Follicular development in European ground squirrels (*Spermophilus citellus*) in different phases of the annual cycle

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Abstract

In seasonally breeding mammals, in particular hibernators, reproduction underlies severe energetic and temporal constraints to enable the allocation of sufficient body fat reserves before winter. Thus, the timing of conception in spring can be crucial in terms of reproductive success. This study investigates follicular development in European ground squirrels (*Spermophilus citellus*) in three phases of the annual cycle: at vernal emergence, after weaning the offspring and shortly before hibernation. The animals were kept in outdoor enclosures within the natural habitat of the species. They were captured in weekly intervals, weighed and reproductive status was determined. Unilateral ovariectomy was scheduled such that the three periods were sampled. Numbers and diameters of tertiary follicles (TF) and corpora lutea (CL) in each ovary were determined, and plasma oestradiol and progesterone levels were analysed. The highest numbers of TF, including Graafian follicles, were found in ovaries at vernal emergence. During post-lactation, the number of TF was lower and active CL appeared in the investigated ovaries. Shortly before hibernation, active CL were present, but luteolysis had started in some individuals. Both oestradiol and progesterone secretion peaked after the termination of lactation and decreased before hibernation. The results demonstrate a second oestrus cycle in European ground squirrels after weaning, including an active luteal phase. This non-reproductive oestrus cycle with its endocrine output is an intriguing phenomenon. It may positively affect both prehibernatory fattening and reproduction in the subsequent season.

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Introduction

Follicular development of a seasonal hibernating species like the European ground squirrel is of particular interest, because hibernation puts both physiological and time constraints on this process. Females have to complete mating, gestation, lactation and prehibernatory fattening during a short active season of 4–5 months. Therefore, like in most hibernating small mammal species, female *Spermophilus citellus* produce only one litter per year (Kenagy *et al.* 1990, Risch *et al.* 1995, Rieger 1996, Huber *et al.* 1999). Studies on free-ranging European ground squirrels showed that females became oestrous and mated shortly after they had terminated hibernation between late March and mid-April; they gave birth after a 4-week gestation period (Millesi *et al.* 1999a). Juveniles emerged from the breeding burrow at an age of about 4 weeks, approximately reflecting the time of weaning (Huber *et al.* 2001). The immergence sequence of the sex cohorts in adult European ground squirrels differed from the general pattern of hibernating sciurids (Michener 1984). Adult female European ground squirrels entered hibernation first (late July–early August), followed by adult males (late August–early September) and juveniles (mid-September–

early October; Millesi *et al.* 1999b). Thus, females managed to complete preparation for hibernation in a shorter time span than males, despite the higher energetic and temporal constraints caused by maternal investment.

The fact that females can become oestrous within 1 day after vernal emergence implied that follicular development was at a progressed state at the onset of surface activity (Millesi *et al.* 1999a). Follicular development includes oocyte enlargement, proliferation of the supporting granulosa cells, as well as theca and antrum formation (Greenwald & Roy 1994). Follicular maturation is a lengthy process involving gonadotrophin and oestradiol priming, lasting from ~20 days in rats and hamsters (Chiras & Greenwald 1977, Hirshfield & Midgley 1978, Butcher & Kirkpatrick-Keller 1984, Insler *et al.* 1990) to 6 months in the ewe (Goodman 1994).

Interactions with other seasonal activities can profoundly affect these processes. For example, secretion of luteinising hormone (LH) is highly suppressed during lactation, inhibiting the LH-dependent processes of follicular development (Taya & Greenwald 1982, McNeilly 2001). Hypothermia inhibits gonadal development, which in hibernators, therefore, is restricted to short euthermic periods (Barnes 1986, Barnes *et al.* 1987,

Shvareva & Nevretdinova 1989). Hence, follicular development can either occur before hibernation or during arousals in winter. In previous studies, we have found evidence for a reinitiation of follicular development in females after they had weaned their offspring in summer (Millesi *et al.* 2000). Plasma oestradiol levels increased considerably during post-lactation and reached even higher levels than during the mating period. Oestradiol secretion decreased before hibernation onset and remained baseline throughout the hibernation period.

Based on these results, in this study, we investigated follicular development simultaneously with both histological and endocrinological methods in adult and juvenile European ground squirrels kept in outdoor enclosures under semi-natural conditions. We compared follicular development and endocrine output in three phases of the annual cycle: at vernal emergence, shortly after the females had weaned their litter and before hibernation onset. In addition, we investigated ovaries collected during hibernation.

Results

During all phases, intact secondary follicles were present in the ovaries and their numbers did not differ significantly among the phases (Fig. 1). The number of intact tertiary follicles (TF) in ovaries of adult females at vernal emergence was higher than during post-lactation and prehibernation (Fig. 1). The mean size of TF, however, did not differ significantly between the three phases (Table 1). We further compared the maximum size of intact TF and found significant differences between the phases (Table 1). Maximum size was larger in samples collected at vernal emergence compared

Figure 1 Mean numbers ($\pm$ s.d.) of intact secondary and tertiary follicles, intact and atretic corpora lutea in adult European ground squirrels at vernal emergence ($n=6$) and after weaning ($n=4$) as well as in adults and juveniles shortly before hibernation onset ($n=6$ adults, 6 juveniles); (secondary follicles: Kruskal–Wallis test, $P>0.4$; tertiary follicles: Kruskal–Wallis test, $P=0.02$; *post hoc* tests, emergence versus post-lactation, $P=0.05$; emergence versus prehibernation, $P=0.04$; post-lactation versus prehibernation, $P=0.1$; corpora lutea: post-lactation versus prehibernation Mann–Whitney *U*-test, $P=0.1$).

with post-lactation. No significant differences were found between maximum follicle size during prehibernation and each of the other two phases (Table 1).

Active corpora lutea (CL) were present in the samples both during post-lactation and prehibernation (Fig. 1). Although the numbers of intact CL did not differ significantly between these two phases, atretic CL, indicating luteolysis, were found exclusively in individuals during prehibernation (Fig. 1). In addition, intact CL were significantly larger during post-lactation than shortly before hibernation (Table 1).

Atretic follicles of all stages were present in the ovaries. No significant differences in the number and size of atretic TF were found between the three phases (Table 1; numbers: emergence 28.5 ± 8.3 , post-lactation 12.6 ± 6.1 , prehibernation 27.0 ± 9.6).

In addition to the samples collected in our outdoor enclosures, we analysed ovaries collected towards the end of hibernation, about 4 weeks before the typical onset of vernal emergence in the natural habitat (Millesi *et al.* 1999b). The ground squirrels had hibernated in constant condition chambers. This permitted follicular development to be compared before and after the end of hibernation. Ovaries collected during hibernation differed from those at vernal emergence, in that they contained fewer (13.8 ± 4.5 , $P=0.04$) but on average larger intact TF (Table 1). Nevertheless, maximum size of TF in hibernating individuals did not differ from those at emergence. Atretic TF in ovaries collected during hibernation were significantly smaller than those in ovaries collected at vernal emergence (Table 1).

Secondary follicles occurred in numbers similar to the other periods (22 ± 17.1). In contrast to the prehibernation period, no CL, neither intact nor atretic, were found in the hibernation samples.

We investigated potential age differences by comparing ovaries of adult and juvenile females during the prehibernation period (Fig. 1). Juveniles had significantly fewer TF than adults and no CL or their remains were detected. Neither the mean nor maximum size of TF differed significantly between the age groups (Table 1).

Plasma oestradiol levels did not differ significantly between vernal emergence and the post-lactation period (Fig. 2a). Thereafter, during prehibernation, oestradiol levels had decreased and were significantly lower than during the post-lactation period.

Plasma progesterone levels increased significantly from vernal emergence to the post-lactation period and, similar to oestradiol, decreased again thereafter (Fig. 2b). However, progesterone secretion in the prehibernation phase was still higher than at vernal emergence.

According to the ovarian development in the prehibernation period, both oestradiol and progesterone levels were significantly lower in juvenile than in adult females (oestradiol: 75.3 ± 33.2 vs 191.6 ± 54.3 pg/ml, $P=0.02$; progesterone: 0.0 vs 0.9 ± 1.7 ng/ml, $P=0.004$, $n=6/6$).

Table 1 Mean and maximum diameter (μm) of intact tertiary follicles (TF) and mean diameter of atretic (TF_{atr}) and active corpora lutea (CL) in European ground squirrels in different phases of the annual cycle.

	Hibernation		Emergence		Post-lactation		Prehibernation		
Age	Adult (5)		Adult (6)		Adult (4)		Adult (6)		Juvenile (6)
TF _{mean}	402.6 ± 24.5	**	355.8 ± 36.1	NS	329.6 ± 40.4	NS	353.2 ± 44.4	NS	327.2 ± 18.2
TF _{max}	524.2 ± 23.4	NS	570.0 ± 62.9	*	355.8 ± 36.1	NS	440.9 ± 92.6	NS	393.3 ± 44.6
TF _{atr}	231.0 ± 39.6	*	321.1 ± 38.8	NS	323.6 ± 58.3	NS	329.5 ± 26.2	NS	311.2 ± 42.8
CL	None		None		1086.3 ± 199.4	*	777.7 ± 209.2		None

Means $\pm$ s.d., sample sizes are given in parentheses (** $P \leq 0.01$, * $P \leq 0.04$, NS, $P > 0.05$).

Discussion

The results of this study demonstrate that, in contrast to other hibernating sciurid species, ovarian activity in European ground squirrels remained activated until shortly before hibernation. High levels of oestradiol during post-lactation and the presence of active CL both during post-lactation and prehibernation were indications for a second oestrus cycle during the non-reproductive part of the season. An alternative explanation is that the CL of previous pregnancy had been maintained and rejuvenated, as has been shown in woodchucks (Concannon *et al.* 1983). Correspondingly, progesterone levels in woodchucks were elevated for up to 2 months *post partum* (Concannon *et al.* 1984). However, increased plasma oestradiol levels in European ground squirrels at weaning demonstrated a second oestrus cycle in summer. This is supported by the analyses of vaginal smear samples: they clearly showed vaginal oestrus, defined by the predominance of cornified epithelial cells, during the mating period and shortly before weaning (Strauss *et al.* 2008). In addition, we investigated the ovaries of a female killed by a predator during early lactation and one sample from Groningen collected during mid-May. Neither individual contained CL nor their remains. These data demonstrate that follicular development was reinitiated after weaning, including ovulation and the formation of a second generation CL in the current season. The second ovulation series must have been spontaneous because males had already regressed their testes and no sexual interactions such as anogenital inspections or mating attempts were observed after the mating period in spring (Millesi *et al.* 1998, Strauss *et al.* 2008). A spontaneous oestrus cycle after lactation may occur due to the lack of inhibitory effects of suckling stimuli, as has been shown in a number of species including humans (Taya & Greenwald 1982, Quesnel & Prunier 1995, McNeilly 2001).

The presence of active CL in ovaries collected both in mid-June and early August suggests that the luteal phase in summer lasted for about 6 weeks and that luteolysis started shortly before immergence into hibernation. Another explanation would be more than one oestrus cycle between weaning and hibernation. However, patterns of oestradiol and progesterone secretion both in the field and the enclosures indicate that this was unlikely. No cyclic patterns were detected in either hormone after the rise

during late lactation (Millesi *et al.* 2000, Strauss *et al.* 2008). Luteolysis had started in some females before the onset of hibernation, indicating that all CL were regressed until females entered hibernation. Accordingly, we found no CL or their remains in the ovaries collected during hibernation.

The non-reproductive oestrus cycle could be a remnant of an ancestral reproductive strategy with multiple litters per year. However, the temporal constraints in hibernating small mammals point to beneficial effects of the second oestrus cycle and the prolonged luteal phase. Progesterone levels in woodchucks decreased during May (Concannon *et al.* 1984), indicating that the maintenance of rejuvenated CL may be temporally limited. In *S. citellus*, however, the post-lactational CL remained active until August. Elevated

Figure 2 (A) Plasma oestradiol and (B) progesterone levels in the experimental females at vernal emergence ($n=6$), during post-lactation ($n=4$) and prehibernation ($n=6$); (oestradiol: Kruskal–Wallis test, $P=0.02$; *post hoc* tests emergence versus post-lactation, $P=0.3$; post-lactation versus prehibernation, $P=0.03$; emergence versus prehibernation $P=0.1$; progesterone: Kruskal–Wallis test, $P=0.01$; *post hoc* tests, emergence versus post-lactation, $P=0.03$; post-lactation versus prehibernation, $P=0.01$; emergence versus prehibernation, $P=0.05$).

progesterone secretion could enhance the progress of prehibernatory fattening. Female rats implanted with progesterone had an increased proportion of total body fat compared with control animals (Shirling *et al.* 1981, Mendes *et al.* 1985). Progesterone-treated rats ate more than controls when they had free access to food and also gained weight more rapidly when consuming the same amount of food as controls (Shirling *et al.* 1981). Apart from permissive effects, for instance, by altering the number or activity of insulin receptors on the cell membrane (Mendes *et al.* 1985), progesterone may directly affect fat cell metabolism (Krotkiewski & Björntorp 1976). Administering progesterone pronouncly increased fat cell size in the parametrial fat depot in rats. This effect could be advantageous in animals that have to store body fat in a limited time span. Adult female European ground squirrels enter hibernation in late July/early August, about 4 weeks earlier than adult males and 6 weeks earlier than juveniles (Millesi *et al.* 1999b). Fattening is thus apparently accelerated in females and they enter hibernation as soon as they have sufficient body fat reserves. Selection should favour early immergence if mortality risk due to predation or other environmental factors exceeds that resulting from the lengthy dependence on body fat deposits (Michener 1984). Juvenile females had no CL prior to hibernation, their oestradiol secretion was lower than in adults and progesterone levels were below detectable levels. The juveniles need time and energy for both structural growth and fattening. Structural growth in juvenile European ground squirrels stops at an age of about 3 months. High-protein diet composition resulted in faster growth but not in absolutely larger individuals at the end of the active season, indicating that structural growth is limited to allow sufficient preparation for hibernation (Strauss *et al.* 2007). Accordingly, juvenile females started to hibernate 2 months later than adults. In spring, yearlings had a longer latency to become oestrous than older females (Millesi *et al.* 1999a).

Luteal function could further serve to maintain development of large TF (Bartlewski *et al.* 2001) while inhibiting growth of smaller antral stages (Mills & Stopper 1989). Although sample sizes were small, the data indicate that follicular development continued during summer. Both mean and maximal diameters were slightly higher in prehibernation versus post-weaning samples. This is supported by earlier studies showing elevated oestradiol levels until late July (Millesi *et al.* 2000). Oestrogen secretion during post-lactation may have priming effects on follicular clusters, stimulating further development (Palter *et al.* 2001). Developing follicles to a preovulatory stage during summer and maintaining them over winter would save valuable time in spring. Seasonal timing is known to be related to reproductive success: females mating early in the season had larger litters and lactated their offspring longer than those with a delayed oestrus (Huber *et al.* 1999, Millesi *et al.* 1999a). Follicular development in hibernating females appeared to be at a progressed state

since few, but large, TF were present. The samples had been taken in the later part of hibernation, about 4 weeks before the onset of vernal emergence in the field (Millesi *et al.* 1999b). We therefore assume that follicular maturation in adult female European ground squirrels is initiated during the previous active season, and presumably completed during euthermic periods in late hibernation or, particularly in yearlings, after vernal emergence. In ovaries of bats, Graafian follicles were maintained during hibernation until spring (Oh *et al.* 1985, Mori *et al.* 1989). Testes in male ground squirrels are activated during the final arousals before the end of hibernation (Barnes *et al.* 1988). Interruption of follicular development during the prehibernation period was also reported for Arctic ground squirrels (Shvareva & Nevretdinova 1989).

At vernal emergence, the largest number of TF, including potential preovulatory Graafian follicles was determined. As females can become oestrous within 1 day post-emergence, the presence of mature follicles was expected (Millesi *et al.* 1999a). However, the individual variation in mean diameter of TF indicates that some individuals need additional time to complete follicular maturation in spring. This is supported by observed latencies to oestrus in free-ranging female *S. citellus* varying from 1 day to 2 weeks post-emergence (Millesi *et al.* 1999a). We found no significant differences in mean diameters of intact TF between the three phases; however, maximum size of TF was largest in spring. This may to some extent reflect the larger number of TF at emergence, including early stages and mature ones.

The non-reproductive oestrus cycle, along with its potential endocrine effects, is an exceptional phenomenon and may represent an intriguing adaptation to the short active period in this hibernating small mammal species. Future studies should provide more insight into interactions between ovarian activity and hibernation.

Materials and Methods

Animals, study area and capture techniques

European ground squirrels (*S. citellus*), living in outdoor enclosures built within the habitat of the species north of Vienna (Austria), were studied from vernal emergence (late March/early April) until the onset of hibernation (early August). The animals were permanently marked with subcutaneous transponders (PIT-tag; Datamars SA, Lugano, Switzerland). For distant recognition, the fur was painted with commercial hair dye in an individual pattern. Females were captured in weekly intervals using Tomahawk live traps baited with peanut butter. At each capture, animals were weighed (± 0.5 g, Sartorius laboratory scale) and reproductive status was determined. Gestation and lactation were identified by teat development and body mass changes. Pregnant females had dark-pigmented teats and showed rapid mass increase (Millesi *et al.* 1999a). Parturition was indicated by an abrupt drop of body mass. During lactation, females had enlarged light-pigmented teats and milk secretion could be induced.

Follicular development in European ground squirrels was investigated in three phases of the annual cycle: (i) at vernal emergence, individuals were captured within 2 days after they had left the hibernaculum for the first time in spring, (ii) during post-lactation, the period when teat regression had started, indicating that the females had terminated lactation. This coincided with the onset of moult, and (iii) during the prehibernation phase, defined as the period when prehibernatory fattening had been completed, as indicated by constant body mass until the onset of hibernation. Prehibernatory fattening was defined by a mean body mass increase of >1 g per day (Millesi *et al.* 1999b).

In addition, we received ovaries of five adult females collected during the hibernation period. These animals had hibernated in a constant condition chamber in Groningen, the Netherlands. They were part of a research programme at the University of Groningen. The ovaries had been collected in late February. We had no information on the previous annual timing and reproductive performance of these individuals.

Blood samples (150–200 μ l) were taken from the femoral vein at capture on the day of ovariectomy. The blood was collected in heparinised capillaries and centrifuged in the field; plasma was stored at -20°C . No blood samples of the individuals that had hibernated were available.

All animal manipulations were approved by the Austrian Federal Ministry of Agriculture, Forestry, Environment and Water Management (GZ 68.210/29Pr/4/2001), and by the Austrian Ethical Committee for Animal Welfare.

Hormone assay

Oestradiol levels were analysed in duplicate after diethyl ether extraction with a biotin–streptavidin enzyme immunoassay, according to *Palme & Möstl (1994)*. The antibody was produced by H H D Meyer (Weihenstephan, Germany) using 1,3,5(10)-estratriene-3,17 β -diol 17 HS:BSA (Steraloids Ltd, Croyden, UK) as the immunogen. Cross-reactivity of the antibody with oestrogens other than oestradiol was 13%. Intra- and inter-assay coefficients of variation were $<10\%$.

For progesterone analysis, the 5 α -20-one enzyme immunoassay was used; details are in *Schwarzenberger et al. (1996)*. Intra- and inter-assay coefficients of variation were less than 15%.

Unilateral ovariectomy

The females were captured and immediately transported to a veterinary clinic. Females were unilaterally ovariectomised in one of three phases of the annual cycle: either at vernal emergence (six adults), after they had weaned their litter (four adults) or during prehibernation (six adults and six juveniles). The adult experimental females were randomly assigned to one of the three groups. Six juvenile females were chosen from different litters. An inhaled anaesthesia (ethrane) was administered and unilateral ovariectomy was performed. The left ovary was chosen in all individuals. The operation lasted about 15 min, and the post-operative females were kept in individual cages (100 $\times$ 30 $\times$ 50 cm) provided with nest boxes. After 10 days, the sutures were removed, and the animals were released to their burrows in the outdoor enclosures. The ovariectomised females were not further

used in this study. All experimental animals recovered quickly, hibernated in the enclosure and reproduced successfully in the subsequent season (*Aschauer et al. 2006*).

In the hibernation samples, we were unable to differentiate between left and right ovaries but we compared both on an individual level. Follicular development did not differ between the ovaries (E Millesi, T Burger, A Strauss & M Walzl, unpublished observations), hence one ovary per individual was randomly chosen.

Histological analysis

The ovaries were fixated immediately in Bouin's fluid for 3–6 h and afterwards stored in 70% ethyl alcohol until analysis. The samples were dehydrated in graded series of ethyl alcohol (70, 80, 90 and 100%), embedded in paraffin and cut in 6 μ m serial sections. The sections were stained with azocarmine-anilin blue (AZAN) according to the 'Heidenhain staining protocol' (*Romeis 1989*). The number, size and stage of developing follicles as well as the number of atretic follicles were classified using a Nikon Eclipse 800 microscope.

Large numbers of primordial and primary follicles were present in all ovaries but were not analysed in this study. Follicles in a multilaminated preantral stage were defined as secondary follicles. In TF, an antrum appeared and granulosa, theca interna and theca externa layers were fully developed. Secondary and TF were defined as atretic when degenerative changes were detected in any part of the follicle (*Bykov 1974*).

CL can be easily identified by their typical spheroidal shape. Large and small steroidogenic luteal cells were found in close apposition to blood capillaries. Luteolysis was detected based on degenerative changes in the corpus luteum (*Koering & Thor 1978*).

The diameter of TF and CL was calculated by measuring the largest diameter in a series of sections. Two dimensions of TF were used as measures. Mean diameter was calculated from individual means in each phase of the annual cycle. In addition, we calculated maximum diameters by using the mean value of the four largest intact TF of each individual. Four follicles were chosen because the mean litter size of European ground squirrels in the enclosures and at the nearby field site during years of high population density was four juveniles (*Millesi et al. 1999a*).

Statistical analysis

Non-parametric tests were applied in all cases. Comparisons between the annual phases were done using Kruskal–Wallis tests, and Mann–Whitney *U*-tests were applied for *post hoc* or independent two-sample comparisons. Statistical significance was set at $P < 0.05$. In case of *post hoc* comparisons, *P* values were calculated using Bonferroni correction. Comparisons between adult and juvenile individuals were only possible during the prehibernation phase. If not stated otherwise, means and s.d. are shown in all data sets.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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