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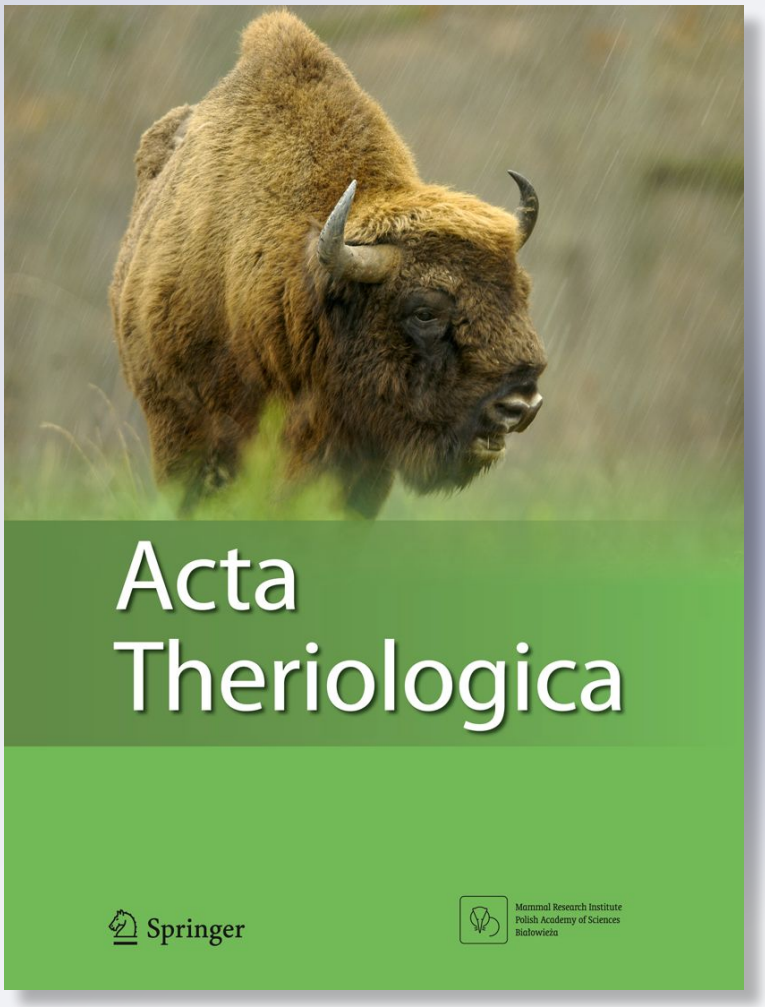


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ORIGINAL PAPER

# Female reproductive behaviour, ovarian hormones and vaginal cytology of the induced ovulator, *Ctenomys talarum*

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**Abstract** In the present study, we evaluated whether reproductive condition affects female reproductive behaviour in the induced ovulator *Ctenomys talarum*. We also explored the effect of the interaction with a male on the reproductive condition of females. To evaluate this, we arranged mating trials and evaluated female reproductive behaviour. Reproductive status of females was evaluated using a combined approach of vaginal smears, urinary progesterone and oestradiol, and ovarian histology. Behaviours denoting attraction ('male sniff' and 'mount attempts') and mutual courtship behaviours ('spin' and copula) were correlated with vaginal cytology before and oestradiol and progesterone levels in urine 12 h after male–female encounter. After 24 h of the interaction, oestradiol levels and vaginal epithelization increased while progesterone levels decreased in soliciting females. *C. talarum* females' reproductive behaviour was related to its physiological reproductive state and vaginal cytology. The kind of male interaction, whether couples copulated or remained indifferent affected the later status of females. Females are induced ovulators by mating but male presence and interaction also affected other components of their reproductive physiology such as ovarian hormones and vaginal cytology.

**Keywords** *Ctenomys* · Estrous behaviour · Induced ovulation · Ovarian hormones · Vaginal cytology

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## Introduction

Successful reproduction relies on a precise neuroendocrine coordination of reproductive physiology and stimuli from social and physical environment. Particularly in mammals, timing of ovulation is crucial for reproductive success. Female mammals can be divided in two groups according to their type of ovulation: those that ovulate spontaneously, in response to hormonal changes and those named induced or reflex ovulators, where ovulation is triggered by stimuli associated with mating activity or male presence (Bronson 1989; Cohen-Parsons and Carter 1988; Hikim et al. 1991; Ramirez and Soufi 1994; Kauffman and Rissman 2006). The underlying difference between those groups is based on the ability to generate (spontaneously or stimulated by the male) the hypothalamic release of Gonadotropin-releasing hormone and the subsequent Luteinizing hormone 'ovulatory' surge (Bakker and Baum 2000; Kauffman and Rissman 2006; Beyer et al. 2007). Tactile stimulation of the vagina during copula appears to be the primary trigger of ovulation, while other cues (e.g. chemical, visual and/or auditive) often enhance (or even trigger themselves) the ovulatory response (Ramirez and Soufi 1994).

Female attractivity (eliciting male response), proceptivity or solicitation (initiating sexual activity) and receptivity (responsiveness needed to achieve copula) are behavioural components of female reproductive behaviour or behavioural oestrous (Beach 1976). While most species of reflex ovulators show short and well defined periods of behavioural oestrus (domestic cat, *Felis catus*, Wildt et al. 1981), others remain in oestrus for up to several months or never display receptivity unless chemical cues from conspecific males are available (e.g. female ferrets, *Mustela furo*, Marshal 1904 in Bakker and Baum (2000)). These examples reveal a wide range of variation in the duration and periodicity of behavioural

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oestrus, which are strongly influenced by environmental (e.g. photoperiod) and social cues (e.g. pheromonal and tactile stimuli from males; Bakker and Baum 2000). A finely tuned coordination of such cues and hypothalamus–pituitary–gonadal axis is required to produce mature ova. These developing follicles produce high levels of oestradiol which act on the brain to promote the display of reproductive behaviour. Whereas in most spontaneous ovulating species, behavioural oestrus is prompted by the action of endogenous oestradiol and progesterone (Kauffman 2010), in induced ovulating species, little evidence is available for progesterone effect on behavioural oestrus. Instead, oestradiol appears to be the main endocrine stimulus acting on the brain to promote reproductive behaviour. Since studies on regulation of behavioural oestrus are scarce for many induced ovulators, additional species should be examined to obtain more generalised conclusions about the role of oestradiol and progesterone (Kauffman and Rissman 2006).

Mating-induced ovulation is found in a growing variety of mammals, indicating that this pattern is as common as spontaneous ovulation (Conaway 1971; Ramirez and Soufi 1994; Kauffman and Rissman 2006). It also appears to be the rule among solitary rodents, occurring at low densities and/or at highly seasonal environments (Zarrow and Clark 1968; Conaway 1971; Milligan 1982). Solitary subterranean rodents are mostly induced ovulators, (Bennett et al. 2000), while social species show both strategies of ovulation (spontaneous ovulators, e.g. *Heterocephalus glaber*; induced ovulators, e.g. *Cryptomys hottentotus pretoriae*, *Cryptomys hottentotus natalensis*, *Fukomys mechowii*, and *Fukomys anselli*; Faulkes et al. 1990, 2010; Willingstorfer et al. 1998; Malherbe et al. 2004; Jackson and Bennett 2005; Hagemeyer 2009). As solitary subterranean rodents are highly aggressive, reproduction represents a short event in which the harsh interactions that frequently occur between intersexual conspecifics become more amicable. During this period, individuals must overcome the limitations imposed by the subterranean environment to communicate their mating purposes (Bennett et al. 2000). Despite most of subterranean species are solitary, the majority of studies on reproductive behaviour refer to social species. Then, reproductive behaviour is almost unknown for solitary subterranean rodents, probably as consequence of their secretive habits in the field and the logistic difficulties of breeding these animals in captivity. Mating behaviour has only been described for a few solitary subterranean species: *Nannospalax ehrenbergi*, *Ctenomys rionegrensis*, *Ctenomys mendocinus*, *Heliophobius argenteocinereus*, and *Ctenomys talarum* (see Nevo 1969; Altuna and Lessa 1985; Shanas et al. 1995; Camin 1999; Bennett et al. 2000; Šumbera 2001; Fanjul and Zenuto 2008a). Furthermore, the physiological basis of oestrus behaviour has not been studied in any species of solitary subterranean rodents exhibiting induced ovulation.

*Ctenomys talarum* (tuco-tuco) is a subterranean herbivore that occupies burrows solitarily (Busch et al. 1989). Ovulation appears to be induced (Weir 1974) since only mated females presented corpora lutea but no details about the source of these data was given. Indeed, several findings support copula-mediated mode of ovulation. Females did not show spontaneous vaginal oestrus over a 6-month period (Fanjul and Zenuto 2008b). Penile morphology is characterised by the presence of spines and spikes (Balbontin et al. 1996), consistent to induced ovulation (Parag et al. 2006). Finally, only mated females that experienced a full copulatory period ovulated (Fanjul and Zenuto 2008a). Breeding season of *C. talarum* extends from autumn up to midsummer (Malizia and Busch 1991) and females adjust their reproductive activity according to temperature and food availability (Fanjul et al. 2006). Populations are characterised by a polygynous mating system (Zenuto et al. 1999). Courtship includes mate assessment involving scents, body contact and specific vocalisations (Zenuto et al. 2002, 2007; Schleich and Busch 2002; Zenuto and Fanjul 2002; Fanjul et al. 2003). Females familiarised with male odours showed some reduction of their aggression during courtship but preferred unfamiliar males as mates (Zenuto et al. 2007). Male odours did not stimulate changes in vaginal smear parameters indicative of receptive condition in females (Fanjul and Zenuto 2008b). Still, male chemical cues or even body contact could cause enhanced follicular development not detected in vaginal smears, as it was found for the social *C. hottentotus pretoriae* (Malherbe et al. 2004). The complex link between the patterns of behavioural oestrous (attraction, solicitation and receptivity), vaginal cytology and hormonal status has not been explored in the genus *Ctenomys*. Moreover, changes in these parameters after male–female interactions are key information to understand the breeding process of solitary subterranean rodents. The present study was aimed to evaluate in *C. talarum* females: (1) whether reproductive status affects a female's reproductive behaviour and (2) whether the interaction with a male affects a female's reproductive condition. We addressed these subjects by using a combined approach of behavioural observations, hormonal analysis, vaginal cytology and ovarian histology. Since vaginal epithelium is responsive to sex steroids (e.g. Bacha and Bacha 2000), an association between vaginal cytology and ovarian hormones was expected. As reported for most induced ovulator species, we predicted a positive relationship between oestradiol, and possibly progesterone, and soliciting female sexual behaviours. In the same way, vaginal cell proportions may also be related to soliciting female sexual behaviours. Moreover, male presence would enhance other components of reproductive physiology, such as hormonal levels and/or the stimulation of follicular development. Corpora lutea might be only found in mated females.

## Material and methods

### Animal capture and housing conditions

Live trapping was used to collect mature *C. talarum* individuals in Mar de Cobo (37°45′S, 57°26′W), Argentina. Sexual maturity of captured individuals was estimated by body weight (Malizia and Busch 1991). Females were captured during their non-breeding season (March to May 2005) to avoid the influence of previous reproductive activity, while males were captured during the reproductive season (June to December). Since males are the dispersal sex (Malizia et al. 1995), genetic relatedness between sexual partners is highly unlikely in the study population which was confirmed by genetic similarity analysis (Zenuto et al. 1999). All animals were transported to the laboratory, where each tuco-tuco was individually housed in a plastic cage measuring 42×34×26 cm, with 3 cm of wood shavings for bedding. Photoperiod and temperature were automatically controlled (12:12 L/D; 25±1°C). Fresh food (carrots, sweet potatoes, catalogna chicory, corn, mixed grasses and sunflower seeds) was provided ad libitum every day to secure water provision since *C. talarum* does not drink free water. Males were allowed to adapt to captivity for at least 5 days before they were used in mating trials. Twenty females and 16 males participated in 20 mating trials. Males were employed in more than one mating trial with at least 10 days interval between subsequent encounters.

### Mating trials

Female reproductive behaviour was evaluated according to Zenuto et al. (2007) and Fanjul and Zenuto (2008a). As it was observed in a previous study (Zenuto et al. 2007), mating trials were characterised mainly by indifference and agonistic behaviours when mating partners were randomly assigned. In contrast, the display of reproductive behaviour was increased when females were allowed to choose between two males, the one that would act as her partner at the time of male–female social interaction (mating trials). To allow each female to show her preference between two males, a transparent acrylic T-maze was connected to the female's box. At the ending of each arm, one male was confined by using a wire mesh barrier. Each female was allowed to explore the maze and select a male. The preferred male for each female was the one to whom more time in close contact with and/or more interest she manifested. The preferred male was assigned to the female as her potential partner during the mating trial. For details of this procedure, please see Zenuto et al. (2007).

The experimental apparatus used during mating trials consisted of three acrylic cages (45×30×30 cm) that were connected to each other by an acrylic tube (10 cm diameter×20 cm length; Zenuto et al. 2007). The test female and its preferred male were individually confined (1-h adaptation time) in one of the cages, which contained soiled shavings from their respective housing cages. At the start of each trial, both animals were allowed to enter to the central cage (neutral space), containing clean shavings. Mating trials lasted 30 min. All tests were performed at mid-afternoon and videotaped under white light conditions. The cages and tubes used were carefully cleaned with odourless detergent, wiped with ethanol, and then allowed to air dry to ensure that no odours from previous trials remained. Latex gloves were worn when handling animals, cages and tubes in order to avoid human scent transfer.

### Vaginal smears

The cytological examination of vaginal smears is a widely used tool to estimate reproductive status based on the proportion of each cellular type found. Vagina is lined by a squamous stratified epithelium and then, its smear contains desquamated epithelial cells but also infiltrated leukocytes from the mucosa layer. Vaginal epithelium is responsive to sex steroids; hormonal variation cause the vaginal epithelium become 'cornified', i.e. epithelial cells suffer from gradual cornification (keratinisation) and nucleus pyknosis. Epithelial cells in different stages can be classified into two basic groups according to their degree of cornification and nucleus presence: nucleated (round cell with nucleus) and cornified (fully cornified cells without nuclei) (Bacha and Bacha 2000).

Smear samples for each female were taken at three times: before starting the mating trial, immediately after it concluded and 24 h after the trial. Smears were taken by inserting a cotton-tipped swab moisturised with sterile physiological saline solution into the vagina and rolled it along the length of a clean glass microscope slide. The smears were fixed in 95° ethanol for at least 5 min and then stained with Giemsa solution (MacFarlane and Taylor 1982). For each smear, at least five fields and a minimum of 100 cells were counted at ×450 magnification. The number of nucleated cells, cornified cells and leukocytes was recorded for each sample. We estimated the degree of epithelization as the proportion of epithelial cells (number of nucleated cells+number of cornified cells/number of epithelial cells+number of leukocytes) and cornification index (CI=number of cornified cells/number of epithelial cells), respectively.

### Urinary hormonal assays

Hormonal levels were estimated by their respective metabolites excreted in urine of females. Urine samples from each female were collected to determine progesterone and oestradiol levels. Samples were taken overnight using metabolic cages similar to that described by Drożdż

(1975) at three times: before (12 h before), after (12 h after) and 24 h after the mating trial. These samples were collected for a period of 12 h in a glass vial containing mineral oil and were discarded if they were contaminated with faecal material. Collected urine was stored at −20°C until hormone determinations were performed. Since urine concentration varies with fluid intake, concentration of oestradiol and progesterone were corrected in relation to creatinine concentrations, which is excreted at relatively constant rate (Schmidt-Nielsen 1997). Hormone concentrations were expressed as nanogram or picogram per milligram creatinine (xg/mg Cr).

Determinations of oestradiol and progesterone were performed by Elecsys 2010 (Roche Dignostics Corp, Indianapolis, IN, USA), an automatized electrochemiluminescence immunoassay method and Cobas (Roche Dignostics Corp, Indianapolis, IN, USA) reagent kit. Urinary oestradiol assays have a sensitivity of 5 pg/ml with an intrassay coefficient of variation (CV) of 1.6% and an interassay CV of 2.3%. Urinary progesterone assays have a sensitivity of 0.03 ng/ml with an intrassay CV of 1.7% and an interassay CV of 3.7%. Assays sensitivities and coefficients of variation are reported as provided by the manufacturers. Automatized Jaffe kinetic method in the Architect 8000 I (Abbott) was employed to estimate creatinine concentration. All assays were performed by Laboratorio Biomédico Dr. Rapela (Argentina).

## Ovarian histology

Qualitative observations were made of the ovaries from a subgroup of females ($n$=17) used in this study. Ovaries were removed the fifth day after the trial, placed in formalin and treated by standard histological techniques (Drury and Wallington 1967): dehydrated in ethanol, embedded in paraffin wax, sections of 6 μm thick were mounted semi-seriated, and stained with hematoxylin-eosin. Both ovaries were examined for follicular development and presence of corpora lutea using a light microscope at ×450 magnification. Follicles were differentiated in primary, secondary, tertiary (Willingstorfer et al. 1998) and luteinized unruptured follicles (LUF; Weir and Rowlands 1974).

## Behavioural observations and data analysis

Females presented a diverse array of behaviours to convey their willingness to mate, approach and attract male attention. To describe this complex process, we recorded female recognition behaviour, individual solicitation behaviours, male attraction behaviours, and mating behaviours showed by the interacting couple during mating trials (Table 1). The absolute frequency of these behaviours was recorded as in previous studies dealing with reproductive and territorial behaviour in *C. talarum* (Zenuto et al. 2007; Zenuto 2010). The relationship between each behaviour with hormonal levels and vaginal smear composition was evaluated by Spearman correlation tests (Zar 2010).

Three levels of reproductive behaviour were defined on the basis of the frequency of solicitation behaviours and copula occurrence recorded during mating trials: (a) mated ($n$=6), in the case that copula was observed; (b) soliciting ($n$=9), when a female displayed at least three different solicitation behaviours with a total frequency over 15 and (c) indifferent ($n$=5), when none of the later two conditions were met (Table 2). To evaluate if females assigned to these three categories differed in their

**Table 1** Courtship behaviours recorded during *Ctenomys talarum* mating trials

| Actor | Type of behaviour | Description |
|---|---|---|
| Female | Recognition | |
| | Sniff rump | Female sniffs the male hindquarters or genitalia |
| | Solicitation | |
| | Follow | Female pursues the male |
| | Push | Female pushes the flank of her partner, promoting close contact to him |
| | Present rump | Female shows her rump when she encounters the male |
| | Female mount | Female mounts the male |
| | Raising tail | Female raises her tail exposing her genitalia to the male |
| Male | Attraction | |
| | Sniff female | Male sniffs the female, mainly the hindquarters or genitalia |
| | Mount attempt | Male mounts the female in an attempt to achieve mating position |
| Couple | Mutual courtship | |
| | Spin | Male and female sniff anogenital area and/or attempt to mount each other at same time resulting in circling movement |
| | Copula | |

**Table 2** Individual occurrence (×) and group mean (±SD) frequency of behaviours displayed by *Ctenomys talarum*; data are presented in three levels of reproductive behaviour identified during mating trials

| Level of behaviour | Female ID | Behaviours | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Recognition Sniff rump | Solicitation Follow | Push | Present rump | Female mount | Raising tail | Male Sniff female | Mount attempt | Couple Spin | Copula |
| Mated | 3j | × | × | × | × | × | × | | × | × | × |
| | 8j | × | | × | × | | × | × | × | | × |
| | 9j | × | × | × | × | × | | × | × | × | × |
| | 17 | × | × | × | × | × | × | × | × | | × |
| | 1 | × | × | × | × | × | × | | × | × | × |
| | 6 | × | | | | | × | × | × | × | × |
| | 15 | × | | × | × | × | × | × | × | × | × |
| Mean±SD | | 16.86±11.48 | 3.57±5.16 | 6.43±6.68 | 10.14±7.51 | 9.43±15.23 | 3.86±2.67 | 40.4±10.71 | 44.71±29.8 | 5.14±7.64 | 2.86±1.95 |
| Soliciting | 3 | × | × | × | × | × | × | × | × | × | |
| | 7 | × | × | × | × | × | × | × | × | | |
| | 16 | × | | × | × | × | × | × | × | | |
| | b | × | × | × | × | × | × | × | × | × | |
| | 11 | × | | × | × | × | × | × | × | × | |
| | 10 | × | × | × | × | × | × | × | × | × | |
| | 12 | | | × | × | | × | × | × | | |
| | 5 | | | × | × | | × | × | × | | |
| | 4 | × | | × | × | | × | × | × | | |
| Mean±SD | 16±10.57 | 1.22±1.78 | 14.55±20.96 | 14.33±7.86 | 1.22±1.39 | 3.77±2.77 | 16.66±10.08 | 8.44±12.26 | 3.25±5.6 | 0 | |
| Indifferent | 9 | × | | | | | | × | | | |
| | 13 | × | × | × | | | | × | | | |
| | 14 | × | | × | | | | × | | | |
| | 8 | × | × | | | | | × | | | |
| | 2 | × | × | | | | | | | | |
| Mean±SD | 9.2±7.52 | 0 | 3.2±3.63 | 1.4±2.6 | 0 | 0 | 8±6.44 | 0 | 0 | 0 | |

hormonal levels (progesterone, oestradiol) and/or vaginal smear composition (cornification index and proportion of epithelial cells) at each time in relation to mating trial, parametric or non-parametric analysis of variances (ANOVAs) were used depending if data fit normality and homocedasticity assumptions (Zar 2010). Also, to evaluate the effect of male interaction during mating trials, we calculated the change of hormonal levels and vaginal smear variables before and after mating trials (thereafter, 'change'). Differences among these groups were analysed using a parametric or non-parametric ANOVA depending if data fit normality and homocedasticity assumptions (Zar 2010). Post hoc multiple comparisons were reported in the figures along with effect sizes (see ahead). Finally, the relationship between hormonal levels and vaginal smear composition was evaluated by a Spearman correlation (Zar 2010). All tests were performed on software STATISTICA 7.0.

For all tests, the critical significance level was set at $p \leq 0.05$. Along with the exact $p$ values, we reported the observed effect sizes (ES) according to the suggestions of Nakagawa (2004) for behavioural sciences. Cohen (1992) defined effect sizes as 'a scale-free value that measure, in terms appropriate to it, the discrepancy between $H_0$ and the $H_1$'. Here, we reported effect size estimators from $r$ family (Ellis 2010). In the case of ANOVAs, we reported ES Cohen's $f$ (Ellis 2010). For each post hoc multiple comparison, we estimated Cohen's $d$ and transform it to $r$ (Ellis 2010). In three cases, we run the non-parametric homologous test; since there is no way to estimate effect size for this non-parametric test, we estimate ES for post hoc multiple comparison as $r=Z/(n)^{1/2}$ (Rosenthal 1994). For correlation analysis, Spearman correlation coefficient ($r_s$) constitutes itself an estimator of effect size (Ellis 2010).

We assessed differences in the proportion of females that presented a given ovarian structure (tertiary follicles, LUFs or corpora lutea) using a multiple pair comparison within each level of reproductive behaviour (indifferent, soliciting and mating) by a binomial test (Zar 2010).

## Ethical note

We adhered to the Guidelines for the Use of Animals in Research and Teaching (ASAB/ABS 2003). The present project was approved by Argentina's research agency, the Consejo Nacional de Investigaciones Científicas y Técnicas, and the Universidad Nacional de Mar del Plata.

## Results

Male–female interactions started with substantial amount of agonistic approaches (threatening and showing incisors)

**Table 3** Spearman rank correlation matrix for frequency of behaviours and urinary oestradiol, progesterone and vaginal smear parameters at different times of mating encounters

| | | Progesterone | | | | Oestradiol | | | | Cornification index | | | | Epithelization | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Actor | Behaviours | Before | After | Change | 24 h | Before | After | Change | 24 h | Before | After | 24 h | | Before | After | 24 h | |
| Female | Sniff rump | 0.13 | 0.27 | 0.06 | −0.36 | −0.01 | 0.30 | 0.25 | 0.21 | −0.16 | 0.40 | −0.38 | | 0.04 | 0.26 | 0.38 | |
| | Follow | −0.22 | 0.04 | 0.27 | −0.45* | −0.20 | 0.10 | 0.19 | 0.55** | 0.03 | 0.38 | −0.03 | | 0.08 | 0.11 | 0.22 | |
| | Push | 0.16 | 0.28 | −0.01 | −0.48* | −0.06 | 0.37 | 0.40 | 0.10 | −0.28 | 0.13 | −0.53* | | −0.13 | −0.05 | 0.14 | |
| | Present rump | −0.28 | 0.01 | 0.23 | −0.30 | −0.41 | 0.02 | 0.30 | 0.23 | 0.01 | 0.14 | −0.15 | | 0.00 | 0.10 | 0.51* | |
| | Female mount | −0.33 | 0.31 | 0.50* | −0.54** | −0.36 | 0.33 | 0.43* | 0.60** | 0.17 | 0.44* | 0.23 | | 0.28 | 0.41 | 0.57** | |
| | Rise tail | 0.00 | 0.02 | 0.14 | −0.22 | −0.08 | 0.04 | 0.15 | 0.22 | −0.14 | −0.08 | −0.19 | | −0.14 | 0.05 | 0.41 | |
| | Total frequency of solicitation | −0.12 | 0.26 | 0.35 | −0.48* | −0.332 | 0.26 | 0.45* | 0.45* | −0.05 | 0.20 | −0.20 | | −0.03 | 0.11 | 0.60** | |
| Male | Sniff female | 0.12 | 0.63** | 0.26 | −0.16 | −0.11 | 0.60** | 0.47* | 0.04 | 0.49* | −0.02 | −0.23 | | 0.19 | 0.32 | 0.49* | |
| | Mount attempt | −0.06 | 0.46* | 0.44* | −0.42 | −0.12 | 0.41 | 0.36 | 0.22 | 0.13 | 0.52* | −0.46 | | 0.22 | 0.46* | 0.49* | |
| Couple | Spin | −0.20 | 0.55** | 0.69** | −0.45* | −0.26 | 0.58** | 0.64** | 0.24 | −0.00 | 0.43 | −0.5* | | 0.15 | 0.22 | 0.61** | |
| | Copula | −0.12 | 0.42 | 0.37 | −0.04 | −0.14 | 0.16 | 0.07 | 0.07 | 0.53** | 0.42 | −0.01 | | 0.56** | 0.73** | 0.38 | |
| $n$ | | 20 | 20 | 20 | 19 | 20 | 20 | 20 | 19 | 20 | 19 | 17 | | 20 | 19 | 18 | |

For each relationship, Spearman correlation coefficient ($r_s$) is presented

*$p \leq 0.05$

**$p \leq 0.01$

followed by recognition behaviours, mainly sniffing its partner's rump area. This recognition phase was followed by behaviours that denoted the female soliciting mating or the male attraction. Soliciting behaviour in females included: snout pushes, following the male, mounting the male, exposing the posterior area, and raising her tail exposing the genitalia. Much less frequently, and then not included in further analysis, females presented their neck and scent marked by rubbing their anogenital area on the tubes or different parts of the boxes (1.37±1.54 and 1.31±2.18, respectively, for soliciting females). The females that showed no interest for the male (indifferent) remained still and avoided the encounters with the male. When both partners were willing to mate, a more advanced phase of courtship took place by mutual courtship behaviours as spin and/or copula. Please see Table 2 for more details.

Progesterone and oestradiol levels in urine samples taken before the male–female encounter were not correlated to the frequency of any recorded behaviour. Both, the cornification index and the proportion of epithelial cells before the interaction correlated positively with the frequency of copulations (Table 3). After the encounter, both progesterone and oestradiol levels in urine showed a positive relationship with the frequency of attraction behaviours and/or mutual courtship behaviours. The same pattern was detected for the cornification index and the proportion of epithelial cells. The change detected in hormone levels in urine samples collected before and after mating trials was positively correlated to female mounting behaviour, male mounting attempt and 'spin' behaviour. After 24 h of interaction, progesterone and cornification index were negatively correlated to several soliciting behaviours and/or mutual court 'spin' behaviour. Oestradiol level in urine and epithelial cell proportion were positively related to several soliciting behaviours and mutual court behaviours. Please see Table 3 for detailed results.

Epithelization of vaginal smears, but not progesterone, oestradiol levels or cornification index, prior to the male–female encounter revealed that reproductive status is related to reproductive behaviour (mated, soliciting and indifferent) in *C. talarum* females. Progesterone and oestradiol levels showed no differences in relation to the female behaviour (Fig. 1a and b, ANOVA, $F=0.938$, $df=2$, $p=0.410$, Cohen's $f=0.332$; $F=1.411$, $df=2$, $p=0.271$,

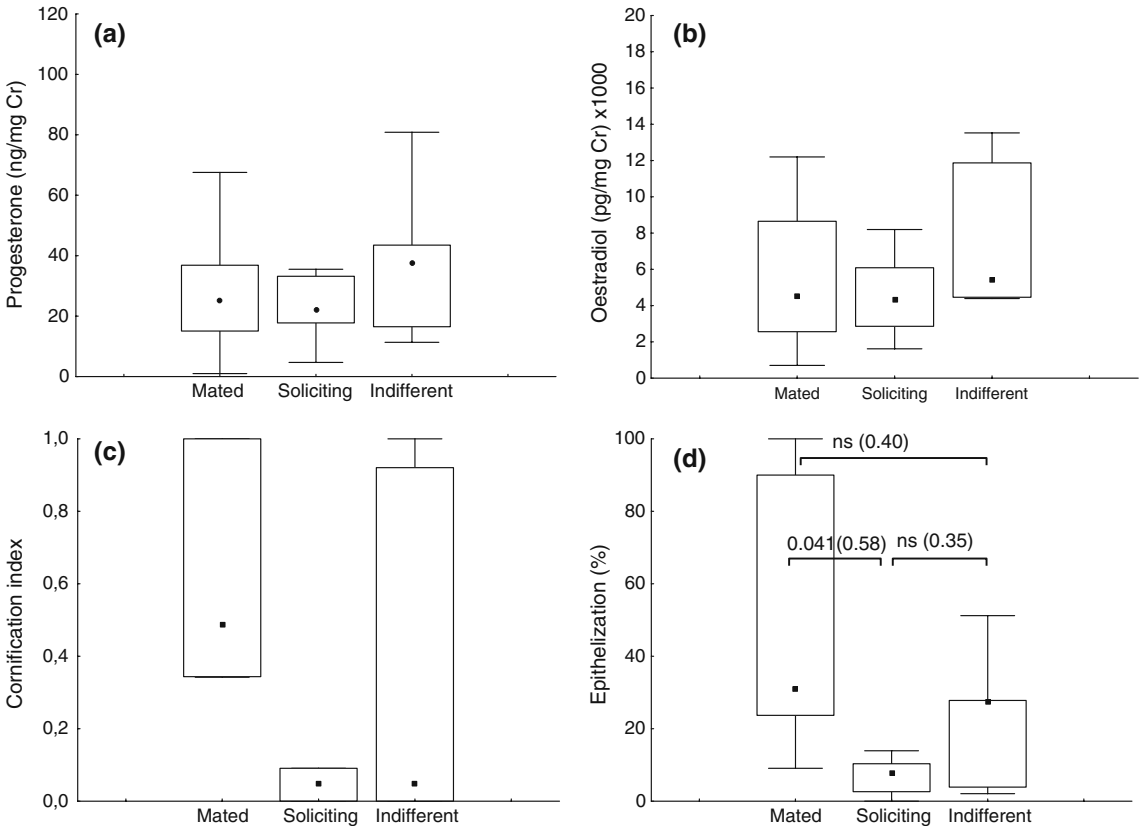


**Fig. 1** Urinary level of progesterone (**a**) and oestradiol (**b**), vaginal smear cornification index (**c**), and percentage of epithelization (**d**) for *C. talarum* females before mating encounters. Here and thereafter: mated, soliciting and indifferent represents levels of female reproductive behaviour displayed during the mating encounters. *Boxes* represent 25th and 75th percentiles, *vertical lines* represent non-outlier range, and *point inside boxes* represents the median. Post hoc multiple comparisons are reported when corresponds indicated by lines along with $p$ values ≤0.05 and effect sizes' $r$ (within parenthesis)

Cohen's $f$=0.407, respectively). Cornification index did not present significant differences either (Fig. 1c, $F$=1.523, $df$=2, $p$=0.246, Cohen's $f$=0.423). However, the percentage of epithelial cells differed between groups (Fig. 3d, $F$=5.593, $df$=2, $p$=0.013, Cohen's $f$=0.811). Mated females showed significant differences with soliciting ones but not with indifferent females nor between soliciting and indifferent females (please see post hoc multiple comparison results at Fig. 1d).

After the interaction with males, progesterone concentrations differed between groups ($F$=3,838, $df$=2, $p$=0.042, Cohen's $f$=0.671) and significant differences were detected for mated females in relation to the soliciting and indifferent groups, but not between the two last ones (please see post hoc multiple comparison at Fig. 2a). Oestradiol concentrations did not vary in a significant way ($F$=2.060, $df$=2, $p$=0.158, Cohen's $f$=0.492); but mated and soliciting females showed higher oestradiol concentrations than those detected in indifferent females (Fig. 2b). There were no significant changes in the cornification index (Kruskal–Wallis, $H$=3.532, $df$=2, $p$=0.171) following the interaction with the males (Fig. 2c). In contrast, degree of epithelization appeared affected ($H$=7.683, $df$=2, $p$=0.021) and significant differences were detected for mated females in relation to the soliciting and indifferent groups, but not between the two last ones (please see post hoc multiple comparison at Fig. 2d).

We also analysed the changes in the urinary hormone levels as well as vaginal smear composition. Progesterone concentrations differed significantly among groups ($F$=5.656, $df$=2, $p$=0.013, Cohen's $f$=0.815). However, significant differences were detected only between the indifferent and mated females (please see post hoc multiple comparison at Fig. 3a). Oestradiol concentrations also varied significantly among groups ($F$=4.117, $df$=2, $p$=0.035, Cohen's $f$=0.695). Significant differences were detected between indifferent and mated females (please see post hoc multiple comparison at Fig. 3b). No significant changes in the cornification index and percentage of epithelial cells were detected after the interaction with males (CI: $F$=0.255, $df$=2, $p$=0.778, Cohen's $f$=0.178; epithelization: $F$=1.152, $df$=2, $p$=0.341, Cohen's $f$=0.379; Fig. 3c and d). Finally, there were no significant differences in hormone levels or vaginal epithelium composition between groups in samples collected 24 h after the interaction (progesterone, $F$=1.391, $df$=2, $p$=0.277, Cohen's $f$=0.417; oestradiol, $F$=0.1949, $df$=2, $p$=0.354, Cohen's $f$=0.275; CI: $H$=1.7333, $df$=2, $p$=0.420; epithelization, $F$=3.623, $df$=2, $p$=0.052, Cohen's $f$=0.695).

We assessed the relationship between urinary hormones and vaginal cytology using Spearman correlations as detailed

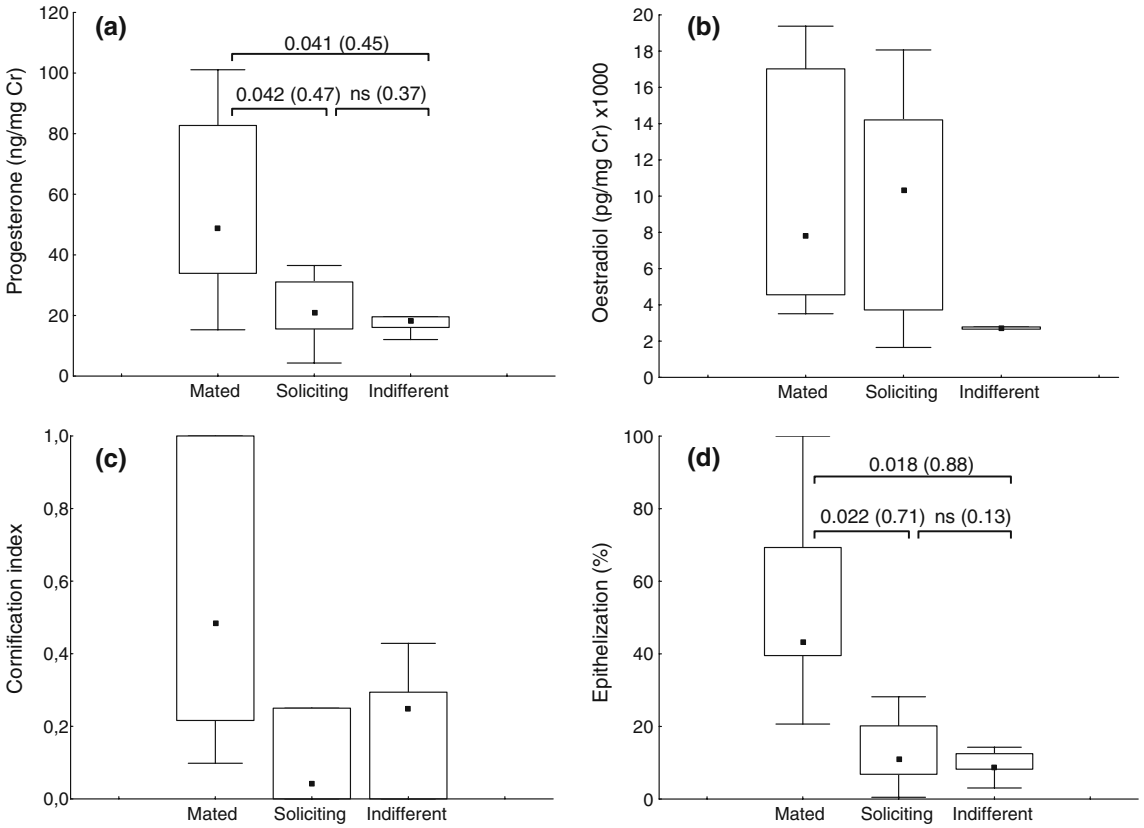


**Fig. 2** Urinary level of progesterone (**a**) oestradiol (**b**), vaginal smear cornification index (**c**), and percentage of epithelization (**d**) for *C. talarum* females after mating encounters

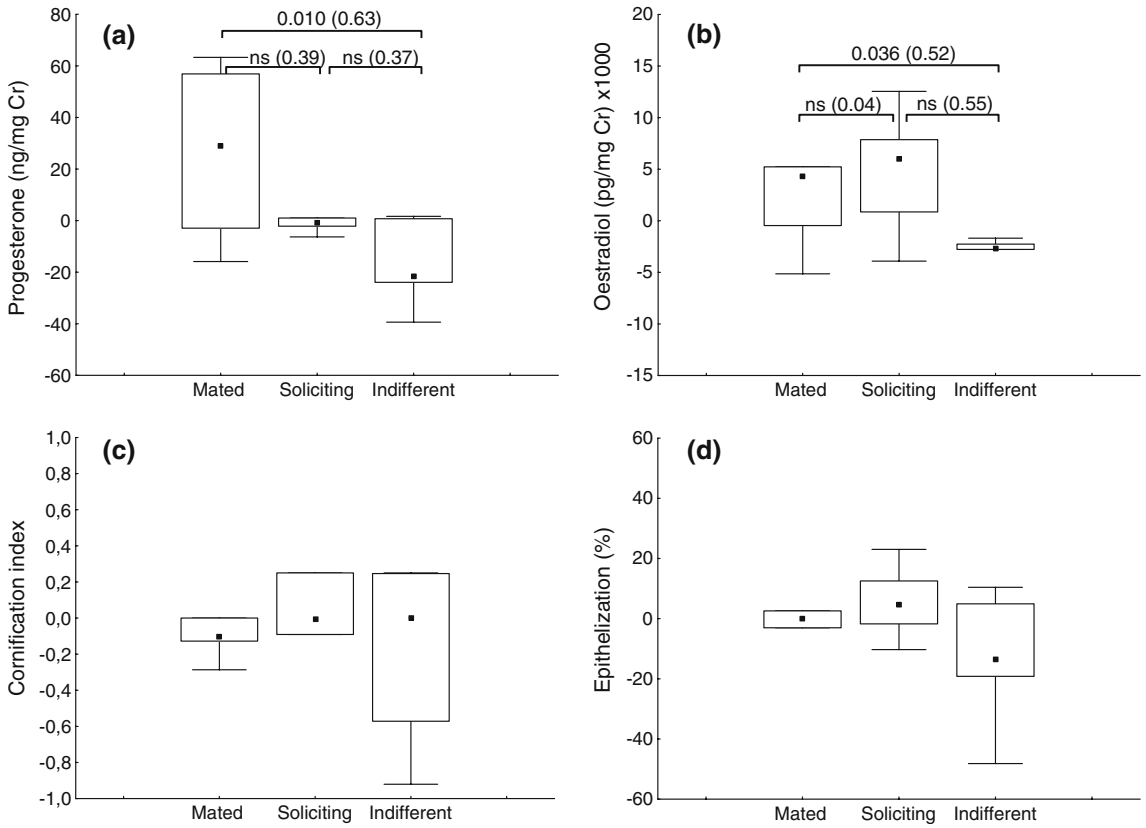


**Fig. 3** Changes (after–before) of urinary level of progesterone (**a**) and oestradiol (**b**), vaginal smear cornification index (**c**), and percentage of epithelization (**d**) for *C. talarum* females

in Table 4. Urine progesterone and oestradiol concentrations were no correlated with cornification index or proportion of epithelization before mating trials. However, positive relationships were detected between the concentrations of both hormones obtained after the interaction with the male and also for the degree of epithelization registered 24 h after the interaction. Oestradiol concentration before the encounter was related to cornification index 24 h after that. Changes in progesterone and oestradiol levels showed a significant correlation with the proportion of epithelization in vaginal smears recorded 24 h after the interaction. In the same way, changes in progesterone levels were correlated to the cornification index after the interaction

Qualitative analysis of ovarian histology showed that all females had primary and/or secondary follicles. No differences were found in the proportion of females showing tertiary follicles (mated vs. soliciting, $p$=0.632; mated vs. indifferent, $p$=0.472; soliciting vs. indifferent, $p$=0.103) and LUFs (mated vs. soliciting, $p$=0.886; mated vs. indifferent, $p$=0.820; soliciting vs. indifferent, $p$=0.275) within each level of reproductive behaviour. However, significant differences (mated vs. soliciting, $p$=0.00001; mated vs. indifferent, $p$=0.00001; soliciting vs. indifferent, $p$=1) and were found for presence of corpora lutea since only mated females presented that structure (Table 5).

## Discussion

*C. talarum* females showed different degrees of reproductive behaviour that were related to their reproductive status as indicated by vaginal cytology and ovarian hormones. Whether mating partners copulated or reached different levels of courtship, affected the later reproductive status of females. Furthermore, the results from the present study strongly support the notion that this species is copula-induced ovulator and also, male presence and interaction with females affected other components of their reproductive physiology, such as hormone levels and vaginal cytology.

Metabolic urinary products of steroid hormones are widely used for reproductive monitoring since they were accurately correlated to plasmatic hormone levels (Shideler et al. 1990; Monfort 2003). In mammals, gonadal steroids excretion occurs usually between the first 12 h from its production (Monfort 2003). We found that behaviours denoting male attraction (‘male sniff’ and ‘mount attempts’) and mutual courtship behaviours (‘spin’ and copula) were positively correlated to vaginal smear composition before male–female interaction. Also, these same behaviours correlated with oestradiol and progesterone levels in urine collected 12 h after encounter, which should correspond to female hormone blood levels at the time of

**Table 4** Spearman rank correlation matrix for urinary oestradiol, progesterone and vaginal smear parameters at different times of mating encounters

| | | Cornification index | | | Epithelization | | |
|---|---|---|---|---|---|---|---|
| | | Before ($n$=20) | After ($n$=19) | 24 h ($n$=17) | Before ($n$=20) | After ($n$=19) | 24 h ($n$=18) |
| Progesterone | Before | −0.05 | −0.28 | −0.29 | −0.01 | −0.217 | −0.12 |
| | After | | 0.39 | −0.28 | | 0.25 | 0.51 * |
| | 24 h | | | 0.23 | | | −0.12 |
| | Change | | 0.45 * | −0.13 | | 0.32 | 0.65 ** |
| Oestradiol | Before | −0.22 | −0.27 | −0.41 | −0.17 | −0.11 | −0.30 |
| | After | | 0.06 | −0.50* | | 0.21 | 0.52* |
| | 24 h | | | −0.24 | | | 0.38 |
| | Change | | 0.18 | −0.25 | | 0.12 | 0.68** |

For each relationship, Spearman correlation coefficient ($r_s$) is presented

*$p \leq 0.05$

**$p \leq 0.01$

the reproductive interaction. The ability of males to accurately perceive female reproductive condition using chemical signals is crucial for their reproductive success (Doty 1986; Ferkin et al. 2004; Achiramana et al. 2010). As in many mammalian species, tuco-tucos are capable to distinguish conspecific scents produced during their reproductive season from those of non-reproductive season (Zenuto et al. 2004). In this study, we showed that male olfactory interest in the female, and the subsequent courtship behaviour, is correlated to female circulating ovarian hormone levels. Excretory products such as urine and faeces serve as a rich source of glandular secretions and metabolic breakdown products that have an important signalling value (Johnston 2003; Hurst 2009).

Solicitation behaviours seem to be important in the progress of the courtship. The cumulative effect of circulating oestradiol and progesterone, rather than a single-day absolute concentration could be responsible for the display of solicitation behaviours. Total frequency of solicitation and frequency of 'female mount' behaviour displayed by the female were related to changes in oestradiol levels. The mount behaviour by the females is a pseudomale behaviour that seems to play an important role conveying her physiological status. In the rabbit, one of most studied induced ovulator, this behaviour has been reported to be linked to large ovarian follicles and sexual receptivity (Beyer et al. 2007).

Vaginal smear cytology has been employed to describe the reproductive receptivity in different species with spontaneous and induced ovulation (e.g. Hikim et al. 1991). For the black-footed ferret (*Mustela nigripes*), an induced ovulator, same-day correlations of faecal oestradiol and vaginal cornification were significant, but they were not as great as those, based on several-days mean oestradiol correlations (Young et al. 2001). For *C. talarum*, there were no same-day correlations of urinary progesterone and oestradiol levels and cornification index or epithelization, while there were those based on the change (increase or decrease) in progesterone or oestradiol urinary levels. Thus, together with previous studies, this finding suggests that hormone levels variation rather than absolute concentrations could be responsible for the changes of vaginal epithelium. Furthermore, hormones mostly affected the vaginal epithelization but to a lesser extent its cornification. Although cornification index is generally employed to estimate the receptivity period, in species such as *Microtus agrestis*, females with nucleated smears were also receptive to copulate (Breed 1967). Moreover, in *N. ehrenbergi*, copula occurred only in females that presented cornified vaginal smears but only 11% of females with vaginal cornification copulated, thus suggesting that the presence of cornified smears is an insufficient criterion to evaluate receptivity in mole rats (Shanas et al. 1995).

Mating-induced ovulation is the most important effect of males on reproductive physiology of *C. talarum* females

**Table 5** Results of histological analysis of *Ctenomys talarum* ovaries, showing the number of females that presented different stages of follicular development and corpora lutea

| | Indifferent ($n$=5) | Soliciting ($n$=8) | Mated ($n$=4) |
|---|---|---|---|
| Tertiary follicles | 3 | 6 | 3 |
| Luteinized unruptured follicles | 3 | 3 | 1 |
| Corpora lutea | 0 | 0 | 3 |

All females presented primary and/or secondary follicles

detected in the present study. Nonetheless, male presence or interaction with females did not cause enhanced follicular development. Corpora lutea, a clear evidence of ovulation (Hinds and Smith 1992 in Malherbe et al. 2004), were only found in mated females, consistent with Weir (1974), but not in females that only experienced body contact or even courtship to the males. Nearly all solitary subterranean rodents are induced ovulators, as most solitary mammals. Intuitively, ovulation in response to mating appears to be the more efficient system to ensure egg fertilisation in non-gregarious species, for which encounters between potential partners may not be frequent (Zarrow and Clark 1968). Induced ovulation in subterranean rodents was related to their social organisation and seasonality of breeding (Bennett et al. 2000). Recently, Faulkles et al. (2010) proposed for solitary African mole rats that a selective pressure for seasonal reproduction coupled with induced ovulation may occur in species that inhabit regions with predictable rainfalls. Solitary seasonal breeders *Georychus capensis* and *Bathyergus suillus* are induced ovulators (Van Sandwyk and Bennett 2005; Parag et al. 2006). In females of the social and aseasonal breeders *C. hottentotus natalensis* and *Cryptomys hottentotus prettoriae*, contact with male odours slightly increased ovarian activity and progesterone levels, while mating produced an ovulatory response, as was denoted by corpora lutea and a conspicuous increase of progesterone levels (Malherbe et al. 2004; Jackson and Bennett 2005).

Twenty four hours after the interaction with the male, oestradiol levels and vaginal epithelization increased while progesterone levels decreased in soliciting females. This oestradiol increase would be due to follicle development. Other subterranean-induced ovulators presented follicular growth and development when males and females were allowed to interact through a mesh (Malherbe et al. 2004; Jackson and Bennett 2005). The presence of LUFs in *C. talarum* ovaries of indifferent and soliciting females could be interpreted as lack of copula-induced ovulation. But this fact seems to be associated to a normal follicular development of hystricomorph species (Weir and Rowlands 1974). Luteinized unruptured follicles were also described for non-reproductive females of social *F. anselli* (Willingstorfer et al. 1998) and females of solitary mole rats *Georychus* and *Bathyergus* (Bennett and Jarvis 1988; Herbst et al. 2004). In these species it also appears not to be products, causes, or correlates of their social structure and reproductive suppression (Willingstorfer et al. 1998). Although no clear functions were identified for LUFs, they could be involved in the progesterone secretion required to sustain long pregnancies typical of this group (Willingstorfer et al. 1998; Spinks et al. 1999).

To sum up, *C. talarum* is a copula-mediated induced ovulator. The manifestation of different degrees of reproductive activity would be the result of their reproductive physiology (i.e. hormonal levels), but also the exercise of female choice may be considered in future studies. Male attractivity was highly related to female receptivity, suggesting that behavioural and non-behavioural cues (pheromones) are involved in female assessment by males. Moreover, male presence and interaction with females affected hormone levels (progesterone and oestradiol) and vaginal cytology but did not stimulate follicular development.

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