rains in Kenya and Malawi, respectively. The litter size of *Heliophobius* from Tanzania was higher compared with the value of 3.2 ± 0.9 observed in *Heliophobius* from Malawi (Šumbera et al. 2008). Birthing of pups during periods of good rainfall ensures the acquisition of quality food resources for lactating females and their young, as the soil is softened and burrowing is made more efficient. Precipitation is important for the re-sprouting, regeneration, and an overall increase in plants succession and vegetation cover (Bennie 1991; Pregitzer and King 2005). Juvenile and (or) subadults were found in the maternal burrow system during the months of the short rains and dispersal of subadult mole-rats probably occurs from December onwards, as no juveniles were caught with their mothers after this time. This observation implies that the timing of dispersal of subadult mole-rats from their natal burrow systems occurs prior to the onset of the next long rains to ensure that the offspring are fit enough and have access to maximal vegetation and food resources for independence when setting up their own tunnel system. This breeding strategy and the associated dispersal from natal burrows are documented for other solitary subterranean rodents from South Africa (e.g., *G. capensis*: Jarvis and Bennett 1991; *Bathyergus* species: Bennett and Faulkes 2000; Herbst et al. 2004). The dispersal of subadults during periods of rain when food is plentiful (Rado et al. 1992; Le Galliard et al. 2012) and the soil is workable (Williams and Cameron 1984) enables subadult *Heliophobius* to successfully establish their own independent burrow system (Jarvis and Sale 1971; Bennett et al. 1991; Herbst et al. 2004).

Our findings from the reproductive hormone profiles, histological assessment of gonadal characteristics, and field observations have clearly revealed that this population of *Heliophobius* is a strictly seasonal breeder, having a single period of reproductive activity within a year. *Heliophobius* from Tanzania shows peaks of reproductive hormone concentrations, pregnancies, births, and subadult dispersals concomitant with peaks of precipitation. This suggests that precipitation is the key factor triggering their seasonal reproduction. The long gestation period precludes the production of a second litter during the short rains. In contrast, *T. splendens* is capable of such fecundity because of the much shorter gestation period of around 38 days (Katandukila et al. 2013). The restriction of reproduction to a single event a year

makes this species susceptible to climate instability owing to the action of El Niño and El Niña weather oscillations in the Indian Ocean. These weather phenomena may bring about either higher or lower rainfall in regions of Tanzania occupied by *Heliophobius* (McSweeney et al. 2010*a*, 2010*b*).

Additional studies on other members of the *Heliophobius* species complex are required to understand how reproduction varies between species and localities. As these species are cryptic, it is important that these initiatives are conducted within a phylogeographical framework.

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