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„Prehibernation fattening in female European ground squirrels (*Spermophilus citellus*) with and without luteal activity during the non-breeding period"

verfasst von / submitted by

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"I am learning to understand rather than immediately judge or to be judged. I cannot blindly follow the crowd and accept their approach. I will not allow myself to indulge in the usual manipulating game of role creation. Fortunately for me, my self-knowledge has transcended that and I have come to understand that life is best to be lived and not to be conceptualized. I am happy because I am growing daily and I am honestly not knowing where the limit lies. To be certain, every day there can be a revelation or a new discovery. I treasure the memory of the past misfortunes. It has added more to my bank of fortitude."

Bruce Lee

Acknowledgements

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Abstract

In previous studies, a second oestrus cycle including an active luteal phase during the non-breeding season in summer has been documented in both free ranging and captive female European ground squirrels. As the onset of prehibernation fattening and peak progesterone levels coincided, potential positive effects of progesterone secretion on the course of the fattening phase were assumed. In the season of 2014, we monitored reproductive performance of 9 females kept in semi-natural enclosures. In contrast to previous years, none of the studied females showed elevated progesterone titres except during gestation, although results of vaginal cytology demonstrated the occurrence of a vaginal oestrus phase in most individuals during summer. This may indicate ovarian activity without the formation of active *corpora lutea* as a main source for progesterone. Reproductive outcome was very low, as only 3 out of 9 females reproduced and 2 of them weaned their litter successfully. In the others, no indications of advanced gestation or lactation could be detected. Due to the restricted sample size, this study was focused on non-reproductive females. Additionally, our data were compared to unmated females of a previous study which were kept in the same outdoor enclosure without any contact to males. Individuals lacking a pronounced progesterone secretion during summer showed a prolonged fattening phase with significantly lower body mass increase rates compared to females of the previous study with active *corpora lutea*. Although females with low progesterone concentrations exhibited a prolonged fattening phase, immergence body mass did not differ between the compared years. Thus, reaching a certain threshold in body fat content might initiate the hibernation period. The result on lower fattening rates in non-reproductive females of this study were reflected by feeding experiments showing no differences in foraging behaviour in non-reproductive females before and during the fattening period. Although the reason for reproductive failure is still unclear, the results support the assumption that elevated progesterone titres improve the process of prehibernatory fattening in female European ground squirrels.

Introduction

In obligate hibernators like European ground squirrels (*Spermophilus citellus*), strong time constraints and a strict annual cycle shape the short active season of five to seven months. In this limited time period females have to accomplish mating, gestation, lactation and prehibernatory fattening (Huber et al. 1999, Millesi et al. 1999a, b). Nevertheless, the females' active season is shorter than that of males. In spring, females emerge from their burrows about four weeks later than males in late March/early April and immerge into their hibernacula already in late July/early August, while adult males emerge in early March and start to hibernate in late August/early September (Matějů 2008, Millesi et al. 1999b). Mating starts shortly after female emergence in spring. After a gestation period of four weeks, the females give birth in late April/early May. Juveniles remain in the natal burrow for four weeks and weaning starts around mid June depending on the mother's condition and litter size (Aschauer et al. 2006, Huber et al. 2001, Millesi et al. 1999a, b). European ground squirrels rely exclusively on body fat during winter (Millesi et al. 1998). Hence, the preparation for hibernation is characterised by a rapid body mass increase (Millesi et al. 1999b). Interestingly, prehibernatory fattening is completed earlier in females than in males, despite both temporal and energetic costs of gestation, parturition and lactation (Millesi et al. 2008b).

Millesi et al. (2008b) suggested that prehibernatory fattening in females could be accelerated by progesterone secretion during the luteal phase of a second non-reproductive oestrus cycle. Starting around the weaning phase, reinitiated ovarian activity with the formation of active *corpora lutea* has been documented in female European ground squirrels (Millesi et al. 2008b, Strauss et al. 2009). This so-called summer oestrus was characterised by elevated oestradiol and progesterone secretion during late lactation and after weaning (Millesi et al. 2008a, b, Strauss et al. 2009). In addition, previous studies showed that in unmated females, the lack of reproduction led to a shift in the seasonal timing, particularly a prolongation of the vaginal oestrus in spring. Furthermore, the summer oestrus started earlier in unmated than in mated females and hormone titres remained elevated until mid-June. Comparatively, mated females exhibited high progesterone levels from late May until late June. Correspondingly, the onset of fattening started earlier in unmated compared to reproductive females (Divjak 2009, Millesi et al. 2008a). Additionally, non-reproductive females entered their hibernacula about four weeks earlier than reproductive females (Millesi et al. 2008a). However, the course of progesterone excretion during summer as well as the course and extent of fattening was similar in both breeding and non-breeding females, but shifted to an earlier onset in unmated individuals (Millesi et al. 2008a). In European ground squirrels, achieving a certain threshold in body fat content seemed to trigger the termination of above ground activity

(Millesi et al. 2008a, b). Thus, non-reproductive females did not use the additional time to get fatter than those that reproduced successfully, but immerged into their hibernacula as soon as they had accumulated sufficient body fat reserves (Millesi et al. 2008a).

Interestingly, the summer oestrus appeared to be a non-reproductive cycle that occurred spontaneously probably due to the lack of inhibitory effects of suckling offspring, which has been shown in numerous species (Fortune et al. 1986, McNeilly 2001, Quesnel & Prunier 1995, Taya & Greenwald 1982). This is supported by the fact that the males' testes started to regress in late April and remained inactive for the rest of the season (Millesi et al. 1998). Additionally, female European ground squirrels give birth to only one litter per year, as has been documented in most hibernating small mammal species (Everts et al. 2004, Huber et al. 1999, Kenagy & Barnes 1988, Millesi et al. 2004, Pilastro 1992, Rieger 1996).

Facing these facts, potential benefits of the non-reproductive summer oestrus cycle could be assumed. It was hypothesised that the summer oestrus with the extended progesterone secretion period could facilitate and accelerate prehibernatory fattening and might allow females to increase body mass rapidly after weaning (Millesi et al. 2008b, Strauss et al. 2009). Progesterone-treated female rats show upregulated appetite and an increased food intake, body weight and adipose tissue mass, due to an increase in lipogenic enzyme genes expression in adipose tissue (Stelmanska & Swierczynski 2013) and in neuropeptide genes expression in the hypothalamus (Stelmanska & Sucajtys-Szulc 2014). Thus, prehibernatory fattening may be accelerated by the summer oestrus, allowing females to immerge into hibernation about four weeks prior to males and six weeks earlier than juveniles, despite their costly maternal investment and preparation for hibernation (Millesi et al. 1999a, b).

Additionally, the summer oestrus may have a priming effect on follicles, allowing them to develop into a more progressed antral stage before hibernation (Millesi et al. 2008b). This may accelerate follicular development after spring emergence (Millesi et al. 2008b), enabling females to become sexually receptive shortly after emergence (Millesi et al. 1999a, 2000). It has been shown that mating early in the season correlated with larger litter size and better survival of the pups (Huber et al. 1999, 2001, Millesi et al. 1999a, 2004, Rieger 1996). Therefore, a potential priming effect on follicles during the summer oestrus may positively affect reproductive success in the subsequent year.

Seasonal timing, body mass changes and progesterone excretion were repeatedly examined in both free-ranging female European ground squirrels and individuals living in an outdoor enclosure within the natural habitat of the species (Divjak 2009, Hoffmann et al. 2003a, b, Huber et al. 1999, Millesi et al. 1999b, 2000, 2008a, b, Strauss et al. 2009). In this study, we examined the course of progesterone excretion, vaginal cytology and the course of body mass changes throughout the active

season of female European ground squirrels kept in outdoor enclosures under semi-natural conditions. In addition, feeding experiments were carried out to determine possible changes in foraging behaviour between the prefattening and fattening phase. We aimed at documenting the summer oestrus and the subsequent luteal phase and relate the endocrine changes to the course and extent of prehibernatory fattening and foraging behaviour. These results were compared to that of Divjak (2009), which had been obtained in the same semi-natural enclosures.

Material and Methods

Animals, study site and capture techniques

In this study, European ground squirrels, 9 females and 6 males living under semi-natural conditions in an outdoor enclosure built within the natural habitat of the species north of Vienna, Austria (Bisamberg, 48°30′N, 16°38′E), were investigated. The enclosure (185m²) was situated at 260m.a.s.l. with a south-east exposure. The animals were monitored throughout the active season (March-September) in 2014.

The individuals were captured with Tomahawk live traps baited with carrots and peanut butter and were permanently marked with transponder chips injected subcutaneously in the neck region. For visual recognition, the fur was painted with commercial hair dye in a unique and recognisable pattern. Animals were weighed at each capture (± 1g, digital scale). Females were captured in daily to weekly intervals to record reproductive status, body mass, foot length, head length and state of moult.

Vulval swelling and opening were classified on a four-point scale (0: closed and not swollen, 1: small opening and light swelling, 2: medium state, and 3: maximum vulval swelling and opening about 5mm in diameter) (Strauss et al. 2009).

Teat development was classified using a three-point scale, regarding size (1: small and inconspicuous, 2: large and swollen, 3: very large and swollen) and teat pigmentation (1: light pigmented areola, 2: light pigmented teat tip, 3: dark pigmented) (Strauss et al. 2009).

Pregnant females showed rapid body weight gain and had dark-pigmented teats. Parturition was indicated by an abrupt body mass decrease. During lactation, females had large, light-pigmented teats, and milk secretion could be induced. Weaning started with the first emergence of a female's offspring, followed by postlactation, characterized by regressed teats and no signs of lactation (Millesi et al. 1999b).

All females were kept with contact to males during the mating season. Nevertheless, not all females reproduced successfully. Non-reproductive females showed no indication of an advanced gestation, parturition or lactation, as teats were small, inconspicuous and dark pigmented, and body mass changes indicated no successful reproduction.

The onset of prehibernatory fattening is reflected in a pronounced body mass increase and has been defined as the time period after breeding when individual body weight gain exceeded 1g/d, beginning with the first day showing an explicit body mass increase (Millesi et al. 1999b). Prehibernatory fattening was completed, when body mass has plateaued and stayed constant until

onset of hibernation (Millesi et al. 2008b). Immergence date was defined as the last sighting of an individual during summer when hibernation was expected (Millesi et al. 2008a).

Vaginal cytology

For further information about ovarian activity, vaginal smears were collected at capture using cotton swabs tinctured in 0.9% saline solution. Vaginal smears were fixed with polyethylene glycol and stained using the Papanicolaou technique (Papanicolaou 1954). Cell numbers and ratios were determined microscopically (Reichert Biovar) in three 0.5mm X 0.5mm sections per sample. Each smear was assigned to defined phases of the oestrus cycle, based on the predominance (≥70%) of one cell type. Prooestrus was defined as the predominance of nucleated epithelial cells. Vaginal oestrus was characterised by polygonal-shaped, cornified cells without nuclei as the main cell type. Metoestrus showed a high abundance of leukocytes. As the anoestrus phase represents a resting sexual cycle, cell numbers were generally low and all cell types were usually present (Strauss et al. 2009).

Hormone assay

For analysing faecal progesterone metabolites (FPM) fresh faeces was collected at each capture. Faecal samples were stored at -20°C until analysis. Prior to analysis the samples were dried at 60°C for 24h and powdered. 0.2g of each sample was suspended in 80% methanol. FPM concentrations were determined using a biotin-streptavidin enzyme immunoassay (Möstl et al. 2002). FPM concentrations were given in ng/g faeces. Intraassay coefficient of deviation was 8.97% and the interassay coefficient of deviation was 4.97%.

Foraging experiments

Foraging experiments were conducted from early May until mid August. This time span was chosen to enable a comparison between the prefattening and fattening phase. In reproductive females, prefattening and fattening phases coincided with lactation, respectively late lactation and weaning of the young. 40g of commercial grain feed (Funny Meal) were offered throughout the observation period of 30 minutes. Foraging experiments were carried out both before (between 09:00 and 10:00 AM) and after (between 14:00 and 16:00 PM) capturing. Timing of foraging experiments was set within the diurnal activity pattern of European ground squirrels, as they emerge from their burrows 3.9h after civil twilight at dawn with a forenoon peak between 09:00 and 11:00, and retreat 3.2h before civil twilight at dusk. At midday (12:00 and 14:00), the lowest activity pattern was

recorded, leading to a bimodal daily activity pattern with two peaks (Everts et al. 2004, Hut et al. 1999, Váczi et al. 2006). Data points from before and after capturing were pooled, as no significant differences were found. The food bowl was placed in a central, observable spot within the enclosure with particular emphasis on assuring an equal distance to each female's burrow. The bowl placement was randomised at each trial within a diameter of 2m. Parameters recorded included the latency until the first approach to the bowl for each specimen after the 30 minute-interval started, total number of individuals visiting the bowl, arrival order, and total duration of food intake for each female, which was defined as total time the individual lowered its head into the bowl or sat in front of the bowl chewing. Additionally, the total number of individual approaches to the bowl was recorded. Furthermore, frequency of agonistic interactions, including threat, displacement and fighting (Quanstrom 1968, Scott 1956, Yeaton 1972) was counted, but excluded from analysis, because counted datapoints were negligible. Given the fact that European ground squirrels retreat in their burrows during periods of rainfall (Everts et al. 2004, Hut et al. 1999, Spoelstra et al. 2000), days with rainfall occurring during foraging experiments were excluded from the analysis.

Comparison 2009-2014

We compared our findings with results from an earlier study (Divjak 2009) on 10 female European ground squirrels in the same outdoor enclosure on Bisamberg without any contact to males. Divjak (2009) studied seasonal timing, body mass changes and progesterone excretion using the same capturing and marking methods. Procedures for collecting vaginal smears and faecal samples were equal in both years, allowing a comparison between unmated females kept without any contact to males in 2009 and non-reproductive females showing no signs of reproduction in 2014. In this study, data of Divjak (2009) is referred to as "2009", representing the reference year for our data "2014".

Statistics

All statistics were calculated using IBM SPSS Statistics 22. Shapiro-Wilk tests were applied for tests of normality. Student's t-tests were performed to compare normally distributed data and Mann-Whitney-U-Tests for not normally distributed data. For paired data, paired samples t-tests were applied. Significance values were received from two-tailed statistics and the statistical significance was set at $p < 0.05$. Variations in sample sizes for measured parameters are due to females' emergence/immergence. Additionally, not all individuals were captured at each stage. Due to a restricted sample size during the foraging experiment, statistical analysis could be solely

performed in non-reproductive females between the prefattening and fattening phase. Datasets with sample sizes ≤3 are represented using descriptive data analysis.

Ethical note

This study was approved by the Municipal Department 22 – Environmental Protection in Vienna (MA22-1854/2012).

Results

Reproductive output

2 out of 9 females weaned their litter successfully and were therefore classified as reproductive. In one female, gestation and parturition, as well as signs of lactation, were identified, but no juveniles emerged from the mother's burrow. This specimen was also classified as reproductive. 6 females showed no indication of an advanced gestation, parturition or lactation and thus were defined as non-reproductive. Due to the small sample size of reproductive females, our study is focused on non-reproductive females.

Seasonal timing, body mass and prehibernatory fattening

In the season 2014, the first non-reproductive females were captured on 27th March, the last one on 5th August. In 2009, the active season started on 26th March and ended on 6th July (Fig. 1). No significant difference was found in the onset of the active season (first day of observation) between non-reproductive females in 2009 and 2014 (Tab. 1). Additionally, the duration of the active season between 2009 and 2014 did not differ significantly (2009: 89d ± 5.6d; 2014: 106d ± 6.8d; t=-1.843, p=0.083) (Fig. 1). Non-reproductive females in 2014 showed a significantly higher body mass at vernal emergence than those in 2009 (Tab. 1).
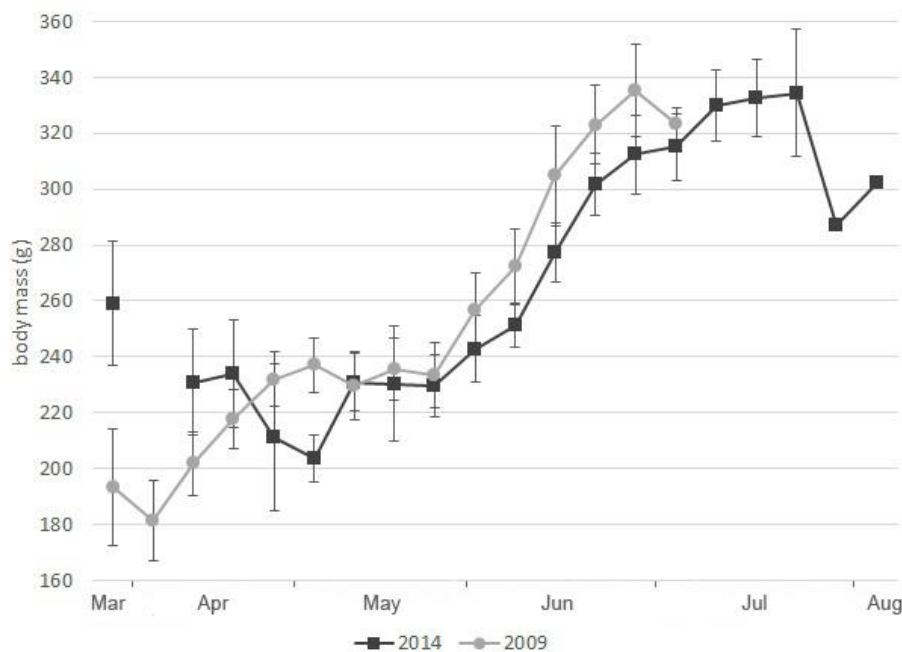

Figure 1. Mean ± SEM of body mass changes (g) of non-reproductive females during the observation period 2009 (n=4,7,10,9,10,10,9,9,10,9,10,9,10,9,9,7,3) and 2014 (n=3,2,3,4,5,3,6,5,5,6,5,5,5,5,4,3,1,1).

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Table 1. Emergence date and body mass at emergence of non-reproductive females in 2009 and 2014 (mean ± SEM).

	Emergence date	Emergence body mass (g)
2009	5th April ± 1.95d (n=11)	184.3 ± 11.01 (n=10)
2014	8th April ± 5.61d (n=6)	236 ± 15.42 (n=6)
t	-0.604	-2.787
p	0.555	**0.015**

The onset of prehibernatory fattening (d) did not differ between non-reproductive females in both years (2009: 140.4d ± 0.72d, n=10; 2014: 142.8d ± 3.38d, n=5; U=-0.732, p=0.513). Although sample sizes were small, data indicated a later onset of prehibernatory fattening in reproductive than in non-reproductive females (2009: 171.3d ± 6.3d, n=3; 2014: 162.7d ± 5.6d, n=3). Non-reproductive females started prehibernatory fattening between 19th May and 25th May in 2009 as well as in 2014 (Fig. 1). The ensuing course of body mass changes throughout the fattening period, however, differed between non-reproductive females of 2009 and 2014. The process of fattening showed a steeper increase in 2009, therefore the daily body mass gain during prehibernatory fattening was significantly higher compared to 2014. Hence, non-reproductive females in 2009 had significantly higher body mass increase rates during prehibernatory fattening than non-reproductive females in 2014 (2009: 3.6g/d ± 0.3g/d; 2014: 2.2g/d ± 0.18g/d; t=3.913, p=0.002) (Fig. 2).
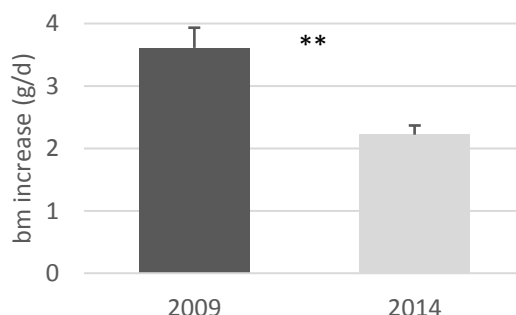
Figure 2. Mean ± SEM of body mass increase (g/d) during prehibernatory fattening of non-reproductive females 2009 (n=10) and 2014 (n=5).

Females exhibited a prolonged fattening phase in 2014, indicated by a constant body mass increase for about 3 weeks longer than females of 2009. Correspondingly, females needed significantly more time to gain weight in 2014 than females in 2009 (2009: 30.2d ± 2.1d; 2014: 50.4d ± 4.6d; t=-4.63, p<0.001) (Fig. 3).
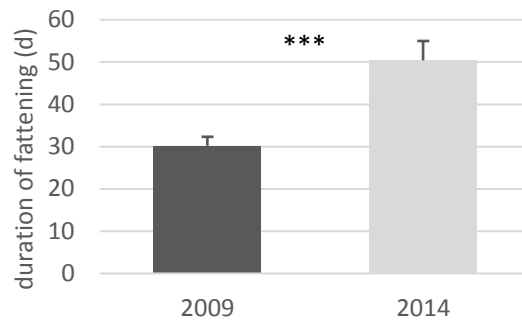
Figure 3. Mean ± SEM of duration of fattening (d) of non-reproductive females 2009 (n=10) and 2014 (n=5).

In 2014, non-reproductive females terminated their above ground activity later than those in 2009 (Tab. 2). However, despite the fact that non-reproductive females in 2014 entered their hibernacula later in the season, immergence body mass did not differ between the two groups (Tab. 2).

Table 2. Immergence date and body mass at immergence of non-reproductive females in 2009 and 2014 (mean ± SEM).

	Immergence date	Immergence body mass (g)
2009	1st July ± 4.32d (n=10)	338.2 ± 8.34 (n=10)
2014	23rd July ± 4.18d (n=6)	337.2 ± 13.98 (n=5)
t	-3.219	0.065
p	**0.007**	0.949

Progesterone analysis

The patterns of FPM and plasma progesterone concentrations differed between years in both reproductive and non-reproductive females. In 2009, both breeding and non-breeding females showed a conspicuous progesterone peak in early summer, representing the luteal phase following the summer oestrus. However, FPM concentrations in 2014 showed a more or less constant decline without a notable peak in both, reproductive and non-reproductive females (Fig. 4, Fig. 5).

During pregnancy, reproductive females showed high levels of FPM, respectively plasma progesterone concentrations in both years, which dropped in early May, representing parturition. In 2009, the summer oestrus with elevated progesterone secretion started during postlactation in the end of May, followed by a constant decrease of plasma progesterone concentrations before hibernation. In comparison to 2009, reproductive females in 2014 showed no signs of elevated

progesterone secretion during postlactation, but a rather constant decrease of FPM levels until the females entered their hibernacula (Fig. 4).

Figure 4. FPM concentrations (ng/g) of reproductive females 2014 (n=1,2,3,3,3,3,3,3,3,2) and plasma progesterone concentrations (ng/ml) of reproductive females 2009 (n=3) during the active season in a two-week-interval.

Non-reproductive females of 2009 showed low levels of plasma progesterone concentrations until the onset of prehibernatory fattening in late April, thereafter progesterone secretion increased and remained elevated until mid June followed by a steep decrease of plasma progesterone concentrations until immergence into the hibernacula. In 2014, non-reproductive females showed higher levels of FPM concentrations during the mating period than unmated females of 2009. In addition, progesterone secretion decreased constantly over time with no notable summer peak related to prehibernatory fattening in 2014 (Fig. 5).
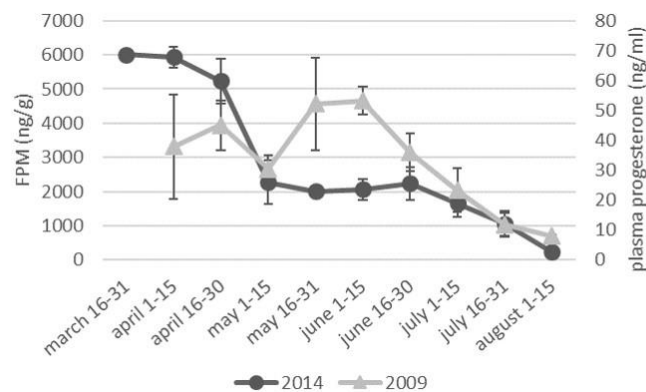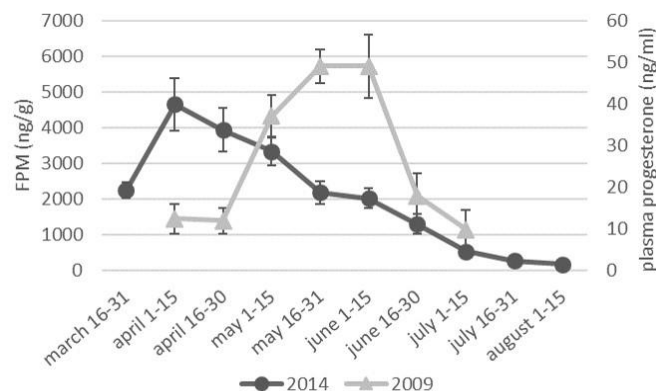

Figure 5. FPM concentrations (ng/g) of non-reproductive females 2014 (n=2,3,5,5,6,6,5,5,4,1) and plasma progesterone concentrations (ng/ml) of reproductive females 2009 (n=10) during the active season in a two-week-interval.

In 2009, progesterone levels were baseline during April, followed by a rapid progesterone increase until levels peaked in late May/early June. Increasing progesterone levels coincided with the beginning of prehibernatory fattening in late May (Fig. 6).

In 2014, progesterone titres were elevated during April. Thereafter, FPM concentrations showed a constant decrease until they reached baseline levels prior to immergence. Prehibernatory fattening started in late May, but no concomitant progesterone secretion was detected, as progesterone levels declined without a notable peak indicating a summer oestrus (Fig. 7).
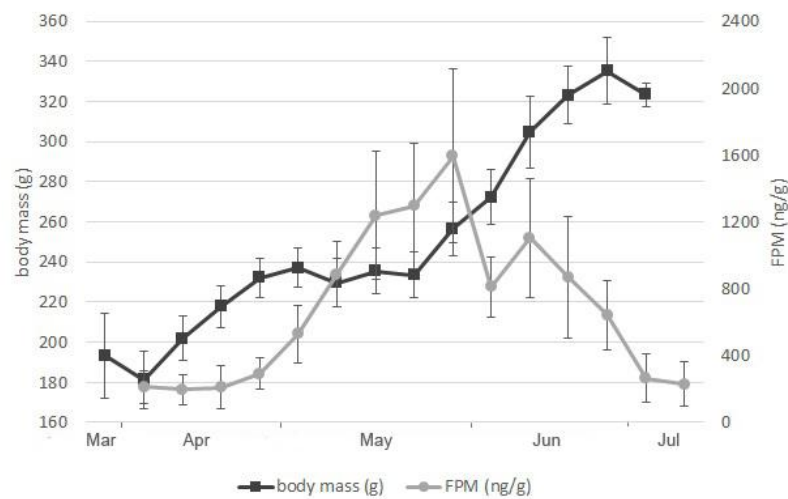
Figure 6. Mean ± SEM of body mass changes (g; n=4,7,10,9,10,10,9,9,10,9,10,9,9,7,3) and FPM concentrations (ng/g; n=4,4,6,5,9,10,8,9,8,9,9,7,9,7,3) of non-reproductive females 2009.
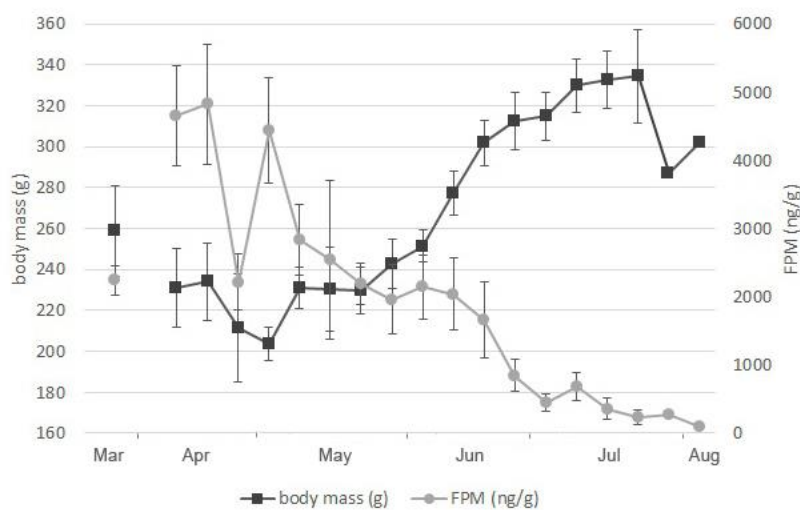
Figure 7. Mean ± SEM of body mass changes (g; n=3,0,3,2,3,4,5,3,6,5,5,6,5,5,5,5,4,3,1,1) and FPM concentrations (ng/g; n=2,0,3,2,3,4,5,2,6,5,6,6,5,5,5,5,3,3,1,1) of non-reproductive females 2014.

To determine changes of progesterone concentrations of unmated females in 2009 and non-reproductive females in 2014, we used the Δ-value, calculated by the difference of FPM concentrations between the prefattening and fattening phase for each year. In 2009, FPM concentrations increased by 50.03% ± 23.95% between the defined phases. In comparison, FPM concentrations of non-reproductive females in 2014 decreased by 52.49% ± 41.93% between the prefattening and fattening phase (2009: prefat: 718.88ng/g ± 255.21ng/g, fat: 1078.55ng/g ± 316.33ng/g, U=-2.69, p=0.007; 2014: prefat: 3391.61ng/g ± 485.19ng/g, fat: 1611.38ng/g ± 281.74ng/g, t=3.998, p=0.007) (Fig. 8).
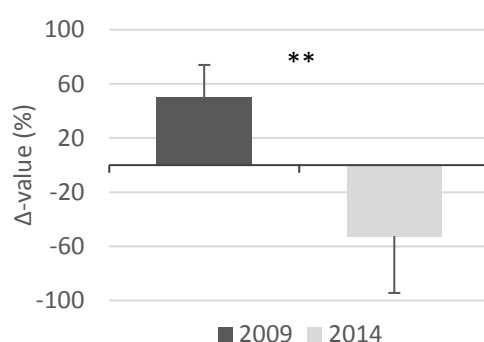
Figure 8. Δ-value (%) of changes of FPM concentrations between the prefattening and fattening phase of unmated (2009; n=10) and non-reproductive females (2014; n=6).

Vaginal cytology

For the analysis of ovarian activity, we collected vaginal smear samples during the active season, following the course of changes in cell composition. At the beginning of the active season and during the mating period, vaginal oestrus could be detected in one non-reproductive and one reproductive female. After the mating period, metoestrus dominated the smears in both reproductive and non-reproductive females. Non-reproductive females reentered prooestrus and oestrus during the second half of May (Fig. 9). In reproductive females, most specimen reentered vaginal oestrus within the weaning period or shortly thereafter during postlactation in the second half of June (Fig. 10).
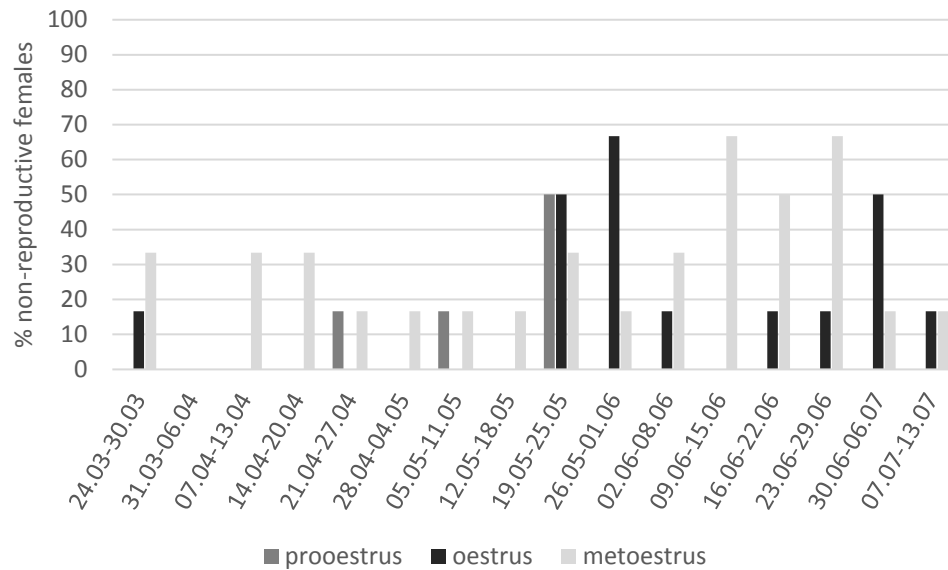
Figure 9. Percentage of non-reproductive females with smear samples classified as prooestrus, oestrus and metoestrus throughout the active season 2014 in weekly intervals. Females whose oestrus cycle switched during one weekly interval are represented in each of the cycle phases (n=6). Anoestrus samples were excluded from the analysis.
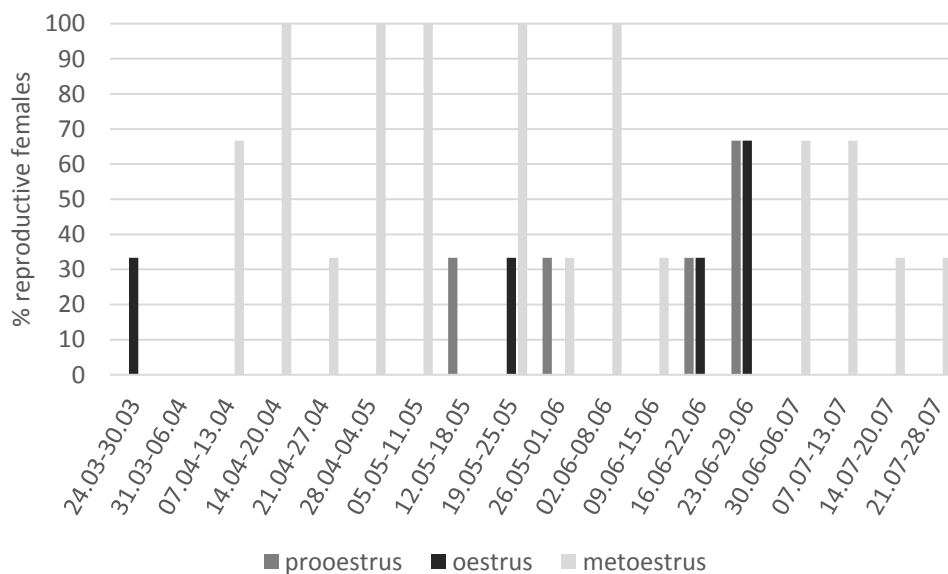
Figure 10. Percentage of reproductive females with smear samples classified as prooestrus, oestrus and metoestrus throughout the active season 2014 in weekly intervals. Females whose oestrus cycle switched during one weekly interval are represented in each of the cycle phases (n=3). Anoestrus samples were excluded from the analysis.

Foraging experiments

Two phases for each individual were determined and compared: (i) the prefattening and (ii) the fattening phase.

No significant differences were found between prefattening and fattening of each individual in the latency until the first approach to the bowl (paired samples t-test, t=0.977, p=0.373), nor in the total feeding time (paired samples t-test, t=1.045, p=0.331).

Total feeding time of non-reproductive females did not differ significantly between the two phases (Tab. 3). Data indicated a rather faster approach to the bowl in non-reproductive females during fattening than during prefattening. Data of reproductive females varied in the range of that of non-reproductive females (Tab. 3).

Table 3. Mean ± SEM of total feeding time (s) and latency (s) of non-reproductive (Non-rep) and reproductive (Rep) females during the prefattening and fattening phase 2014.

	Feeding time (s)		Latency (s)	
	Non-rep	**Rep**	**Non-rep**	**Rep**
prefattening	139.5s ± 63.32s (n=5)	150.67s ± 35.32s (n=3)	1398.11s ± 231.14 (n=3)	371.05s ± 189.48s (n=3)
fattening	122.89s ± 21.69s (n=5)	103.05s ± 27.45s (n=3)	769.25s ± 96.76s (n=5)	546.62s ± 111.69s (n=3)
t	0.379	-	-	-
p	0.724	-	-	-

Discussion

Reproductive output

Although all females were kept with contact to males, only 3 out of 9 female European ground squirrels reproduced in 2014 and 2 weaned their litter successfully. The low reproductive outcome in 2014 represents the initial point of discussion about potential negative influences on reproductive success in European ground squirrels.

In this study, reproductive females were exclusively adult individuals, raising the question whether age effects are the reason for the low reproductive outcome. However, Millesi et al. (1999a) showed that over 90% of all yearling females weaned their litter successfully. The age of females solely influenced reproductive outcome concerning litter size: yearling females often produced smaller litters. However, the overall reproductive output was not affected by a female's age (Millesi et al. 1999a). Hence, age effects can be ruled out as an influencing parameter on reproductive success.

Another possible factor affecting reproductive success in European ground squirrels is the yearly fluctuation in environmental conditions, especially spring weather, as has been shown in other *Spermophilus* species (Morton & Shermann 1978, Smith & Johnson 1985) and various mammal species (Merritt et al. 2001). Those studies showed that climate can easily lead to a shift in the seasonal timing and therefore affects reproductive outcome. Hufnagl et al. (2011) documented an exceptionally harsh winter in 2005/06 in Vienna that appeared to lower reproductive success in Common hamsters by 50% compared to previous seasons. However, Millesi et al. (1999b) illustrated variations in spring emergence of male European ground squirrels depending on weather conditions, but recorded almost no fluctuations in spring emergence of females. Thus, females seem to have a strict timetable for reproduction that is merely influenced by weather conditions (Millesi et al. 2000).

Failed mating seems to be a mentionable factor, especially when it comes to a low reproductive outcome (Vasilieva & Tchabovsky 2015). However, some non-reproductive females showed signs of an early aborted gestation, leading us to the conclusion that the low reproductive output may be caused at a later stage of reproduction, during gestation.

Previous studies showed that social stress, such as high population densities or frequent territorial behaviour, initiates a stress response associated with glucocorticoid secretion (Creel et al. 2013), influencing both behaviour and physiology in birds (Carere et al. 2001, Carere et al. 2003, Kotrschal et al. 2000) and rodents (Meerlo et al. 1996, Meerlo et al. 1997). Especially high density populations are often associated with social stressors (Christian 1971) and may lead to territorial

behaviour in females (*S. armatus*: Morse 1978, Slade & Balph 1974). This has been shown to affect reproductive success in several species (*Melospiza melodia*: Arcese & Smith 1988, *Microtus agrestis*: Ergon et al. 2011, *Microtus fortis*: Fowler 1981, Han et al. 2014). Millesi et al. (1998) described a population density of 41-68 reproductive European ground squirrels/ha in Austria as high. Referring to this, 9-15 individuals/0.02ha in our study (450-750 individuals/ha, respectively) exceeded this data by far. Nevertheless, the density in the outdoor enclosure was similar to that in earlier studies carried out in the same enclosure, in which all studied females weaned their litters successfully (Millesi et al. 2000, Millesi et al. 2008a, Strauss et al. 2009). Therefore, neither population density with its associated social stressors, nor housing conditions appeared to be a factors affecting on reproductive success of European ground squirrels in our study.

However, altricial species like *S. citellus*, that use burrow systems for rearing their offspring, show female territoriality in order to protect their vulnerable young (Festa-Bianchet & Boag 1982, Wolff & Peterson 1998). According to the offspring-defense hypothesis (Wolff & Peterson 1998), the main function of territoriality in females is to avoid infanticide. Interestingly, burrow systems were largely connected subterraneously in the outdoor enclosure, enabling neighbouring females to easily invade natal burrows, which might have led to an increased stress response in females of our study.

Gaasch (2016) documented a similar low reproductive output in the same population during the subsequent season of 2015. Despite the fact that all females in 2015 showed signs of gestation and parturition, only one out of 5 females reproduced successfully, whereas no lactation was documented in the other females (Gaasch 2016). Hence, infanticide was considered as the most plausible explanation (Gaasch 2016). Infanticide seems to be a common behavioural trait in rodents (Blumstein 1997, Labov et al. 1985), and has been confirmed in some species of the genus *Spermophilus* (*S. beecheyi*: Trulio 1996, *S. columbianus*: Hare 1989, Waterman 1984, *S. beldingii*: Sherman 1982, *S. citellus*: pers. obs.).

Gaasch (2016) documented higher faecal cortisol metabolites as measurable indicators for stress response (Möstl & Palme 2002) during the expected time of parturition in females that lost their juveniles compared to the successfully reproducing female. This indicated higher stress levels accompanied by losing their litters due to abandoning or killing the young, either by the mother or an intruder as a consequence of easily accessible natal burrows. In contrast to findings by Gaasch (2016), non-reproductive females of our study showed solely signs of an early gestation. This raises the idea of an aborted gestation due to stress-induced hormonal changes as a result of easily intruded burrows by neighbouring females. Those stressful social interactions are known to evoke endocrine responses including changes in prolactin-, gonadotrophin- and sex steroid-secretion

(Marchlewska-Koj 1997, Rivier & Rivest 1991, Tilbrook et al. 2000, Yonetani et al. 1974). Prolactin stimulates progesterone secretion by the *corpora lutea* that is necessary for the maintenance of pregnancy (Bole-Feysot et al. 1998, Niswender et al. 2000). Social stress acts on the neurohormonal system, inhibiting the release of prolactin (Marchlewska-Koj 1997) and, thus, decreasing progesterone titres in inseminated female rodents (*Microtus pennsylvanicus*: Clulow & Langford 1971, *Mus musculus*: Bruce 1960, Chipman & Fox 1966, Marchlewska-Koj 1977). This leads to a pregnancy blocking by the prevention of blastocyst implantation (Rosser et al. 1989). In alpine marmots, for example, reproductive failure despite clear signs of early gestation was associated with social stressors, like male takeovers, during gestation leading to a block of pregnancy (Hackländer & Arnold 1999).

Considered all aspects above, the architecture of subterraneously connected burrow systems seems to affect the endocrine balance of steroids that might result in reproductive failure.

Progesterone analysis

Progesterone is mainly synthesised and secreted by c*orpora lutea* in order to prepare the reproductive tract for maintenance of pregnancy in mammals (Niswender et al. 2000). Millesi et al. (2008b) provided evidence of intact *corpora lutea* in female Eurpean ground squirrels during both postlactation and prehibernation. Interestingly, *corpora lutea* of the pregnancy seemed not to be maintained and rejuvenated, but built up after weaning as a second generation *corpora lutea* (Millesi et al. 2008b). However, hormonal analysis revealed no second progesterone peak during summer, as has been documented in European ground squirrels (Millesi et al. 2008b, Strauss et al. 2009). Generally, the summer oestrus is associated with mainly two beneficial effects: (i) the priming effect on follicles and (ii) the enhancement of prehibernatory fattening (Millesi et al. 2008b, Strauss et al. 2009).

Endocrine influence of the summer oestrus

Earlier studies emphasise possible advantageous influences of the summer oestrus, along with its accompanying elevated steroid titres, on follicular development (Millesi et al. 2008b, Palter et al. 2001, Strauss et al. 2009). Progesterone and oestrogen excretion during late lactation may stimulate follicular clusters to develop into higher antral stages (Palter et al. 2001). Therefore, it is assumed that follicular maturation is initiated during summer oestrus and can be rapidly reactivated and completed at vernal emergence (Millesi et al. 2008b). Hence, females are receptive immediately between 2 and 13 days after spring emergence (Millesi et al. 1999a, 2000). Earlier

reproduction positively influences litter size and survival of the pups, as females can invest more time in lactation (Huber et al. 1999, 2001, Millesi et al. 1999a, Rieger 1996). Hence, elevated oestradiol and progesterone titres during late lactation correlate positively with litter size in the subsequent season (Huber et al. 1999). Thus, females exhibiting high oestradiol and progesterone levels during the second half of lactation reproduce in higher quantities in the following year. Accordingly, we assume that the missing progesterone excretion within the period of summer oestrus inhibited the possible priming effect on follicles during late lactation. This may negatively affect the reproductive output in the subsequent year, which is corroborated by the low reproductive outcome of the same population at Bisamberg in the following year (Gaasch, 2016). The time of conception, however, seemed not to differ conspicuously between 2009 and 2014.

In addition to hormonal analysis, we documented cytological changes in vaginal smears throughout the active season to record ovarian activity. Given the fact that vaginal oestrus could be assigned to only one non-reproductive and one reproductive female after emergence and during mating, we assume that spring oestrus had been completed in most females in 2014 before data acquisition started. Most reproductive females reentered prooestrus and oestrus, indicated by a high abundance of cornified cells, within the weaning period or during postlactation during the second half of June. Due to the lack of reproductive effort, seasonal events are shifted in non-reproductive females (Millesi et al. 2008a). Therefore, most non-reproductive females reentered prooestrus and oestrus 4 weeks prior to reproductive females. These results suggested an ovulation, followed by follicular atresia and spontaneous luteolysis, leading to a destruction of *corpora lutea* as a main source for progesterone (Gaytán et al. 2001, Quirk et al. 1986).

Summer oestrus and its influence on the enhancement of prehibernatory fattening

Besides the stimulation of follicular development, progesterone and its endocrine effects seemed to be beneficial in order to facilitate and accelerate prehibernatory fattening (Strauss et al. 2009). As shown in rats, progesterone directly influenced genes expression in both adipose tissue and hypothalamus (Stelmanska & Sucajtys-Szulc 2014, Stelmanska & Swierczynski 2013). Therefore, a summer oestrus cycle with elevated progesterone titres might facilitate the accumulation of body fat reserves in order to reach the required threshold in a shorter time span. Accordingly, females immerged into hibernation about four weeks prior to males, despite both time and energy expenditure due to gestation, parturition, lactation and prehibernatory fattening (Matějů 2008, Millesi et al. 1999b, Millesi et al. 2008b).

As the onset of prehibernatory fattening of non-reproductive females did not differ between 2009 and 2014, the course of the following fattening phase in the respective years could be compared. The enhanced fattening period of unmated females in 2009, indicated by a more pronounced body mass gain in half of the time span than non-reproductive females in 2014, represented valuable clues to the importance of progesterone excretion during prehibernatory fattening. The missing progesterone excretion during summer in non-reproductive females in 2014 may have negatively influenced prehibernatory fattening. Given the fact that non-reproductive females in 2014 needed about 3 weeks longer for fattening and therefore gained about half as much weight per day than unmated females in 2009, we corroborate the hypothesis that the summer oestrus cycle is essential for the enhancement of prehibernatory fattening.

Physical parameters and seasonal timing

Although no differences were found in the first day of observation between non-reproductive females in 2009 and 2014, the body mass at emergence was significantly higher in females in 2014 than that in 2009. Nevertheless, individual body mass of females was within the range of documented body mass of European ground squirrels in both years (Matějů 2008). All studied animals had access to additional food supplies to ensure sufficient food resources. However, Millesi et al. (1999b) showed that food supplementation during the monitored season had no effect on the range of body mass changes in comparison to free-living European ground squirrels. Therefore, our provided additional food supplies can be ruled out as a reason for higher body mass of females in 2014. At the end of April, mean body mass decreased. This putative body mass loss was caused by one yearling female, as a yearling's emergence body mass differ to that of older females (Millesi et al. 1999b). Both, non-reproductive females in 2009 and 2014 started prehibernatory fattening between 19th May and 25th May. In 2009, unmated females showed a higher increase of body weight gain per day and completed fattening about 3 weeks earlier than non-reproductive females in 2014, resulting in an earlier termination of above ground activity. However, irrespective of the shortened active season in non-reproductive females in 2009, body mass shortly before immergence did not differ between the two years. This supports the assumption that European ground squirrels enter their hibernacula as soon as they have reached a certain threshold in body fat content (Millesi et al. 2008a, b). Thus, females terminate their above ground activity once they have obtained sufficient body fat reserves necessary to survive hibernation, instead of using the additional time to get fatter (Millesi et al. 2008a). Michener (1984) proposed that an early immergence seems to be advantageous, as an extended active period could lead to a higher

mortality risk due to predation. Therefore, we emphasise the importance of reaching a certain threshold in body mass as an initiator of aboveground activity termination.

Foraging experiments

We detected no significant behavioural changes in non-reproductive females between the prefattening and fattening phase. Nonetheless, it is shown that many sciurid hibernators choose their food more selectively and increase their food intake rates prior to hibernation (Dark 2005, Fall 1971, Florant & Healy 2012, Frank 1994, Frank et al. 1998). Mrosovsky & Boshes (1986) showed that mean frequency as well as total food consumption of Golden-mantled ground squirrels increased about twofold during the hyperphagic period compared to the prefattening phase.

Feeding patterns and fluctuations in body mass are affected by several hormonal factors (Florant & Healy 2012) including gonadal steroids, such as progesterone and prolactin that are typically considered lipogenic (Saleh et al. 2011). The luteotrophic hormone prolactin is released from the pituitary in response to suckling stimulation (Pi & Voogt 2001) and is considered to trigger both an increased food intake (hyperphagia) and body weight gain in birds (Buntin et al. 1999) and rats (Sauvé & Woodside 1996, Woodside 2007). However, due to restricted sampling, we analysed solely the gonadal hormone progesterone. As discussed above, progesterone is known to evoke an upregulated appetite and an increased food intake in rats (Stelmanska & Sucajtys-Szulc 2014, Stelmanska & Swierczynski 2013). The summer oestrus with its elevated progesterone secretion in European ground squirrels is hypothesised to allow females an accelerated body fat accumulation prior to hibernation (Strauss et al. 2009). We could not find any pronounced changes in foraging behaviour between the prefattening and the fattening phase. This could perhaps be a captivity effect, as high-quality food was available for all individuals. Nevertheless, increased food intake during fattening would have been expected. On the other hand, the lack of peak progesterone levels and low body mass increase rates during the fattening period in the studied females would support the hypothesis that progesterone positively affects feeding behaviour in this species and by that accelerates the fattening process.

Relating to reproductive females, sample sizes were too small for statistical analysis, but descriptive data analysis points in the direction of an increased foraging behaviour in lactating females during the prefattening phase that coincides with the lactation phase. These results are substantiated by findings of Kenagy et al. (1989) that Cascade ground squirrels increase their foraging time from the mating phase to the lactation phase. The necessity for higher daily energy intake in reproductive

females may be responsible for the increase in foraging behaviour, as lactation is generally the time of maximal energy intake in mammals (Daan et al. 1991, Kirkwood 1983).

Since the sample size was comparatively small, it might be valuable to regard existing data more as a trend than as a definite result – this underlines the importance of further studies. Nevertheless, present data emphasise that lactation is an energy-demanding phase, in which female European ground squirrels incur the nutritional needs of their young. Additionally, non-reproductive females without elevated progesterone levels showed no behavioural changes regarding feeding patterns between prefattening and fattening, which lays the foundation for further studies investigating interactions between progesterone and foraging behaviour.

Conclusion

Considering all aspects discussed above, we emphasise the possible beneficial effects of the non-reproductive summer oestrus cycle. Given the results, the summer oestrus seems to be an important factor on the effectiveness of prehibernatory fattening and perhaps on reproductive success in the subsequent season.

Given the alarming decline of European ground squirrels within the last decades, conservation actions are needed to stabilise current populations (Coroiu et al. 2008, Hoffmann et al. 2003a, b, Kryštufek et al. 2012). Short-grass steppe, natural or anthropogenic meadows and pastures represent the species' specific habitat requirements (Hoffmann et al. 2003b). However, European ground squirrels are vulnerable to both the urbanisation (Hoffmann et al. 2003b) and the loss of short-grass meadows due to increased intensification of agriculture (Spitzenberger 2005). Therefore, finding a promising approach to implement conservation action plans is crucial for the populations' survival. A cornerstone for future studies on specific mechanisms linked to effectiveness of prehibernatory fattening was laid, but to which extent maximal support and maintenance of reproductive success and therefore improving inclusive fitness is realisable, is still unclear and should be addressed in further studies.

References

Arcese P, Smith JNM (1988): Effects of population density and supplemental food on reproduction in song sparrows. *Journal of Animal Ecology* 57: 119-136.

Aschauer A, Hoffmann IE, Millesi E (2006): Endocrine profiles and reproductive output in European ground squirrels after unilateral ovariectomy. *Animal Reproduction Science* 92: 392-400.

Blumstein DT (1997): Infanticide among golden marmots (*Marmota caudata aurea*). *Ethology Ecology & Evolution* 9: 169-173.

Bole-Feysot C, Goffin V, Edery M, Binart N, Kelly PA (1998): Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. *Endocrine Reviews* 19(3): 225-268.

Bruce HM (1960): A block to pregnancy in the mouse caused by proximity of strange males. *Journal of Reproduction and Fertility* 1: 96-103.

Buntin JD, Hnasko RM, Zuzick PH (1999): Role of the ventromedial hypothalamus in prolactin-induced hyperphagia in Ring Doves. *Physiology & Behavior* 66: 255-261.

Carere C, Welink D, Drent PJ, Koolhaas JM, Groothuis TGG (2001): Effect of social defeat in a territorial bird (*Parus major*) selected for different coping styles. *Physiology & Behavior* 73: 427-433.

Carere C, Groothuis TGG, Möstl E, Daan S, Koolhaas JM (2003): Fecal corticosteroids in a territorial bird selected for different personalities: daily rhythm and the response to social stress. *Hormones and Behavior* 43: 540-548.

Chipman RK, Fox KA (1966): Oestrous synchronization and pregnancy blocking in wild house mice (*Mus musculus*). *Journal of Reproduction and Fertility* 12: 233-236.

Christian JJ (1971): Population density and reproductive efficiency. *Biology of Reproduction* 4: 248-294.

Clulow FV, Langford PE (1971): Pregnancy block in the Meadow vole, *Microtus pennsylvanicus*. *Journal of Reproduction and Fertility* 24: 275-277.

Coroiu C, Kryštufek B, Vohralík V, Zagorodnyuk I (2008): *Spermophilus citellus*. The IUCN Red List of Threatened Species 2008. *www.iucnredlist.org*. Accessed 24.08.2016

Creel S, Dantzer B, Goymann W, Rubenstein DR (2013): The ecology of stress: effects of the social environment. *Functional Ecology* 27: 66-80.

Daan S, Masman D, Strijkstra AM, Kenagy GJ (1991): Daily energy turnover during reproduction in birds and mammals: its relationship to basal metbolic rate. In: Everts LG, Strijkstra AM, Hut RA, Hoffmann IE, Millesi E (2004): Seasonal variation in daily activity patterns of free-ranging European ground squirrels (*Spermophilus citellus*). *Chronobiology international* 21(1): 57-71.

Dark J (2005): Annual lipid cycles in hibernators: integration of physiology and behavior. *Annual Review of Nutrition* 25: 469-497.

Divjak A (2009): Verlauf der aktiven Saison und ovarielle Aktivität bei unverpaarten weiblichen Europäischen Zieseln (*Spermophilus citellus*). Masterarbeit, *Universität Wien*, Vienna.

Ergon T, Ergon R, Begon M, Telfer S, Lambin X (2011): Delayed density-dependent onset of spring reproduction in a fluctuating population of field voles. *Oikos* 120: 934–940.

Everts LG, Strijkstra AM, Hut RA, Hoffmann IE, Millesi E (2004): Seasonal variation in daily activity patterns of free-ranging European ground squirrels (*Spermophilus citellus*). *Chronobiology international* 21(1): 57-71.

Fall MW (1971): Seasonal variations in the food consumption of woodchucks (*Marmota monax*). *Journal of Mammalogy* 52(2): 370-375.

Festa-Bianchet M, Boag DA (1982): Territoriality in adult female Columbian ground squirrels. *Canadian Journal of Zoology* 60: 1060-1066.

Florant GL, Healy JE (2012): The regulation of food intake in mammalian hibernators: a review. *Journal of Comparative Physiology-B* 182: 451-467.

Fortune JE, Wissler RN, Vincent SE (1986): Prolactin modulates steroidogenesis by rat granulosa cells: II. effects on estradiol. *Biology of Reproduction* 35: 92-99.

Fowler CW (1981): Density dependence as related to life history strategy. *Ecology* 62: 1077-1090.

Frank CL (1994): Polyunsaturate content and diet selection by ground squirrels (*Spermophilus lateralis*). *Ecological Society of America* 75(2): 458-463.

Frank CL, Dierenfeld ES, Storey KB (1998): The relationship between lipid peroxidation, hibernation, and food selection in mammals. *American Zoologist* 38(2): 341-349.

Gaasch J (2016): Reproductive performance, stress load and prehibernatory fattening in European ground squirrels under semi-natural conditions. Master's thesis*, University of Vienna*, Vienna.

Gaytán F, Bellido C, Morales C, Sánchez-Criado JE (2001): Cyclic changes in the responsiveness of regressing *corpora lutea* to the luteolytic effects of prolactin in rats. *Reproduction* 122: 411-417.

Hackländer K, Arnold W (1999): Male-caused failure of female reproduction and its adaptive value in Alpine marmots (*Marmota marmota*). *Behavioral Ecology* 10(5): 592-597.

Han Q, Zhang M, Guo C, Shen G, Wang Y, Li B, Xu Z (2014): Effects of population density on reproduction in *Microtus fortis* under laboratory conditions. *Acta Biologica Hungarica* 65: 121-131.

Hare JF (1989): Intraspecific killing of preweaned young in the Columbian ground squirrel, *Spermophilus columbianus*. *Canadian Journal of Zoology* 69: 797-800.

Hoffmann IE, Millesi E, Huber S, Everts LG, Dittami JP (2003a): Population dynamics of European ground squirrels (*Spermophilus citellus*) in a suburban area. *Journal of Mammalogy* 84(2): 615-626.

Hoffmann IE, Millesi E, Pieta K, Dittami JP (2003b): Anthropogenic effects on the population ecology of European ground squirrels (*Spermophilus citellus*) at the periphery of their geographic range. *Mammalian Biology* 68: 205-213.

Huber S, Millesi E, Walzl M, Dittami JP, Arnold W (1999): Reproductive effort and costs of reproduction in female European ground squirrels. *Oecologia* 121: 19-24.

Huber S, Hoffmann IE, Millesi E, Dittami JP, Arnold W (2001): Explaining the seasonal decline in litter size in European ground squirrels. *Ecography* 24: 205-211.

Hufnagl S, Franceschini-Zink C, Millesi E (2011): Seasonal constraints and reproductive performance in female Common hamsters (*Cricetus cricetus*). *Mammalian Biology* 76: 124-128.

Hut RA, Van Oort BEH, Daan S (1999): Natural entrainment without dawn and dusk: the case of the European ground squirrel (*Spermophilus citellus*). *Journal of Biological Rhythms* 14: 290-299.

Kenagy GJ, Barnes BM (1988): Seasonal reproductive patterns in four coexisting rodent species from the Cascade Mountains, Washington. *Journal of Mammalogy* 69(2): 274-292.

Kenagy GJ, Sharbaugh SM, Nagy KA (1989): Annual cycle of energy and time expenditure in a Golden-mantled ground squirrel population. *Oecologia* 78: 269-282.

Kirkwood JK (1983): A limit to metabolisable energy intake in mammals and birds. In: Everts LG, Strijkstra AM, Hut RA, Hoffmann IE, Millesi E (2004): Seasonal variation in daily activity patterns of free-ranging European ground squirrels (*Spermophilus citellus*). *Chronobiology international* 21(1): 57-71.

Kotrschal K, Dittami J, Hirschenhauser K, Möstl E, Peczely P (2000): Effects of physiological and social challenges in different seasons on fecal

testosterone and corticosterone in male Domestic geese (*Anser domesticus*). *Acta ethologica* 2: 115-122.

Kryštufek B, Glasnovic P, Petkovski S (2012): The status of a rare phylogeographic lineage of the vulnerable European souslik *Spermophilus citellus*, endemic to central Macedonia. *Fauna & Flora International*, *Oryx* 46(3): 442-445.

Labov JB, Huck UW, Elwood RW, Brooks RJ (1985): Current problems in the study of infanticidal behavior of rodents. *The Quarterly Review of Biology* 60: 1-20.

Marchlewska-Koj A (1977): Pregnancy block elicited by urinary proteins of male mice. *Biology of Reproduction* 17: 729-732.

Marchlewska-Koj A (1997): Sociogenic stress and rodent reproduction. *Neuroscience and Biobehavioral Reviews* 21(5): 699-703.

Matějů J (2008): Ecology and space use in a relict population of the European Ground Squirrel (*Spermophilus citellus*) at the north-western edge of its distribution range. *Lynx*-39(2): 263–276.

McNeilly AS (2001): Lactational control of reproduction. *Reproduction, Fertility and Development* 13: 583-590.

Meerlo P, Overkamp GJF, Daan S, Van Den Hoofdakker RH, Koolhaas JM (1996): Changes in behaviour and body weight following a single or double social defeat in rats. *Stress* 1: 21-32.

Meerlo P, Overkamp GJF, Koolhaas JM (1997): Behavioural and physiological consequences of a single social defeat in Roman high- and low-avoidance rats. *Psychoneuroendocrinology* 22(3): 155-168.

Merritt JF, Lima M, Bozinovic F (2001): Seasonal regulations in fluctuating small mammal populations: feedback structure and climate. *Oikos* 94: 505-514.

Michener GR (1984): Age, sex and species differences in the annual cycle of ground-dwelling sciurids: implications for sociality. In: Murie JO, Michener GA (eds.): The biology of ground dwelling squirrels: annual cycles, behavioral ecology, and sociality. *University of Nebraska Press*, Lincoln: 81-107.

Millesi E, Huber S, Dittami JP, Hoffmann IE, Daan S (1998): Parameters of mating effort and success in male European ground squirrels, *Spermophilus citellus*. *Ethology* 104: 298-313.

Millesi E, Huber S, Everts LG, Dittami JP (1999a): Reproductive decisions in female European ground squirrels: factors affecting reproductive output and maternal investment. *Ethology* 105: 163-175.

Millesi E, Strijkstra AM, Hoffmann IE, Dittami JP, Daan S (1999b): Sex and age differences in mass, morphology, and annual cycle in European ground squirrels, *Spermophilus citellus*. *Journal of Mammalogy* 80: 218-231.

Millesi E, Huber S, Pieta K, Walzl M, Arnold W, Dittami JP (2000): Estrus and estrogen changes in mated and unmated free-living European ground squirrels. *Hormones and Behavior* 37: 190-197.

Millesi E, Hoffmann IE, Aschauer A, Franceschini C (2004): Reproduction and hibernation in females: a comparison of two sympatric ground-dwelling rodents. In: Life in the Cold: Evolution, Mechanisms, Adaption, and Application. 12th International Hibernation Symposium. *Biological Papers of the University of Alaska* 27: 367-375.

Millesi E, Divjak A, Strauss A (2008a): Seasonal timing and pre-hibernation fattening in breeding and non-breeding European ground squirrels. In: Lovegrove BG, McKechnie AE (eds.): Hypometabolism in animals: torpor, hibernation and cryobiology. *Interpak Books*, Pietermaritzburg: 291-296.

Millesi E, Strauss A, Burger T, Hoffmann IE, Walzl M (2008b): Follicular development in European ground squirrels (*Spermophilus citellus*) in different phases of the annual cycle. *Reproduction* 136: 205-210.

Morse TE (1978): A description and analysis of behavior patterns among Uinta ground squirrels. All Graduate Theses and Dissertations, *Utah State University*, Logan.

Morton ML, Sherman PW (1978): Effects of a spring snowstorm on behavior, reproduction, and survival of Belding's ground squirrels. In: King WJ, Festa-Bianchet M, Hatfield SE (1991): Determinants of reproductive success in female Columbian ground squirrels. *Oecologia* 86: 528-534.

Möstl E (2002): Measurement of cortisol metabolites in faeces of ruminants. *Veterinary Research Communications* 26: 127–139.

Möstl E, Palme R (2002): Hormones as indicators of stress. *Domestic Animal Endocrinology* 23: 67-74.

Mrosovsky N & Boshes M (1986): Meal patterns and food intakes of ground squirrels during circannual cycles. *Appetite* 7: 163-175.

Niswender GD, Juengel JL, Silva PJ, Keith Rollyson M, McIntush EW (2000): Mechanisms controlling the function and life span of the corpus luteum. *Physiological Reviews* 80(1): 1-29.

Palter SF, Tavares AB, Hourvitz A, Veldhuis JD, Adashi EY (2001): Are estrogens of import to primate/human ovarian folliculogenesis? *Endocrine Reviews* 22: 389-424.

Papanicolaou GN (1954): Atlas of exfoliative cytology. *Harvard University Press*, Cambridge.

Pi X, Voogt JL (2001): Mechanisms for suckling-induced changes in expression of prolactin receptor in the hypothalamus of the lactating rat. *Brain Research* 891: 197-205.

Pilastro A (1992): Communal nesting between breeding females in a free-living population of fat Dormouse (*Glis glis* L.). *Bolletino di zoologia* 59: 63-68.

Quanstrom WR (1968): Some aspects of the ethoecology of Richardson's ground squirrel in eastern North Dakota. Dissertation*, University of Oklahoma*, Norman.

Quesnel H, Prunier A (1995): Endocrine bases of lactational anoestrus in the sow. *Reproduction Nutrition Development* 35: 395-414.

Quirk SM, Hickey GJ, Fortune JE (1986): Growth and regression of ovarian follicles during the follicular phase of the oestrous cycle in heifers undergoing spontaneous and PGF-2α-induced luteolysis. *Journal of Reproduction and Fertility* 77: 211-219.

Rieger JF (1996): Body size, litter size, timing of reproduction, and juvenile survival in the Uinta ground squirrel, *Spermophilus armatus*. *Oecologia* 107: 463-468.

Rivier C, Rivest S (1991): Effect of stress on activity of the hypothalamic-pituitary-gonadal axis: peripheral and central mechansims. *Biology of Reproduction* 45: 523-532.

Rosser AE, Remfry CJ, Keverne EB (1989): Restricted exposure of mice to primer pheromones coincident with prolactin surges blocks pregnancy by changing hypothalamic dopamine release. *Journal of Reproduction and Fertility* 87: 553-559.

Saleh J, Al-Wardy N, Farhan H, Al-Khanbashi M, Cianflone K (2011): Acylation stimulating protein: a female lipogenic factor?. *Obesity Reviews* 12(6): 440-448.

Sauvé D, Woodside B (1996): The effect of central administration of prolactin on food intake in virgin female rats is dose-dependent, occurs in the absence of ovarian hormones and the latency to onset varies with feeding regimen. *Brain Research* 729: 75-81.

Scott JP (1956): The analysis of social organization in animals. *Ecology* 37(2): 213-221.

Sherman PW (1982): Infanticide in ground squirrels. In: Wolff JO, Peterson JA (1998): An offspring-defense hypothesis for territoriality in female mammals. *Ethology Ecology & Evolution* 10: 227-239.

Slade NA, Balph DF (1974): Population ecology of Uinta ground squirrels. *Ecology* 55: 989-1003.

Smith GW, Johnson DR (1985): Demography of a townsend Ground squirrel population in southwestern Idaho. *Ecology* 66: 171-178.

Spitzenberger F (2005): *Spermophilus citellus* (Linnaeus, 1766), Ziesel. In: Zulka KP (ed.): Rote Liste der Säugetiere Österreichs. In: Ramos-Lara N, Koprowski JL, Kryštufek B, Hoffmann IE (2014): *Spermophilus citellus* (Rodentia: Sciuridae). *Mammalian Species* 46(913): 71-87.

Spoelstra K, Strijkstra AM, Daan S (2000): Ground squirrel activity during the solar eclipse of August 11, 1999. *International Journal of Mammalian Biology* 65: 307-308.

Stelmanska E, Swierczynski J (2013): Up-regulation of lipogenic enzyme genes expression in inguinal white adipose tissue of female rats by progesterone. *Journal of Steroid Biochemistry & Molecular Biology* 134: 37-44.

Stelmanska E, Sucajtys-Szulc E (2014): Enhanced food intake by progesterone-treated female rats is related to changes in neuropeptide genes expression in hypothalamus. *Endokrynologia Polska* 65: 46-52.

Strauss A, Hoffmann IE, Walzl M, Millesi E (2009): Vaginal oestrus during the reproductive and non-reproductive period in European ground squirrels. *Animal reproduction science* 112(3-4): 362–370.

Taya K, Greenwald GS (1982): Mechanisms of suppression of ovarian follicular development during lactation in the rat. *Biology of Reproduction* 27: 1090-1101.

Tilbrook AJ, Turner AI, Clarke IJ (2000): Effects of stress on reproduction in non-rodent mammals: the role of glucocorticoids and sex differences. *Reviews of Reproduction* 5: 105-113.

Trulio LA (1996): The functional significance of infanticide in a population of California ground squirrels (*Spermophilus beecheyi*). *Behavioral Ecology and Sociobiology* 38(2): 97-103.

Váczi O, Koósz B, Altbäcker V (2006): Modified ambient temperature perception affects daily activity patterns in the European ground squirrel (*Spermophilus citellus*). *Journal of Mammalogy* 87: 54-59.

Vasilieva N, Tchabovsky A (2015): A shortage of males causes female reproductive failure in yellow ground squirrels. *Science advances* 1(9): 1-6.

Waterman JM (1984): Infanticide in the Columbian Ground Squirrel, *Spermophilus columbianus*. *Journal of Mammalogy* 65(1): 137-138.

Wolff JO, Peterson JA (1998): An offspring-defense hypothesis for territoriality in female mammals*. Ethology Ecology & Evolution* 10: 227-239.

Woodside B (2007): Prolactin and the hyperphagia of lactation. *Physiology & Behavior* 91: 375-382.

Yeaton RI (1972): Social behavior and social organization in Richardson's ground squirrel (*Spermophilus richardsonii*) in Saskatchewan. *Journal of Mammalogy* 53(1): 139-147.

Yonetani S, Jojima M, Suzuki Y (1974): Blockade of ovulation in rats by forced immobilization for surgical treatment. *Endocrinologia japonica* 21(1): 61-68.

Zusammenfassung

Ein strikter Jahresrhythmus prägt die aktive Saison von Winterschläfern, wodurch enge Zeitfenster für diverse saisonale Prozesse entstehen. Um sowohl die Reproduktion als auch das Überleben zu sichern, müssen Winterschläfer die Fortpflanzung sowie die Vorbereitung für den Winterschlaf innerhalb der kurzen aktiven Saison von fünf bis sieben Monaten meistern. Demzufolge ziehen die meisten obligaten Winterschläfer, wie das Europäische Ziesel (*Spermophilus citellus*), nur einen Wurf pro aktiver Saison groß. Dieser wird Ende April/Anfang Mai geboren. Die Entwöhnung der Jungtiere erfolgt, abhängig von Wurfgröße und mütterlicher Verfassung, etwa Mitte Juni. Studien haben gezeigt, dass zu dieser Zeit ein zweiter, nicht reproduktiver Ovarialzyklus beginnt. Dies führt nach der Ovulation zur Entwicklung neuer Gelbkörper, deren Aktivität sich in einem ausgeprägten Anstieg der Progesteronkonzentration widerspiegelt. Die gesteigerte Progesteronsekretion des Sommer-Östrus ist zeitlich an den Beginn des Auffettens für den Winterschlaf gebunden. Es wird vermutet, dass dies den Prozess des Auffettens bei Weibchen erleichtert bzw. beschleunigt, wodurch sie schneller ihr Zielgewicht für den Winterschlaf erreichen und somit ihre aktive Saison vor den Männchen beenden. Bei nicht-reproduzierenden Weibchen konnte in früheren Studien ebenfalls ein Sommer-Östrus nachgewiesen werden, wobei die Progesteronsekretion während des Frühsommers ein Monat vor der der reproduzierenden Weibchen ihr Maximum erreicht.

In dieser Studie wurden endokrinologische Schwankungen und Gewichtsveränderungen als Parameter für die Winterschlafvorbereitung, in Zusammenhang mit Fressverhalten vor und während des Auffettens analysiert.

Nur 3 der 9 untersuchten Weibchen reproduzierten, 2 davon zogen ihren Wurf erfolgreich groß. Die restlichen 6 Weibchen zeigten keine Anzeichen von fortgeschrittener Gestation, Geburt oder Laktation. Daher lag der Fokus bei dieser Studie auf den nicht-reproduzierenden Weibchen. Diese wurden mit unverpaarten Weibchen einer früheren Studie verglichen, welche 2009 im selben Gehege ohne Zugang zu Männchen untersucht wurden.

Anders als in früheren Studien zeigte keines der in 2014 untersuchten Weibchen erhöhte Progesteronwerte, die auf einen Sommer-Östrus hindeuten würden, obwohl in der Auswertung der Vaginalabstriche zu dieser Zeit Anzeichen eines Vaginalöstrus gefunden werden konnten. Der Beginn des Auffettens bei nicht-reproduzierenden Weibchen wies zwischen dem Jahr 2009 und 2014 keine Unterschiede auf, weshalb die anschließende Gewichtsveränderung in beiden Jahren miteinander verglichen werden konnte. Weibchen ohne erhöhte Progesteronsekretion zeigten eine signifikant längere Phase des Auffettens, wodurch die Rate der täglichen Gewichtszunahme deutlich geringer war. Die länger andauernde Winterschlafvorbereitung der nicht-reproduzierenden Weibchen 2014

führte zu einem signifikant späteren Winterschlafbeginn als im Vergleichsjahr. Interessanterweise gab es dennoch keinen Unterschied im Gewicht der nicht-reproduzierenden Weibchen 2014 und der unverpaarten Weibchen 2009 kurz vor Eintreten des Winterschlafes. Frühere Studien dokumentieren eine kritische Gewichtsschwelle, deren Erreichen die Hibernation einleitet. Das verlängerte, weniger effiziente Auffetten in 2014 könnte somit das Erreichen der notwendigen Gewichtsschwelle, und damit den Hibernationsbeginn, deutlich verzögert haben. Zwischen der Phase vor und während des Auffettens wurden keine Unterschiede im Fressverhalten innerhalb der nicht-reproduzierenden, bzw. reproduzierenden Weibchen festgestellt. Vor Beginn des Auffettens tendierten reproduzierende Weibchen jedoch dazu, sich eher dem bereitgestellten Futter anzunähern als nicht-reproduzierende Weibchen. Dies könnte auf den erhöhten Energieverbrauch der reproduzierenden Weibchen hindeuten, da zu dieser Zeit die Juvenilen noch im mütterlichen Bau gesäugt werden.

Diese Studie zeigt, dass nicht-reproduzierende Weibchen mit fehlender Progesteronsekretion während des Frühsommers eine verlängerte sowie weniger effiziente Auffettphase aufweisen als unverpaarte Weibchen mit einem Sommer-Östrus. Dieses Ergebnis unterstreicht die mögliche positive Wirkung des Sommer-Östrus auf die notwendige Körperfettanlagerung vor dem Winterschlaf.