Folia Zool. – 58 (Suppl. 1): 65–74 (2009)

The influence of olfactory stimulus and sexual activity on gonadal steroids in eusocial mole-rats

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Received 1 December 2008; Accepted 1 April 2009

A b s t r a c t . The two studied sibling species of African mole-rats, *Fukomys anselli* and *F. kafuensis*, are blind, subterranean rodents that live in an eusocial families with only a single pair breeding whilst its offspring exhibit prolonged philopatry and do not breed. The reproductive skew is caused by incest avoidance through individual recognition of family members. The aim of the study is to contribute to basic understanding of priming capacities of olfactory stimuli and reproductive activity on biological state of a female. We compared hormonal profiles (normalized urinary estradiol- and progesterone concentration (mg/crea) and their temporal changes in females throughout three different test phases: I. Five week phase without manipulation; II. Five day phase with olfactory stimulation with odours of potential reproductive partners; III. Five day phase with reproductive stimulation with respective partners. Colpocytology was performed to correlate spontaneous or induced estrus with vaginal cytological findings. There was a strong correlation between sexual activity of females (queens) on one side and high mean estradiol and progesterone levels on the other side as well as estradiol increase triggered by mating. No correlation was found between estrus phase and typical estrus like cells in colpocytological examinations.

Key words: estrus, colpocytology, *Fukomys*

Introduction

Ansell's and Kafue mole-rats, *Fukomys anselli* and *F. kafuensis* (formerly designated as *Cryptomys*, cf. K o c k & I n g r a m 2006) further called here as *Fukomys* mole-rats, are subterranean rodents (Bathyergidae) from Zambia, living in large, multigenerational families founded by a single pair monopolizing the breeding. The offspring remain in the family as helpers to their parents and younger siblings, and do not breed. Compared to helpers known in monogamous mammals or in birds (e.g., S c h o e c h et al. 1996), the "workers" in mole-rats show long lasting philopatry resulting in overlap of several generations (= litters) of siblings occurring in the nest. Although the "workers" in *Fukomys* do not reproduce, they are physiologically fertile. In the ovaries of adult non-breeding female Ansell's mole-rats, all stages of follicular development up to tertiary follicles were found (W i l l i n g s t o r f e r et al. 1998). Many unruptured luteinized follicles, yet missing corpura lutea indicated that ovulation was absent. In actively breeding females, however, true corpora lutea were recorded. Apparently, the Ansell's mole-rat is an induced ovulator (W i l l i n g s d o r f e r et al. 1998, B u r d a 1999) like the Natal mole-rat (*Cryptomys natalensis*) and the Highveld mole-rat (*C. pretoriae*) (M a l h e r b e et al. 2004). Anovulation may therefore be result of lack of (mechanical) stimulation through copulation rather than a consequence of reproductive inhibition (B u r d a 1999).

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Chemosignals influence reproductive behaviour and neuroencdocrine function in many mammals, e.g. in mouse (Vandenbergh 1969, Novotny et al. 1986, Mucignat-Caretta et al. 1995, Wersinger & Rissman 2000, Brennan 2004), prairie vole (Williams et al. 1992, DeVries et al. 1997), pine vole (Solomon et al. 1996, Schwab et al. 2004), and hamster (Reasner & Johnston 1988). The urinary odor of unfamiliar breeding males is an adequate stimulus to accelerate growth and puberty in juvenile females (Reasner & Johnston 1988, Mucignat-Caretta et al. 1995). Chemosignals are also of relevance in *Fukomys* mole-rats in different contexts; individual discrimination for ano-genital odors was demonstrated across species of this genus (Heth et al. 2002, 2004). Furthermore, *Fukomys* non-reproductive males discriminate and significantly prefer ano-genital odours of reproductive females (queens) to those of non-reproductive females (Hagemeyer, unpubl.). *F. anselli* males prefer the ano-genital odours of unrelated females to those of their unfamiliar sisters and also prefer to mate with unrelated females (Heth et al. 2004).

Here we studied the priming capacity of chemosignals and the effect of sexual activity upon cycling reproductive steroids (normalized urinary estradiol- and progesterone concentration (mg/crea)) in sexually unexperienced females. In parallel we examined vaginal cytology (colpocytology), looking for signs of ovulation, with and without olfactory or sexual stimulation. Thus far, neither chemosensory and hormonal assays nor colpocytology, as a marker for ovulation, have been reported for any *Fukomys* mole-rat species (but see endocrinological studies in *F. damarensis,* Snyman et al. 2006).

Material and Methods

Animals

Three "categories" of females of two sibling species of *Fukomys* mole-rats (*Fukomys anselli, F. kafuensis*) and their hybrids (family Bathyergidae) were studied: (I) reproducing (i.e. pregnant and/or lactating) sexually active queens (rQ); (II) non-reproducing queens (nrQ), i.e. dominant, sexually experienced females with a history of breeding which, however, at time of testing, did not show breeding activity like copulating, pregnancy and/or lactation.; (III) non-reproductive, and sexually unexperienced female "workers" (nrf). Altogether 12 females (5 rQ, 2 nrQ and 5 nrf) were involved in the control test (steroid measurement and colpocytological investigations without experimental behavioural manimpulation). Six of these females (1rQ, 2nrQ and 3 nrf) were involved also in following two experimental test phases (olfactory stimulation and mating; Table1).The reproductively active females were involved only in the control test phase because they already had been succesful queens (except for female rQ5). Female rQ5 was wild-caught and its last successful reproduction was one year before the study started, although it copulated regularly with one male. Still, she did not monopolize the king, as another female in the group was also mating with this king. Non reproductively active females (nrQ and nrf) were involved in all the three test phases, except for female nrf1, because of animal husbandry reasons (cf. Table1).

All the animals were housed in glass terrariums, filled with a thick layer of horticultural peat as ground substrate, in a room with a natural dark-light cycle and ambient temperature ranging throughout the year between 20°C and 25°C. Mole-rats were fed ad libitum with fresh carrots, potatoes, supplemented with apples, lettuce and cereals.

Table 1. Composition of (mating) pairs in the third test phase. Females are categorized as nrf = non-reproductive females, rQ = reproductive queens and nrQ = non-reproductive queens. Males are categorized as nrm = non-reproductive males and nrK = non-reproductive kings.

		mating partners		
number	**female**	**male**	**mating success**	**breeding**
1	rQ5	nrm2	mounting, sperms	+
2	nrQ1	nrK1	mounting, sperms	+
3	nrQ2	nrm1	mounting, no sperms	-
4	nrf2	nrm4	mounting, no sperms	-
5	nrf3	nrm5	no mounting, male aggression	-
		nrm6	mounting, sperms	+
6	nrf4	nrm3	no mounting, no aggression	-

Colpocytology and urine collection

Except for two nrf, all females were subjected to the vaginal smear every early morning. To avoid contamination, disposable gloves were worn. The animal was picked up by gently holding it with two fingers on its backside with the head pointing down. The cotton bud (prepared of a tooth pick, and soaked with 0.3% NaCl solution) was carefully inserted into the vagina, thoroughly rotated and smeared onto a microscope slide which was then air-dried for at least three minutes, fixed for 5 minutes in methanol, again air-dried, and Giemsa-stained for 20 minutes. Each slide was examined at a 25 to 100fold magnification under light microscope. The amount of cells was classified as follows: stage 1 = solitary/insular, stage 2 = sporadic, stage 3 = few, stage 4 = many, stage 5 = lots of, stage 6 = plentiful cells. Together with intermediate stages we got a twelve-stage system ranging from 1 over 1.5 to 6.0. As nucleated cells are the most frequent in the smear of rats at proestrus, and develop into cornified cells, we tried to find some periodical changes in representation of these two cell types. Furthermore, we looked for correlation between the estradiol levels and the amount of nucleated cells on the one side and second between progesterone levels and cornified cells on the other side.

Parallel to colpocytology, urine was collected almost daily: An animal lifted up while being held at the skin fold on its back urinates almost immediately, and urine can be collected directly into a 1.5 ml Eppendorf-tube. Alternatively, an animal was put into a clean bucket, where it urinated within seconds or few minutes: in few cases when an animal did not urinate spontaneously within few minutes, it was returned to its colony and we abstained from urine sample for that particular day. Urine was immediately collected with an Eppendorf pipette into an Eppendorf-tube, centrifuged at 8 000 rpm for 8 seconds and the supernatant was pipetted into a fresh Eppendorf-tube to avoid contamination with dirt from the animal's fur. Collected urine was frozen and kept under –20°C until analysis.

Hormones and creatinine assessment

All steroid (estradiol and progesterone) concentrations in urine were related to creatinine concentration. The creatinine concentration (Cr) was measured on the Bayer ADVIA1650 system (Siemens Medical Solutions Diagnostics, Fernwald, Germany) according to the Jaffé method using reagents supplied by the manufacturer. At least 100 μl of urine was necessary for analysis. Hormones were determined on the Bayer Centaur system (Siemens Medical

Solutions Diagnostics, Fernwald, Germany) using immunoassay. Between 500 and 700 μl of urine was required for the analysis. If available, urine of every second day was analyzed.

Urinary steroid samples were calibrated to the Bayer Centaur system, since the automatic assays are optimized to human plasma samples. From each steroid, four dilutions (1:5, 1:10, 1:20, 1:50) were measured three times and proved by a regression curve ($R^2 > 0.98$). Thus, the inter- and intra-assay variations were limited allowing comparison between urine concentrations of different individuals.

Test-procedure

The first phase of the study, control assessment, covered 39 days (19.05.2005-26.06.2005), thus encompassing a larger part of the estrus cycle, the length of which was estimated to be 30 to 45 days in some bathyergids species (Faulkes et al. 1990, Snyman et al. 2006). All the females were involved in the control test phase.

During the second phase (day 40–45), non-reproductively active females (cf. Table 1) were exposed (for six hours each day) to the odouros compound, composed of male body secretions, urine and feces odour(see below). Among the males - five non-reproducing males (nrm) and two non-reproducing kings (nrK) were used as donors of odours and as sex partners. One non-reproducing male (nrm6) was only involved in the third test phase, since his predecessor, nrm5, was aggressive to his female mate (nrf 3, cf. Table 1).

To create an odouros mixture, the respective sex partners were put into a clean glass terrarium with 300 g horticultural peat, two pieces of tissue paper and one carrot for 12 hours overnight in the course of six subsequent nights. Every early morning the inhabitant of cages were exchanged, i.e females were put into the cage of a male odour donor and vice versa for six hours.Thus the subjects were exposed to the odours left from the former inhabitant of that particular cage. After six hours, urine and vaginal smear were collected (see above) and the subjects were removed to their respective family group for next 6 hours in order to keep family contact. Before the next night, test-subjects were put into a freshly prepared cage (see above). The glass terraria of exchange partners were of the same size.

The day following the last olfactory stimulation, the third test phase started. The respective odour partners (Table 1) were put together into one cage and the frequency of copulation within the first 30 minutes was recorded. The first vaginal smear from mated females was collected 0.5 h, 4 h, and 10 h after copulation. Subsequently, vaginal smear was collected twice a day, every twelve hours, for five days. Urine was collected from each subject after mating and then collected daily for five subsequent days.

We compared steroid values only from females in which mating led to reproduction i.e. if four requirements were fullfilled: 1. we observed mounting and thrusting movements (defined as copulation), 2. we detected sperms in vaginal smear after mating, 3. females became pregnant and gave birth about 100 days later and 4. females were not attacked by their partner,(as happened in female nrf3) (cf. Table 1). The mean steroid values during the first test phase, however, were assessed in all the 12 females.

Ethical note

Efforts were taken to minimize stress of the animals. Animal husbandry and all the experimental procedures complied with the German regulations on the care and use of experimental animals. The animals were tame, accustomed to handling and were not apparently stressed by vaginal smear collection and urine sampling.

Statistical analysis

Non-parametric statistical tests were used, since data did not fit normal distribution (Kolmogorov-Smirnov-test) and variances were not homogeneous (Levene-test). To test for an increase of urine steroid concentration in the course of the three test phases, we used the Friedman´s rank-test. Significant differences of pairwise data were tested by the Wilcoxon-rank-test for paired samples (e.g. mean concentration of urinary estradiol (nmol/mg Cr) in test-phase 1 vs. 2, in test-phase 2 vs.3 and in test-phase 1 vs.3. The similar proceduree was applied to analyses of urinary progesterone). To compare mean steroid concentrations between different categories of females (e.g. urinary estradiol concentration of sexually active rQs vs. sexually inactive nrQs and nrfs) during the control phase, Mann-Whitney U-test for independent samples was used. The similar procedure was applied for the assessment of urinary progesterone.

The significance level was set to $p < 0.05$. Only outliers from the mean were excluded to produce comparable data. There were partly large differences in numbers of samples between respective test phases, due to their different length (1st test phase = 39 days, 2nd test-phase two = 5 days). All statistical analyses were performed with SPSS, version 11.

Results

Colpocytology

Cytological estrus changes, like the exclusive presence of cornified cells during the estrus phase, were not detected. There was no correlation between olfactory stimulus and/or the mating session afterwards on one side and induced estrus on the other side.

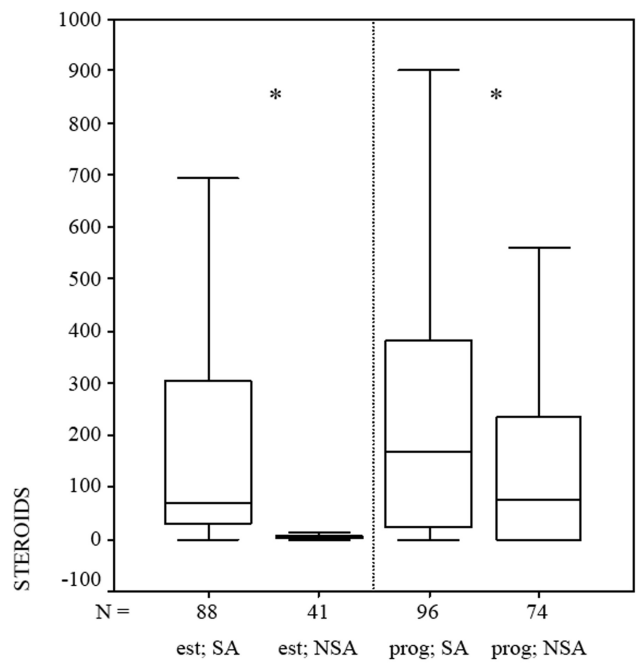

Fig. 1. Boxplot of Mann-Whitney U-test for independent samples: Mean urinary steroid concentration (estradiol (nmol/mg Cr) and progesterone (ng/mg Cr*10) in females of different reproductive activity. Significance level; * = $p < 0.001$, N = analyzed samples, est = estradiol, prog = progesterone, SA = sexual active, NSA = not sexually active.

Mean steroid concentrations in the first test phase

Mean urinary estradiol concentrations depicted in Fig. 1 were significantly higher ($p < 0.001$; Mann-Whitney U-test) in sexually active females (228,059 nmol/mg Cr, n = 88) than in sexually inactive females (4,536 nmol/mg Cr; n = 41).

The mean urinary progesterone concentrations also depicted in Fig. 1 were significantly higher ($p = 0.001$, Mann-Whitney U-test) in sexually active females (all rQs = 35.1 ng/mg Cr, n = 96) than in sexually inactive females (all nrQs and nrfs = 12.9 ng/mg Cr; n = 47).

Steroids in non-reproductive females

The successful mating resulting in pregnancy was recorded in the females nrQ1 and rQ5. Female nrf3 was attacked by the male in the first mating session but was successfully mated afterwards with a different partner (Table 1).

The female nrQ1 and its male partner nrK1 copulated 51 times within the first half an hour. This pair showed the whole range of mating behaviour (nose to nose contact, lifting and presenting vagina etc.). Sperms were found in the first vaginal smear after the first half an hour in large amount (stage 6, cf. analogy to cell stages in colpocytology). Mating led to pregnancy and giving birth to two pups 101 days later. The mean estradiol level of this female increased after mating significantly ($p = 0.043$, Wilcoxon-rank-test), 37-fold, compared to the control, from 2,355 (range 0–9,762) to 89,233 (range 4,405–181,553) nmol/mg Cr (control phase n = 18, third phase n = 5; Fig. 2, Fig. 3). Olfactory stimulation (the second test phase) did not affect the mean estradiol level compared to control (Wilcoxon-rank-test: n = 4, $p = 0.068$; first phase = 2,355; second phase = 2,329; range 435–3,052;

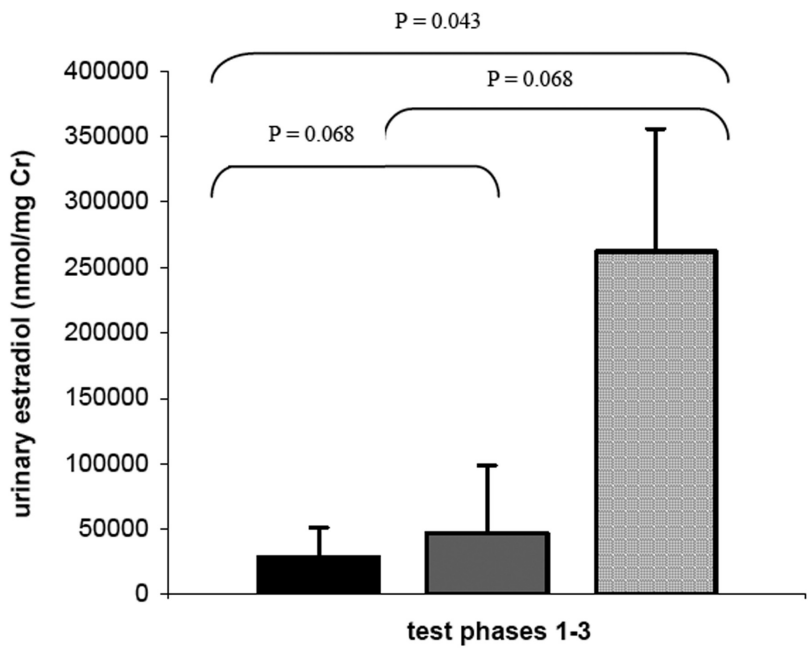

Fig. 2. Mean concentrations of urinary estradiol concentration in rQ5 measured during consecutive test-phases (test phase one to three) (Wilcoxon-rank-test ; Significance level $p < 0.050$): Black column = test phase one, grey column = test phase two, lightgrey column = test phase three.

Fig. 3). The mean progesterone concentration was not influenced by olfactory stimulation or by mating (Friedman´s rank test: n = 4, p = 0.174; Fig. 3).

Female rQ5 showed significant increase (p = 0.043, Wilcoxon-rank-test) in estradiol concentration following mating, with levels rising from 28,407 (range 809–91,849, n = 20) in the control phase to 262,059 (range 159,173–397,741, n = 4) nmol/mg Cr in the third test phase. The olfactory stimulation of female rQ5 tendentially influenced the estradiol concentration (Friedman's rank test: n = 4, p = 0.050; Fig. 2, Fig. 3). Although the progesterone increase was not significant (Friedman´s rank test: n = 4, p = 0.779) from 2nd test phase to the 3rd test phase there was a tendency towards progesterone concentration increase with a maximum peak on day 48 (Wilcoxon-test: first phase n = 20, second phase n = 4, p = 0.061, first phase = 24.3, range = 0–90,0, second phase = 38.9 ng/mg Cr, range = 19,7–33,5).

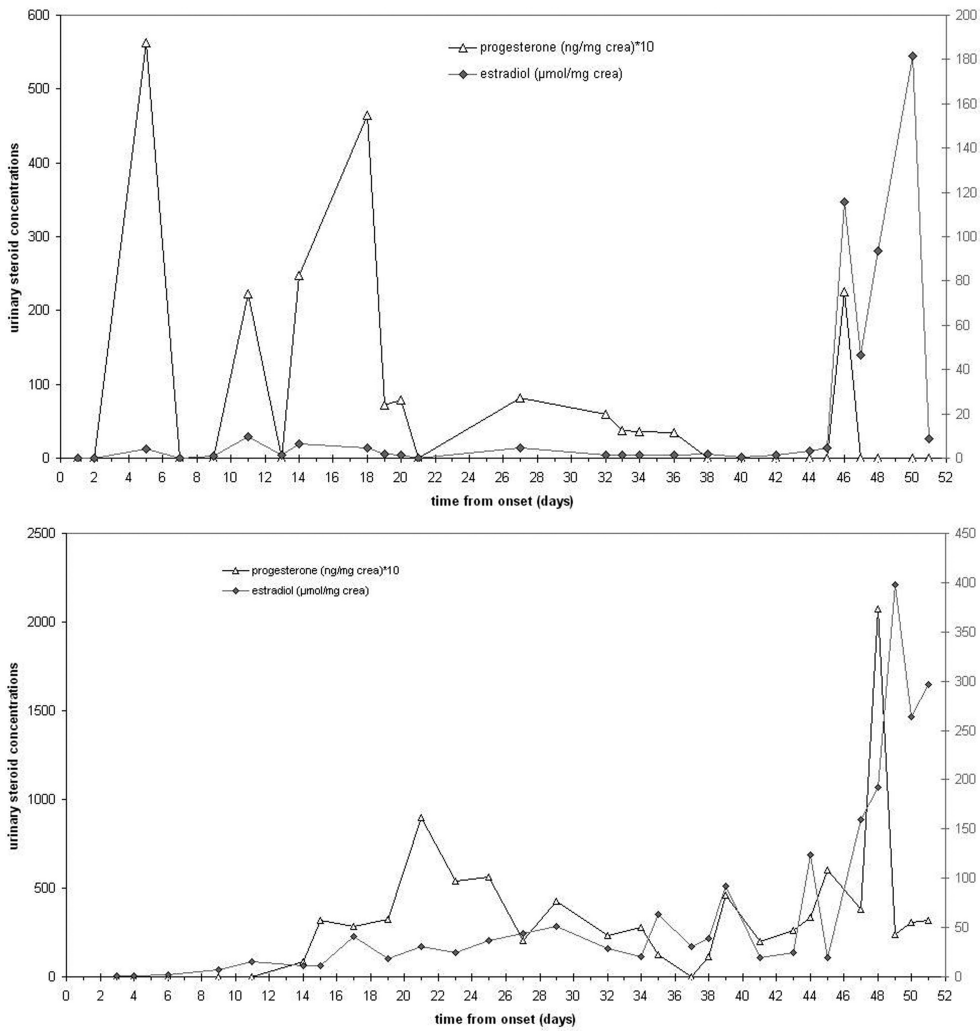

Fig. 3. Increasing urinary progesterone and estradiol concentration of two successfully mated females during consecutive test-phases.

Female nrf 3 mated successfully with its second partner, yet the levels of steroids did not change significantly. However, the steroids were measured only for a short period, namely for the last five days of the first test phase.

Sexually experienced females, rQ5 and nrQ1, were apparently primed through mating much faster than nrf3.

Discussion

Because the number of suitable animals was constrained, we studied mole-rats of two sibling species (*Fukomys anselli* and *F. kafuensis*) and their hybrids. It should be noted that these species were distinguished and formally described on chromosomal, allozyme and molecular grounds only recently (Filippucci et al. 1994, Burda et al. 1999, Ingram et al. 2004) but they do not apparently differ morphologically, behaviourally, or ecologically. Despite differences in their karyotypes (2n=58 in *F. kafuensis* and 2n=68 in *F. anselli*) they interbreed in the laboratory and produce viable offspring.

Our study demonstrates a strong correlation between high mean estradiol- and progesterone values and sexual activity. To date, there have been no reports on estrogen concentrations in *Fukomys* mole-rats. Endocrine studies in naked mole-rats (*Heterocephalus glaber*) revealed low urinary estrogen concentrations, confirming lack of ovarian cyclicity and ovulation. This block to ovulation was assumed to be due to inadequate concentrations of circulating LH (Faulkes et al. 1990, Westlin et al. 1994). Likewise non-breeding, sexually abstinent *Fukomys* mole-rat females do not have elevated estradiol levels (this study) although they exhibit all stages of follicular development (Willingstorfer et al. 1998). Although the LH-levels have not been studied here, we recorded in one (rQ5) of two successfully mated females that olfactory stimulation tend to have an increasing effect on urinary estradiol concentration (most probably via having primary effect upon increase of LH levels; Fig. 2). Chemosensation thus may have a preparing effect on estradiol production, which eventually, following successful mating, results in a strong estradiol increase, typical for ovulation (Fig. 3). Our findings demonstrate that the lack of copulation could lead to low estradiol (and progesterone – see below) levels, whereas the whole range of sexual activity resulted in an increase of circulating estradiol level.

Our analyses are partly consistent with findings of low urinary progesterone levels in non-reproductive females of *Fukomys damarensis* (Molteno & Bennett 2000), which all were housed together with their parents and were apparently sexually quiescent. Progesterone levels in non-reproductive *F. damarensis* females increased, when emancipated from their respective mothers, the queens. This finding differs from our results. In our sample, progesterone was also low in non-breeding females, however, irrespective whether they were queens (nrQ) or workers (nrf). Decisive was the fact, whether they had mating opportunities (and were mated) or not. In *F. damarensis,* the queen's absence seemed to be sufficient to increase progesterone levels (Molteno & Bennett 2000). Apparently, in *F. anselli* and *F. kafuensis* elevated circulating progesterone in reproductive active females reflects primarily their sexual behaviour. The primary effect of sexuality upon higher progesterone level is strongly supported by findings in the copulating but non-breeding female queen, rQ5 (Fig. 3). Two progesterone peaks may be indicative for ovulation (initiating the luteal phase of the estrus) triggered by repeated copulation. It should be noted, that mating opportunity was reflected in generally higher urinary steroid levels in rQ5 as compared to female nrQ1 living in without a male (Fig. 3).

It seems that sexually experienced rQ and nrQ could be primed by sexual and probably also by olfactory stimulus faster than nrf. However, it cannot be excluded that aggressive behaviour of the first male partner suppressed the priming effect in subsequent mating in female nrf3.

Our present findings bring strong support for existence of induced ovulation in *Fukomys* mole-rats and are consistent with previous findings and suggestions by W i l l i n g s t o r f e r et al. (1998) and B u r d a (1999). Assessment of LH levels in females with different sexual experience and activity are needed to complement our understanding.

Since nucleated cells are the major cell population in the smear in rats at proestrus and develop into cornified cells (M a e d a et al. 2000), we tried also to find periodical correlation between occurrence and frequency of nucleated and cornified cells. However, cornified cells were not found to be a reliable indicator in mole-rats. This cell type was always present but never represented the major cell population in smears, probably due to mechanical abrasion of the vaginal epithel. Due to a missing spontaneous ovulation cycle, colpocytology showed no cyclic change of cellular composition. We have not found a cytological picture of an estrus. Even after successful mating, no correlation between induced ovulation and the frequency of typical estrus-like cells (increase of cornified cells, decrease of nucleated cells and decrease of leucocytes in late proestrus and estrus) could be detected. Colpocytology therefore is not an applicable method to examine estrus in the studied *Fukomys* mole-rat species.

In summary, the present study shows marked impact of sexual acitivity on female steroid levels. Although olfactory stimulation of females appears to increase the estradiol level, it is not sufficient to reach high estradiol level registerd after successful mating. This study supports conclusions of previous studies (W i l l i n g s t o r f e r et al. 1998, B u r d a 1999) that *Fukomys* mole-rats are induced ovulators, primed exclusively through repeated and regular sexual activity and not solely through as single copulation or absence from the queen. Prolonged olfactory stimulation could lead to stronger outcomes.

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