MASTERARBEIT | MASTER'S THESIS

Titel | Title

Ovarian acitivity and prehibernatory fattening in reproductive and non-reproductive female European ground squirrels (Spermophilus citellus)

verfasst von | submitted by

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angestrebter akademischer Grad | in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien | Vienna, 2025

Studienkennzahl lt. Studienblatt | Degree programme code as it appears on the student record sheet:	UA 066 878
Studienrichtung lt. Studienblatt | Degree programme as it appears on the student record sheet:	Masterstudium Verhaltens-, Neuro- und Kognitionsbiologie
Betreut von | Supervisor:	ao. Univ.-Prof. Dr. Eva Millesi

Acknowledgements

I would like to express my deepest gratitude to my supervisor Eva Millesi for the opportunity to write my master thesis under her supportive guidance. Thank you for your endless patience, the many helpful comments and your valuable scientific input for my studies. Special thanks go to Carina Siutz for her experienced advice and help with evaluating the vaginal smears and to Elisabeth Barnreiter for her supportive help in the lab. Thanks are due to Dagmar Rotter for assisting me with all kinds of administrative matters.

My diligent colleagues Mariam, Kimberly, Melanie and Arthur I want to thank for the many hours we spent together with field work, data collection and lab work. Without your helping hands my data collection would not have been possible. I am grateful for my brilliant friends Florian and Xaver for their experienced advice on statistics and data presentation and some great laughs along the way.

I want to thank my beloved family, especially my parents, grandparents and parents-in-law for showing me unconditional love as well as their emotional and financial support during my studies. My special thanks go to my dearest friend Victoria for her longstanding friendship during and after our study time together, our motivational conversations and her constructive criticism on earlier versions of this thesis. I want to thank my lovely friend Birgit, who can make me laugh even on the worst days and always knows what I think before I even have to say it out loud – you are the best!

I am deeply grateful for my lovely partner Alex for his unconditional emotional support and being my best friend who always believes in me and makes me smile despite any challenges we may face together. Thank you for being the way you are!

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1 Abstract

As obligate hibernators European ground squirrels (*Spermophilus citellus*) rely exclusively on body fat reserves during hibernation. In previous studies the prehibernatory fattening phase coincided with elevated progesterone and oestradiol levels caused by a second non-reproductive oestrus cycle including an active luteal phase after weaning in summer. The second oestrus cycle and its accompanying endocrine changes have been assumed to positively affect prehibernatory fattening. Histological data revealed advanced follicular maturation shortly before onset of hibernation, thereby enabling females to become receptive and mate within a few days of vernal emergence.

To further investigate possible relationships between ovarian activity, endocrine secretion and body mass changes ten females kept in semi-natural enclosures were monitored in the season of 2021. Four females were classified as reproductive and six as non-reproductive by analysing body mass changes together with teat and vulva development. Faecal hormonal metabolites of progesterone and cortisol were measured from collected faeces. Vaginal smears were analysed to compare timing, duration, cell numbers and cell composition of vaginal oestrus during mating in spring and the non-reproductive period. The focus was on comparing spring and summer oestrus phases regarding physiological changes, particularly progesterone secretion patterns. The oestrus phase during the non-breeding period was further related to the timing and duration of prehibernatory fattening.

A vaginal oestrus was identified in all observed females during the mating period in spring. The second oestrus was observed earlier in non-reproductive females, probably due to the lack of inhibitory effects on ovarian activity by gestation and lactation. The duration, cell number and cell composition were similar in both oestrus phases.

Reproductive females showed a delayed second oestrus without elevated faecal progesterone metabolite concentrations. As shown in previous studies, progesterone levels increased in both groups coincidently with prehibernatory fattening. The main progesterone peak was shifted forward in non-reproductive females.

Course and extent of prehibernatory fattening were similar in both groups, but non-reproductive females were generally heavier and gained significantly more g/day during fattening. We found no evident differences in the duration, the onset and end dates of the fattening phase between the females which reproduced successfully and those that did not. Cortisol metabolite concentrations increased after the mating season and again towards the end of the season in both groups.

Elevated levels of progesterone and glucocorticoids could increase foraging behaviour, allowing females to accumulate sufficient body fat deposits prior to hibernation.
The results demonstrate that a lack of reproductive effort led to a shift in seasonal timing of ovarian activity and enhanced body condition prior to hibernation with possible advantageous effects of the second oestrus in summer on the subsequent reproduction period of the next season.

Key words: Spermophilus citellus, European ground squirrel, progesterone, hibernation, oestrus cycle, body mass, fattening phase, vaginal cytology

2 Introduction

Seasonality of environmental conditions has favoured the evolution of an endogenous circannual clock and annual rhythms in animals. Changing seasons often mean that food is abundant only during a limited time period and ambient temperatures can become so harsh, that animals need to entirely elude these energetically costly conditions to survive (Németh et al., 2009). Some species migrate vast distances to reach more favourable climes, others cache food in hidden spaces to feed on during the cold winter months. Some resident species become dormant for several months by reducing their metabolism to a bare minimum and fall into deep torpor to save energy during the winter months (Geiser, 2004; Williams et al., 2014).

Seasonal breeders schedule their short mating periods according to fluctuating resources, like food and water availability or other challenging environmental constraints, to ensure their offspring´s survival. Like many ground-dwelling sciurids, European ground squirrels (*Spermophilus citellus, Rodentia, Sciuridae*) are obligate hibernators, engage in scramble competition polygyny and are seasonal breeders producing only one litter per year (Ahmad, 2008; Michener, 1984; Millesi, Hoffmann, & Huber, 2004; Németh et al., 2009). Females therefore have to complete mating, gestation, lactation and prehibernatory fattening within a short period of about five months during their active season from late March/April to August (Huber et al., 1999; Millesi et al., 2010; Strauss et al., 2009).

European ground squirrels are medium-sized diurnal sciurids endemic to the grasslands of central and southeastern Europe. They live in colonies of variable population densities and construct individual underground burrows for resting, mating, nursing, hibernation and taking refuge from predators like stoats and birds of prey (Ramos-Lara et al., 2014). Like other ground squirrels, *S. citellus* rely solely on their body fat reserves during hibernation and no food caching behaviour has been documented so far (Ahmad, 2008; Michener, 1984; Millesi et al., 2010, 1999b). Gaining enough body mass, especially in the form of body fat, before entering hibernation can be crucial for overwinter survival and may also positively influence reproductive success as well as gonadal development at vernal emergence in the following season (Huber et al., 1999; Millesi et al., 1999b, 2000b, 2008b).

The species is in decline due to habitat loss and population fragmentation and currently listed as “Endangered” under the Bern Convention, Appendix 2 and included as “Endangered” in the IUCN Red List of Threatened Species 2020 and 2024 (IUCN, 2019, 2023; Slimen et al., 2012).

In most hibernating ground squirrels, adult males are the first to enter hibernation, followed by adult females and juveniles from the previous season. The latter are often called “yearlings”. Juveniles born in the current season are the last ones to enter hibernation.

Timing of cohort hibernation onset dates in *S. citellus* resembles those of related species as vernal emergence of adult males precedes that of adult females and yearlings (Michener, 1983, 1984). But conversely, adult females immerge into hibernation in late July/early August before adult males (late August/early September), followed by yearlings and lastly juveniles in mid-September/early October (Millesi et al., 1999a, 1999b; Ramos-Lara et al., 2014). The juveniles, who are born in May and weaned in mid-June, have only a few months to grow and prepare themselves for their first hibernation period. To achieve sexual maturity and participate in the mating season already after their first hibernation the juveniles need to complete structural growth and gonadal maturation before their first mating season in spring.

Likewise, adults must physiologically prepare for the next mating season that follows the hibernation period. While most females become sexually mature as yearlings after their first hibernation, onset of puberty in males is more flexible, as they can become sexually active either after their first or after their second hibernation period (Millesi et al., 2010, 1999b). According to literature, sexually mature males resume above ground activity in spring, usually early March to early April (Millesi et al., 2008b). They emerge from their hibernacula when temperatures are constantly over 0 °C, approximately two - four weeks before the females, with completely developed testes as well as elevated plasma testosterone levels (Barnes, 1996; Michener, 1983; Millesi et al., 2002, 2010; Williams et al., 2014).

This indicates that follicular development in females as well as testicular maturation in males must theoretically be completed either before hibernation or during brief spontaneous euthermic arousal phases in the hibernaculum. Although there seems to be a conflict between reduced metabolism paired with decreased body temperature during winter dormancy and simultaneous reproductive development. The animals spend 80 - 90 % of the hibernation period in deep torpor, thereby lowering their body temperatures down to a few degrees above ambient soil temperatures, typically ranging from 2 - 10 °C for inhabitants of temperate zones (Carey et al., 2003). Torpor is periodically interrupted by periods of spontaneous euthermic arousal phases lasting for about 12 - 24 hours with normal body temperature and intense metabolic activity (Barnes et al., 1986; Carey et al., 2003; Geiser, 2004). To conserve energy many physiological body functions and metabolic processes are arrested or suppressed during bouts of torpor and reactivated during arousal phases, including respiration, digestion, immune system functions, renal functions, transcription, translation, and mitosis (Ahmad, 2008; Barnes et al., 1986; Carey et al., 2003; Geiser, 2004; Németh et al., 2009). Previous studies on male ground squirrels suggest that rapid gonadal growth and advanced spermatogenesis presumably resume only after entering a prolonged euthermic state at the end of the hibernation period, usually remaining

sequestered in the hibernaculum, after which above ground activity is resumed prior to female emergence (Barnes et al., 1986; Barnes, 1996; Michener, 1983, 1984, 1998).

Evidence for this behaviour has been found in studies on golden-mantled ground squirrels (*Callospermophilus lateralis*) by Barnes et al., (1986) and Richardson´s ground squirrels (*Urocitellus richardsonii*) by Michener (1992, 1998).

However, Strauss et al. (2008) pointed out that testicular growth and spermatogenesis in male European ground squirrels are basically halted during hibernation but to be resumed during brief euthermic arousal periods towards the end of hibernation. Four juvenile males were monitored in climate chambers during their first hibernation period and their testes remained small and were located abdominally along with very baseline testosterone levels for the most part of hibernation. During the last weeks before emergence, the length of torpor bouts shortened as euthermic arousal phases became longer, and rising testosterone levels alongside testis growth were detected. While two males emerged with scrotal testes the other two showed scrotal testes within a few days after emergence. Maximum testis size and pigmentation was reached in all observed animals within three - four weeks after ending heterothermy. Hibernation patterns were identified by monitoring body temperature throughout the study period and revealed that the males did not spend a prolonged euthermic phase pre-emergence but left their hibernacula after the last torpor bout to further improve their body condition above ground in the absence of females (Strauss et al., 2008).

It should be noted that European ground squirrels do not cache food and rely on body fat reserves during hibernation (Ahmad, 2008), which would likely make a prolonged euthermic phase staying sequestered in the hibernaculum too energetically expensive compared to males of other species like *U. richardsonii* and *C. lateralis*, who are both known to cache food in their hibernacula (Barnes et al., 1986; Michener, 1992, 1998; Strauss et al., 2008).

In females the first vaginal oestrus ("spring oestrus") occurs 2 - 13 days after emergence, which usually happens from late March to early April (Millesi et al., 2000b), thus enabling them to mate within a few days after having left their hibernacula (Huber et al., 2001; Michener, 1983; Millesi et al., 1999a; Strauss et al., 2009). Early male emergence is therefore in this context interpreted as the result of intrasexual selection as emerging later than other males bears the risk of reduced breeding opportunities during the mating period, since females are impregnable only for a few days. Resuming above ground activity prior to females is one of several behaviours exhibited by males to enhance their individual reproductive success (Michener, 1983, 1984). Female oestrus in spring can be delayed due to low posthibernatory body mass, resulting in later born, smaller and often female-biased litters (Huber et al., 2001; Michener, 1983; Millesi et al., 1999a; Strauss et al., 2009).

Female European ground squirrels produce one single litter with a litter size ranging from 2 to 11 juveniles/litter each year. Litter size varies with population density and latitude and can also depend on maternal body condition (Millesi et al., 1999a; Ramos-Lara et al., 2014).
Yearling females generally produce smaller litters and deliver later in the season compared to older females (Hoffmann et al., 2003; Millesi et al., 2000a; Huber et al., 1999, 2001). Gestation duration is consistent for yearlings and adults and lasts approximately four weeks. The lactation period is varying from 22 to 61 days and its duration can depend on maternal body mass, age and overall seasonal timing of the female´s reproductive activities (Huber et al., 1999; Millesi et al., 1999a). Previous studies showed that lactation duration was positively correlated with litter size only in adult females, but not in yearling females and that generally early born litters were nursed for longer periods than later born litters (Huber et al., 1999; Millesi et al., 1999a).

After the three-week mating period in spring (end of March to April) gonadal regression in males starts. After recovering from mating effort, males could dedicate the remaining season to accumulate prehibernatory fat depots (Millesi et al., 2002, 2000a, 2000b). Females on the other hand, who had to invest additional time and energy into gestation, lactation and caring for their offspring for several weeks, have less time to achieve sufficient body fat content for the winter months. Non-reproductive females have been reported to enter their hibernacula about four weeks earlier than reproductive females, although immergence body mass was similar in both groups (Millesi et al., 2008b, 2008a).
This led to the conclusion that they would rather start hibernation earlier, after reaching a certain body mass or body fat content, than spending additional time above ground to gain even more body mass. Early immergence may still be the better option as mortality due to an extended hibernation period is assumed to be lower than the risk of predation during the additional time spent above ground, as has been proposed in edible dormice (*Glis glis*) (Ruf & Bieber, 2023). Accordingly, disappearance rates of small mammals during the active season are much higher than overwinter mortality (Michener, 1984; Millesi et al., 2008a, 1999b; Turbill et al., 2011). It can be concluded that reaching a certain body fat content seems to be the trigger for terminating above ground activity in female European ground squirrels (Millesi et al., 2008a; Steinerberger, 2017).
Consequently, this raises the question how reproductive females manage to gain sufficient body mass to enter hibernation approximately four weeks before males and about six weeks prior to juveniles, despite their energetically costly and time-consuming maternal investment (Millesi et al., 1999a, 1999b; Steinerberger, 2017).

It has been documented in both captive and free-ranging animals that adult females undergo a full second oestrus cycle ("summer oestrus"), after having weaned their offspring, including an active luteal phase (Millesi et al., 2008b, 2000b; Millesi & Hoffmann, 2008; Strauss et al., 2009). Previous studies have shown that in female European ground squirrels oestradiol and progesterone levels rise again during late lactation and after weaning and decline again with the onset of the prehibernatory fattening phase (Aschauer, 2003; Millesi et al., 2000b).

The summer oestrus cycle and its accompanying endocrine changes during the non-breeding season are assumed to have beneficial effects on prehibernatory fattening (Steinerberger, 2017; Strauss et al., 2009). The existence of a second vaginal oestrus, defined by the predominance of cornified epithelial cells, after weaning was further supported by the analysis of vaginal smears (Aschauer, 2003; Millesi et al., 2000b; Strauss et al., 2009). During the mating period oestradiol levels increased, peaked around oestrus and decreased during the following metoestrus and dioestrus (Millesi et al., 2000a). In case of fertilization the following gestation period was characterized by declining oestradiol levels and increasing progesterone secretion until parturition, when progesterone levels went down to baseline levels (Huber et al., 1999; Strauss et al., 2009).

In mammals the hormone progesterone is mainly synthesized and secreted by *corpora lutea* in the ovaries after ovulation to prepare the reproductive tract for enabling gestation and maintaining a pregnancy (Bachelot & Binart, 2005; Davis, 2002; Tomac et al., 2011). Progesterone has been suspected to facilitate rapid gain of body mass and thus playing an important role in the surprisingly early retirement of female *Spermophilus citellus* (Steinerberger, 2017; Strauss et al., 2009). In a study by Shirling et al. (1981) progesterone-treated rats ate more than the control group when given free access to food. High progesterone levels facilitate body mass gain even without increased food intake in rats (Lobo et al., 1993; Mendes et al., 1985; Shirling et al., 1981) as well as in mice (Dewar, 1964), assumably also via alterations of the gut microbiome, as has been shown in non-pregnant mice implanted with a progesterone-emitting device (Nuriel-Ohayon et al., 2021).

Millesi et al. (2000b) found that adult females left the hibernacula within 3 days after the last torpor bout with higher oestrogen levels than yearling females. They showed that follicular development shortly before entering hibernation was more progressed in adult than in juvenile females by investigating ovaries retrieved from the animals right before and after entering hibernation in a climate chamber. Differently matured follicles including intact tertiary follicles were found in adult females shortly before hibernation. Ovaries of juvenile females had mainly primordial or atretic follicles before immergence.

During hibernation oestrogen secretion remained low and no further ovarian maturation was detected in both adult and yearling females until termination of hibernation in spring. It can be concluded that follicular development in juvenile females was mostly terminated at an earlier stage leaving them to complete follicular maturation after terminating heterothermy (Millesi et al., 2000b). Previous research suggests positive effects for reproduction in the subsequent season by initiating follicular maturation before hibernation and thus enabling female oestrus and mating within a few days after vernal emergence (Millesi et al., 2000b).

As mentioned, early born litters are weaned longer and have more time to grow and increase body fat depots necessary to survive through their first winter (Huber et al., 1999, 2001). Moreover, early mating females produce larger litters, can presumably initiate summer oestrus sooner after weaning and may benefit from early immergence by being able to invest more energy into next seasons offspring (Millesi et al., 2008b, 1999a). In addition, the lack of reproduction in non-breeding females led to a shift of the seasonal timing in previous studies, resulting in an extended first vaginal oestrus during the mating phase in spring, perhaps to increase the chances of mating. The summer oestrus was induced earlier in unmated females than in mated females, due to the lack of a gestation and lactation period (Divjak, 2009; Millesi et al., 2008a; Steinerberger, 2017).

2.1 Research aims

For this study the reproductive performance and the course of body mass changes, vaginal cell composition changes together with indirect measurement of progesterone and cortisol concentrations via hormone metabolites in faeces was closely monitored in ten female European ground squirrels kept under semi-natural conditions during the active season of 2021. Based on the findings from previous studies the current study investigated potential differences between the first oestrus period in spring, during the mating phase and a second period of ovarian activity during the non-reproductive period. Vaginal smears were taken throughout the season to compare the timing, duration, cell numbers and cell composition of both phases.

We observed the course of progesterone and cortisol metabolites and aimed at documenting potential relationships between endocrine changes and body mass course, in particular the timing and duration of the fattening phase, as well as body mass gain throughout the active season. The main goal was to compare the first and second oestrus phase with regard to vaginal cell composition, progesterone levels and body mass changes within individual females.

3 Methods

3.1 Housing conditions

The data collection for this study was conducted from the 10th of March 2021 to the 25th of September 2021 at Bisamberg, Vienna, Austria. The animals were housed in two separate outdoor enclosures within the natural habitat of the species, located on a slope facing to the south-east and surrounded by vineyards and orchards. Both enclosures were built in a similar way, with a metal anti-rust grid buried about three meters beneath the surface to make sure the animals could not escape. Above ground the metal arc construction reached up approximately three to four meters with a 1.5 meters high metal fencing all around the enclosure (Figure 1 and 2). The fence also had a double bent-over metal sheet edge at the top to prevent the study animals from climbing out and other animals like stoats from invading the enclosure and harming the study animals. Additionally, there was a bird net draped and fixed over the opening of the whole enclosure Nr.1 to keep off arial predators, as soon as the juveniles had been spotted outside their mother´s burrows.

Figure 1 Outdoor enclosure Nr.1 at Bisamberg, Vienna, Austria. (Author´s image).

Figure 2 Outdoor enclosure Nr.2 at Bisamberg, Vienna, Austria with capture equipment box, clipboard, and Tomahawk life traps (Author´s image).

3.2 Feeding regime

Since high grass would have made it difficult to spot the animals and find their borrow holes, the grass inside and around the enclosures has been mowed a few times throughout the season. Additional water in bowls and supplementary food was provided to balance out their diet. The additional food included gras pellets (ssniff Spezialitäten GmbH, V2233-000 Guinea pig maintenance, 4mm), rodent grain food, carrots, salad, herbs like clover and dandelion and occasionally an apple. Protein pellets (Altromin Spezialfutter GmbH & Co, C 1002 Eiweißreiche Diät (II)) were added to their diet at the onset of lactation period and until weaning to meet the increased nutritional requirements.

The onset of the prehibernatory fattening phase for each female was defined by a pronounced body mass increase exceeding 1g/day, excluding the gestation period in reproductive females. Prehibernatory fattening was completed when an individual´s body mass curve had plateaued and stayed constant until immergence (Millesi et al., 1999b; Strauss et al., 2009). Immergence date has been defined as the last sighting of an individual during summer, after which an animal had terminated above-ground activity, and the onset of hibernation could be expected (Millesi et al., 2008b).

3.3 Data collection

In spring open burrows could be detected by fresh digging traces. To determine individual vernal emergence dates all burrow holes in the enclosures were checked daily from beginning of March until all animals had initiated above-ground activity (Millesi et al., 2008a). We attempted to capture the animals every two days, depending on the weather conditions, or at least twice a week, with Tomahawk life traps baited with peanut butter (Mascher, 2008; Steinerberger, 2017; Strauss et al., 2009), (Figure 2). A custom-made tapered denim bag with a hook-and-loop fastener along one edge was tightly pulled over one opening of the trap to release the animal into the bag. This made it easier to handle them and enabled a safe and less stressful examination, as the animals were usually calm inside the bag (Divjak, 2009; Mascher, 2008). First the identity of the animal was checked by scanning the implanted transponder. The animal got chipped and assigned an ID, if it had not been chipped yet. They were chipped in the neck region using “Backhome BioTec® Slim Transponder” (*At.Virbac.Com*). Commercial hair dye was used for painting unique markings on the animal´s backs to facilitate individual identification. Females were marked with red and males with black colour (Divjak, 2009; Strauss et al., 2009).

Parameters recorded at each capture included body mass (± 0.5 g, digital scale), faecal sample number, vaginal smear number, fur change status, and whether it was a re-capture or the first observation of the season or the first time at all capturing the individual. Other recorded categories were the individual animal’s ID as well as the individual transponder code and fur marking along with sex (male or female), and age. The first age category were juveniles, regarding animals born this year. The second category were yearlings, which were animals who were born the season before. The third category were adults, referring to all individuals older than two seasons. Yearlings and adults can be grouped together as non-juveniles (Millesi et al., 1999b). We also noted date, time and weather conditions (temperature, cloudiness, rainfall, wind).

Additionally, the trapping location in the enclosures was noted: enclosure Nr.1 was divided in a grid of three columns (A, B, C) and eight rows (1 - 8), while the smaller enclosure Nr.2 was divided in a grid of three columns (A, B, C) and five rows (1 - 5).

3.4 Group composition

Construction of enclosure Nr.1 had just been complete the previous year. At the beginning of the season all animals were therefore kept in the smaller second enclosure (Nr.2), which was located approximately 150 m downhill from enclosure Nr.1. As the animals gradually emerged from hibernation some were transferred to the empty bigger enclosure Nr.1 to establish two sex-balanced groups. See table 1 for the final distribution of study animals in both enclosures. At the beginning of the season there were ten females and six males aged one to three years. In early April two adult males disappeared, one from each enclosure, leaving us to put another reproductive male in enclosure Nr.2, to assure mating opportunities. In total, there were ten non-juvenile females, two juvenile females and four non-juvenile males left at the end of the season 2021 (Table 1).

3.5 Vulva, teats and lactation

Vulval swelling and opening status was documented on a four-point scale (0: closed and not swollen, 1: small opening and lightly swollen, 2: wide opening and moderately swollen, 3: maximum opening and maximum vulval swelling). Teat size was also documented on a four-point scale (0: small and inconspicuous, 1: small and lightly swollen, 2: large and moderately swollen, 3: very large and maximum swelling). Consequently, teat pigmentation was documented on a four-point scale as well (0: light-pigmented areola, 1: light-pigmented tips, 2: light-pink tips, 3: dark-pigmented teats). Lactation was identified by big swollen teats with light pigmentation, pink tips, and milk residues (Strauss et al., 2009).

Information about the vulva and teats has been used to determine which females were pregnant and gave birth, as only those had a prolonged lactation phase characterized by lightly coloured and greatly enlarged teats as indication for milk production (Gaasch, 2016; Strauss et al., 2009). Lactation occurred from early-May until late-June/early-July in four females (ID´s 804, 903, 1008 and 1018) and lasted 44.75 days on average (± 5.90). Female 804 was lactating for 39 days, female 903 for 47 days, female 1008 for 52 days and female 1018 for 41 days.

3.6 Pregnancy and reproductive output

During the mating season three of the four remaining males (ID´s 901, 1022, 9004) showed scrotal testes and were therefore classified as reproductive.

Reproductive females were classified by monitoring changes in body mass, vulva opening state and teat conditions over the season (Gaasch, 2016; Strauss et al., 2009). Pregnant females had an abrupt loss of body mass around the beginning of May, and bloody vulvas could be observed, indicating parturition. During lactation females show enlarged and light pigmented teats. In this study, however, not all females who had given birth, showed signs of lactation, some only for one or two weeks.

There were four females who showed signs of advanced gestation, parturition and advanced lactation and were therefore defined as productive (ID´s 804, 903, 1008, 1018).

The other six females had no distinct body mass drop and had showed no signs of parturition. Their teats had remained small and dark during the whole season indicating that no lactation occurred. These females were classified as non-reproductive (ID´s 807, 1002, 1011, 1015, 1016, 1020), (Table 1).

Table 1 List of the ten female study subjects: ID, individual fur marking, age in years, enclosure and reproduction status (0 = non-reproductive, 1 = reproductive).

ID	fur marking	age	enclosure	reproduction
807	red circle	3	2	0
1002	red triangle	1	2	0
1011	red dot	1	1	0
1015	red question mark	1	1	0
1016	red line	1	1	0
1020	red H	1	2	0
804	red V	3	1	1
903	red arrow	2	1	1
1008	red plus	1	1	1
1018	red percentage sign	1	1	1

All females had access to males and thus had the chance to mate during the mating season. Although four out of ten females showed signs of pregnancy and parturition, only three juveniles were observed (Table 1). We were unable to assign the juveniles to individual mothers. We therefore distinguished between reproductive and non-reproductive females, even though not all females who showed signs of pregnancy successfully raised offspring in 2021. The term “oestrus” refers to the vaginal oestrus unless stated otherwise in the text. These methods have been proved to be successful for assigning reproductive states in previous studies (Millesi et al., 1999b; Strauss et al., 2009).

3.7 Vaginal cytology

Vaginal smears were taken by carefully inserting a cotton swab soaked in 0.9% medical saline solution into the vagina and twisting the cotton swap gently. Then the samples were spread out on labelled glass slides and fixed using polyethylene glycol (M-FIX® spray fixative for cytodiagnosis). With the Papanicolaou technique (Papanicolaou, 1942; Papanicolaou & Traut, 1944; Traut & Papanicolaou, 1943) the cells on the slides were stained and then cell numbers and cell ratios were evaluated microscopically (Nikon Alphaphot 2-YS2, magnification 100x) in three 0,5 mm x 0,5 mm sections per sample.

Depending on the ratio of three different cell types and overall cell number the samples were assigned to one specific stage of the oestrus cycle, based on the predominance (>70%) of one cell type (Strauss et al., 2009). Transitional stages where the classification was somewhat difficult were assigned to the predominant feature of the smear or otherwise excluded if a classification was not possible.

Pro-oestrus was categorized by the predominance of small round or oval-shaped, blue-stained nucleated epithelial cells ("NEC"). During the following oestrus phase the dominant cell type was keratinized anucleated epithelial cells ("AEC") with sharp edges and stained in an orange-pink colour, although in some keratinized cells a "ghost nucleus" could still be seen (Cora et al., 2015). Metoestrus, also called dioestrus, was defined by the sudden appearance of high numbers of small, blue-stained granular leucocytes, also called neutrophils ("NEU"), which contain no nucleus. The following anoestrus is a phase characterized by generally low cell numbers with no dominant cell type (Cora et al., 2015) and represents a resting phase of the sexual cycle (Strauss et al., 2009).

3.8 Hormone analysis

The faecal samples were stored in Eppendorf Tubes® (*Eppendorf.at*, 2021) and stored in a cooling bag on icepacks during the hours of field work. At the end of each day, they were frozen at -20 °C until further analysis. Because the cooling possibilities for the collected faeces were limited at the beginning of the season collected data before 29th of April were not considered for further analysis. At room temperature transformation of steroid hormone metabolites in faecal samples can happen within a few hours after defecation, which could greatly alter the results obtained from enzyme immunoassay analysis (EIA) (Dantzer et al., 2010; Pettitt et al., 2007). Proper storage and transportation (e.g. freezing at -20°C or drying at 40°C for several hours) of faecal samples is deemed necessary to prevent the degradation of steroid hormone metabolites by naturally occurring faecal bacteria, bacterial enzymes and ultraviolet radiation (Pettitt et al., 2007).

Hormone metabolite concentrations in the faecal samples were analysed via biotin-strepdavidin enzyme-linked immunoassays (EIA) following a protocol by Möstl and Palme (Möstl & Palme, 2019). The faeces were completely dried in a drying cabinet at 60 °C for at least 24 hours and then finely grinded. Afterwards the samples were weighted into laboratory glass tubes, and 80 % methanol was added for hormone extraction (0.1 g = 2 ml, 0.05g = 1 ml, 0.02 g = 0.4 ml). Samples were vortexed for 30 min on a multivortex and centrifuged (3000 rpm; 15 min).
For the faecal cortisol metabolite (FCM) analysis the samples were diluted 1:100 with assay puffer (AP) in Eppendorf Tubes®, while the dilution for the faecal progesterone metabolite (FPM) analysis was 1:10.
The EIA and calculation of results was performed following the protocol of Möstl and Palme (Möstl & Palme, 2019). For FCM-EIA the intraassay was 11.03 % and the interassay for plates 2 -12 was 11.88 % and 0.08 % for plates 13 and 14. For FPM-EIA the intraassay was 4.08 % and the interassay for plates 2 - 9 was 5.20 %.

3.9 Statistical analysis

Statistical analysis was performed using RStudio with R version 4.4.2 (2024-10-31 ucrt).
Data distributions were tested for normality with Shapiro-Wilk-Tests. Most of the retrieved data was not normally distributed and therefore non-parametric tests were applied. Wilcoxon-Mann-Whitney-U-Tests were applied for pairwise comparisons of two independent measurements. For two repeated measurements a paired Wilcoxon-Signed-Rank-Test was performed. We used individual means when repeated measurements were available in a particular phase. In case of normally distributed data a paired student´s t-Test and Cohen´s d for the effect size was used.
Correlation statistics were assessed with Pearson´s r correlation in case of normally distributed samples. Otherwise, Spearman´s ρ was used. For some datasets statistical analysis was not reasonable due to the restricted sample size, when comparing reproductive and non-reproductive females and the fact that not all individuals were captured at each stage. Significance levels were obtained from two-tailed statistics and statistical significance was set at $p < 0.05$. If not stated otherwise, means (± SD) are used to describe datasets.

4 Results

4.1 Duration and timing of vaginal oestrus phases

Classification of vaginal smears into the four stages of the vaginal oestrous cycle revealed the occurrence of a spring oestrus event (1st oestrus) shortly after emergence from hibernation for all females with an average duration of 7.7 days (± 5.6). Reproductive females showed a mean duration of 5.0 days (± 4.9) for the first vaginal oestrus in spring, compared to 9.5 days (± 5.7) in non-reproductive females.

A 2nd oestrus phase was observed in nine females and lasted 4.4 days (± 3.8) on average. In one individual (ID 1002) we could not collect vaginal smears confirming a 2nd oestrus. The 2nd oestrus lasted for 3.5 days (± 3.8) in non-reproductive females and 4.8 days (± 4.1) in reproductive females (Table 2).

Table 2 Onset dates and duration in days for the 1st and 2nd vaginal oestrus phase. Reproductive females are labelled "1" and non-reproductive females are labelled "0".

ID	reproduction	onset 1st oestrus	duration 1st oestrus [days]	onset 2nd oestrus	duration 2nd oestrus [days]
807	0	09.04.2021	8	03.05.2021	1
1002	0	05.04.2021	5	**no data**	0
1011	0	01.04.2021	4	22.07.2021	6
1015	0	31.03.2021	18	14.05.2021	1
1016	0	05.04.2021	7	14.05.2021	3
1020	0	09.04.2021	15	01.05.2021	10
804	1	31.03.2021	11	22.07.20221	6
903	1	02.04.2021	1	22.07.2021	10
1008	1	03.04.2021	7	11.08.2021	2
1018	1	05.04.2021	1	22.07.2021	1

There was no significant difference in the duration between the 1st and the 2nd oestrus phase ($V = 36$, $p = 0.120$). All individuals had their 1st oestrus in late March or early April starting within a 9-day-period (Figure 3, Plot A), whereas a 2nd oestrus could be identified in nine animals either in May (four non-reproductive females) or in July and August (one non-reproductive and four reproductive females) (Figure 3, Plot B).

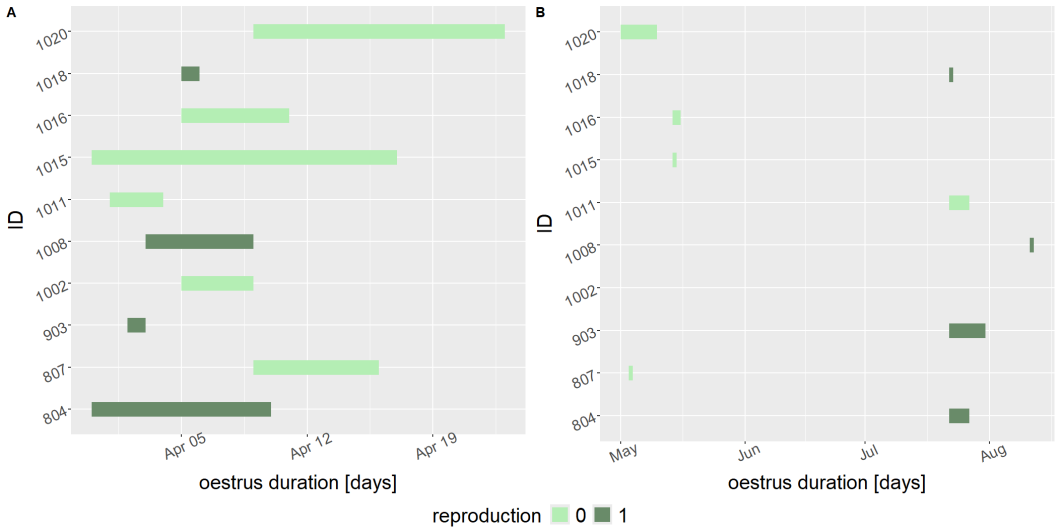

Figure 3 Duration and onset of the first (plot A) and second (plot B) vaginal oestrus phase in days with reproductive females in dark-green ("1") and non-reproductive females in light-green ("0"). One female (ID 807) had a third oestrus-like phase in August, which is not shown in the plot

4.2 Cell number and cell composition

The analysis of vaginal smears allowed to identify the first and second vaginal oestrus and compare the mean cell numbers (all cell types) and cell compositions (percentage of cell types) between the two oestrus phases. The mean total cell number during the 1st oestrus (133.34 cells ± 111.65) was higher than during the 2nd oestrus (81.93 cells ± 54.29), although the difference was tightly above significance level ($t = 2.20$, $p = 0.059$, $d = 0.73$), (Figure 4). There was however a high variation in the mean cell numbers among the ten individuals and between the 1st and 2nd oestrus phase (Table 3).

Table 3 Mean cell numbers of the two vaginal oestrus phases. Reproductive status is displayed as 0 (non-reproductive) and 1 (reproductive).

ID	reproduction	mean cell number 1st oestrus	mean cell number 2nd oestrus
807	0	131	43
1002	0	140	0
1011	0	85	98
1015	0	55	65
1016	0	57	74
1020	0	308	155
804	1	39	32
903	1	341	72
1008	1	217	73
1018	1	162	79

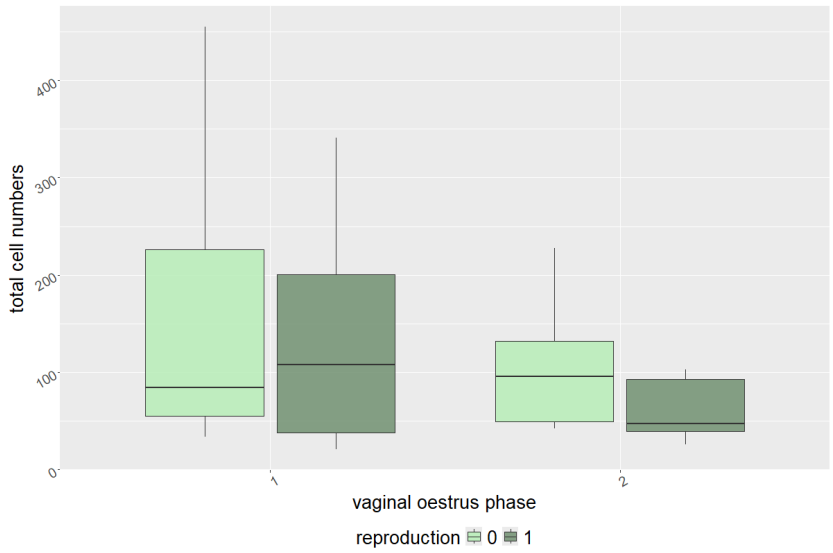

Figure 4 Mean cell numbers of all cell types of the two vaginal oestrus phases for reproductive ("1") and non-reproductive ("0") females.

Percentages of anucleated epithelial cells (AEC), (t = -0.65, p = 0.532, d = 0.22), nucleated epithelial cells (NEC), (t = 0.66, p = 0.528, d = 0.22) and neutrophils (NEU), (t = 0.52, p = 0.615, d = 0.18) were similar in the 1st and 2nd oestrus of individual females (Figure 5).

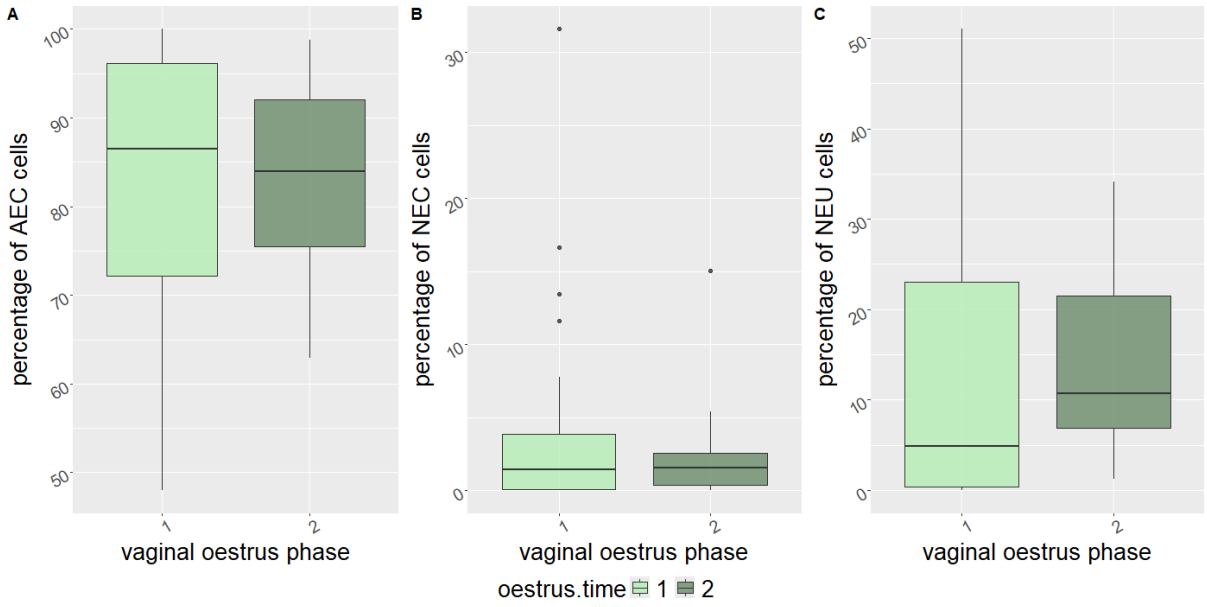

Figure 5 Percentages of three cell types (plot A: AEC, plot B: NEC, plot C: NEU) of the two vaginal oestrus phases ("oestrus.time") for reproductive ("1") and non-reproductive ("0") females.

4.3 Faecal progesterone metabolite (FPM)

The faecal progesterone metabolite (FPM) concentrations ranged between a minimum of 233 ng/g and a maximum of 15 961 ng/g across all ten females over the whole season. For better comparability FPM concentrations were assigned into ten intervals from beginning of May to end of September 2021, each covering a time span of 14 days. For every two-week interval the mean FPM value was calculated separately for every study subject. A series of high mean FPM values was considered as the main progesterone peak of the season for each female. Most females had a distinctive peak in their FPM concentration levels, representing the luteal phase following the second vaginal oestrus in summer (Figure 6 and Figure 7).

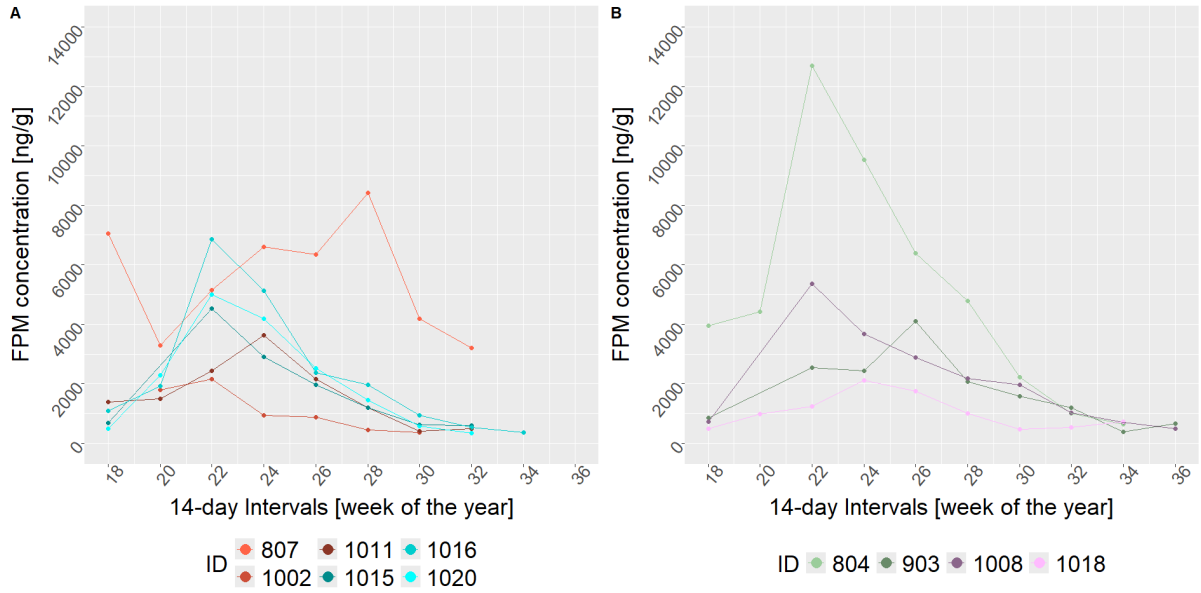

Figure 6 Mean FPM (Faecal progesterone metabolite [ng/g]) concentrations calculated for ten 14-day intervals, from calendar week 18 to week 36, covering the time from May to September 2021 for six non-reproductive females (Plot A) and four reproductive females (Plot B).

In five non-reproductive females the FPM concentration peaked from calendar week 18/20 (early-/mid-May) to week 24/26 (mid-/late-June), representing the luteal phase associated with the second oestrus in May, except for one individual (ID 807), who showed two separated peaks, (one in May, after her second oestrus, starting before calendar week 18, and a second peak stretching from week 20 to week 30 (mid-May to late July), (Plot A, Figure 6)).

In four reproductive females the FPM concentration began to rise during lactation in calendar week 20/22 (mid-/late-May) and decreased again in week 28 (mid-July), with one female (ID 804) showing exceptionally high FPM values up to over 12 000 ng/g (Plot B, Figure 6)

After the summer peak there was a constant decline of FPM concentration levels until the females entered their hibernacula at the end of the season in August/September (Figure 7). The overall course of the FPM concentration did not differ between reproductive and non-reproductive females (Figure 7). There was however a high variation in the course and range of FPM concentrations among the ten individuals (Figure 6).

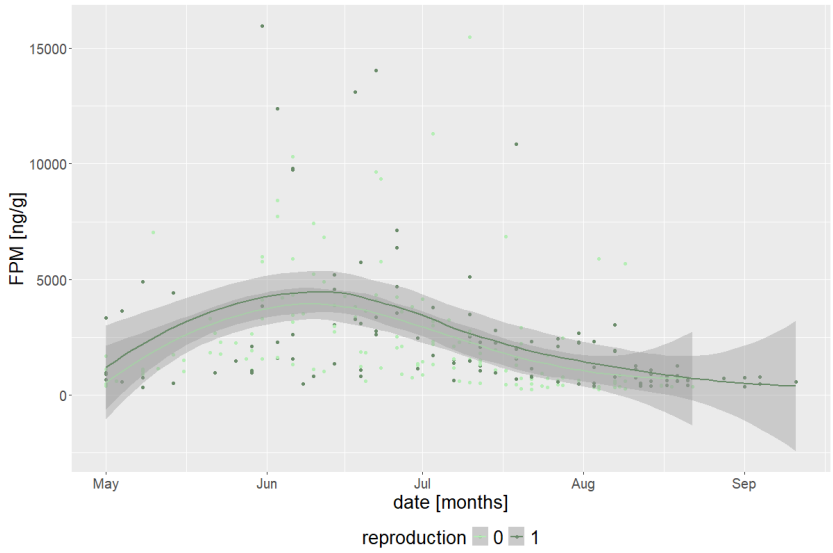

Figure 7 FPM curves [ng/g] for the season of 2021 from May to September for six non-reproductive ("0") and four reproductive ("1") females with confidence intervals (0.95).

4.4 Faecal cortisol metabolite (FCM)

The faecal cortisol metabolite (FCM) concentration levels ranged between a minimum of 4.48 ng/g and a maximum of 954.10 ng/g across all ten females over the whole season of 2021. FCM concentration for each adult female was assigned into ten intervals from beginning of May to end of September, each covering a time span of 14 days. For every two-week interval the mean FCM value was calculated separately for every individual. If there was a series of high mean FCM values this was considered as a cortisol peak. Most individuals showed two or three peaks in their FCM curves (Figure 8).

The FCM course of female 807 differs noticeably from the other individuals, as this female showed a distinct peak at the beginning of the season followed by a steep decrease until a second smaller peak in July/August (week 30 and 32) before entering hibernation. The FCM concentrations of the other five non-reproductive females were elevated in May (calendar week 18 to 22) and then stayed on a higher level with a second peak rising in July (calendar week 26/28), (Figure 8, Plot A).

Reproductive females showed a higher interindividual variation of FCM levels. There were three separated peaks in two females (ID 804 and 1018). In early-May female 804 showed a small peak, then a second bigger peak followed in June (week 20 to 26), and she had a third peak in mid-July to early-August (week 28 – 32). Female 1018 had elevated FCM levels in May (week 22), then a bigger peak in June (week 26 to 28), and a third peak in July (week 32). In female 903 FCM levels constantly increased from May until reaching a peak in late-June (week 26), then dropped and increased again until emergence (week 32 to 34). The FCM course of female 1008 was elevated from early-June (calendar week 22) and stayed on a plateau phase until hibernation, (Figure 8, Plot B).

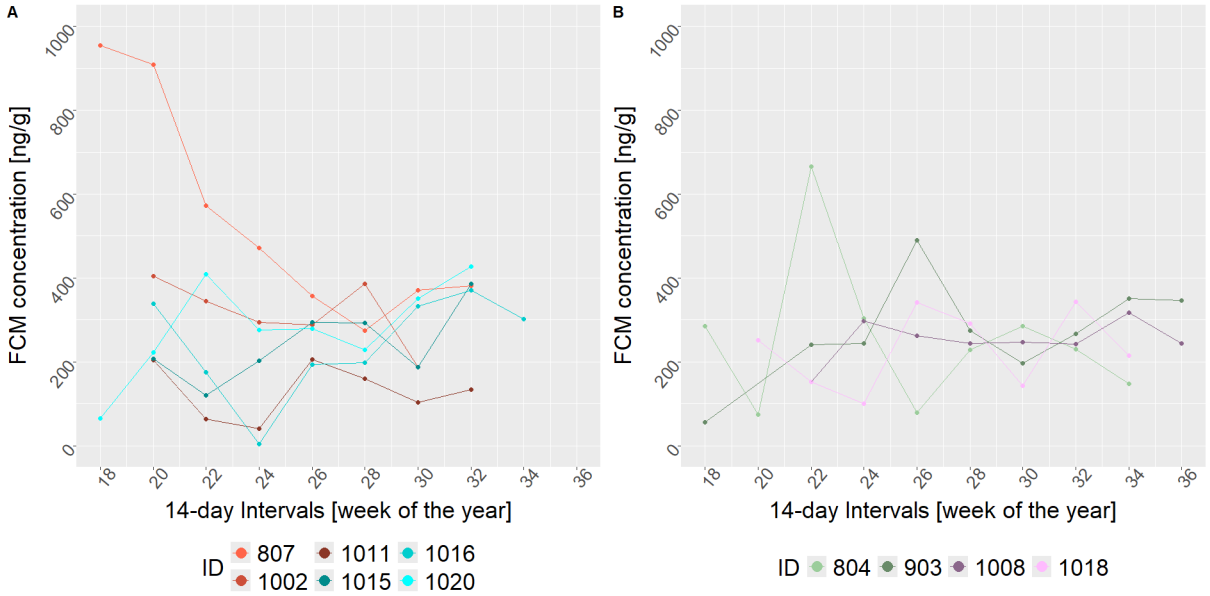

Figure 8 Mean FCM (Faecal cortisol metabolite [ng/g]) concentrations calculated for ten 14-day intervals, from calendar week 18 to week 36, covering the time from May to September 2021 for six non-reproductive females (Plot A) and four reproductive females (Plot B).

In general, both groups showed rather low FCM concentrations at the beginning of the season. In non-reproductive females the peak in May after the second oestrus was marginally higher than the second peak in summer. While reproductive females showed a conspicuous FCM peak rising in May and going on throughout June/July, occurring at the same time as parturition and the lactation phase. Towards the end of the season there was a noticeable increase of FCM levels in both non-reproductive and reproductive females (Figure 8 and Figure 9).

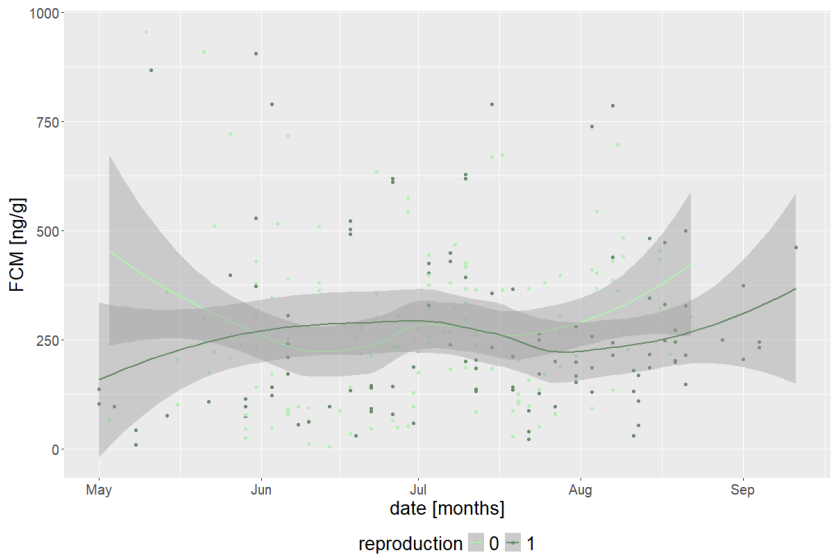

Figure 9 FCM curves [ng/g] for the season of 2021 from April to September for four reproductive ("1") and six non-reproductive females ("0") with confidence interval (0.95).

4.5 Body mass and fattening phase

During the active season the body mass ranged from a minimum of 163 g to a maximum of 389 g across all ten females. Mean body mass right after emergence in spring was 209.9 g (± 30.3) and mean body mass before immergence in summer was 318.4 g (± 26.9), (Table 4).

Table 4 Emergence date (= first observation of the season) and immergence date (= last observation of the season) and corresponding body mass [g]. Reproductive status is displayed as 0 (non-reproductive) and 1 (reproductive).

ID	reproduction	emergence date	emergence body mass [g]	immergence date	immergence body mass [g]
807	0	05.04.2021	210	16.08.2021	339
1002	0	02.04.2021	210	28.07.2021	358
1011	0	01.04.2021	264	07.08.2021	348
1015	0	28.03.2021	163	07.08.2021	295
1016	0	25.03.2021	193	22.08.2021	312
1020	0	09.04.2021	255	16.08.2021	325
804	1	29.03.2021	217	21.08.2021	334
903	1	02.04.2021	201	11.09.2021	274
1008	1	31.03.2021	200	04.09.2021	306
1018	1	29.03.2021	186	21.08.2021	293

The relationship between body mass at vernal emergence and emergence date was positive and marginally significant ($R = 0.62$, $p = 0.057$). Individuals with higher body mass emerged later in spring than lighter ones (Figure 10, Plot A).

There was no correlation between date of emergence and date of immergence (R = - 0.075, p = 0.837), (Figure 10, Plot B). Individuals who were heavier in spring were marginally heavier at immergence in summer (R = 0.59, p = 0.071), (Figure 10, Plot C). Body mass at immergence was negatively correlated with immergence date (R = - 0.71, p = 0.021), indicating that individuals with higher body mass showed an earlier onset of hibernation (Figure 10, Plot D).

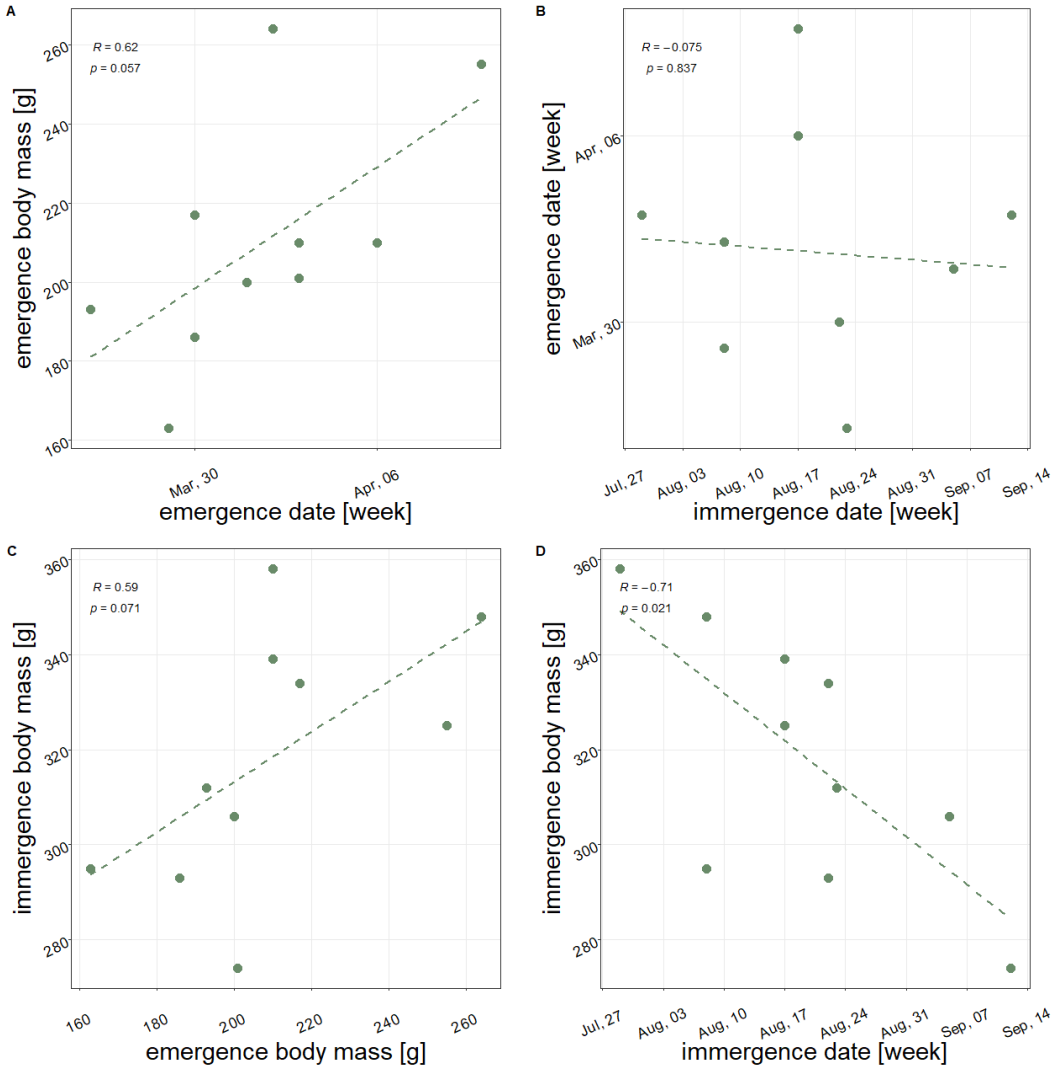

Figure 10 Correlations with Pearson´s correlation coefficient (R) and p-values of body mass [g] and dates of emergence and immergence [week]. Plot A: emergence body mass and emergence date. Plot B: emergence date and immergence date. Plot C: immergence body mass and emergence body mass. Plot D: immergence body mass and immergence date.

All females rapidly gained body mass in spring after initiating above ground activity and in four reproductive females there was a conspicuous drop of body mass in early-May, representing parturition. Their body mass continued to decline due to the energetical costs of lactation until early-June, followed by a phase of rapid body mass increase (= fattening phase) until termination of above ground activity in mid-August to September (Figure 11, Plot B).

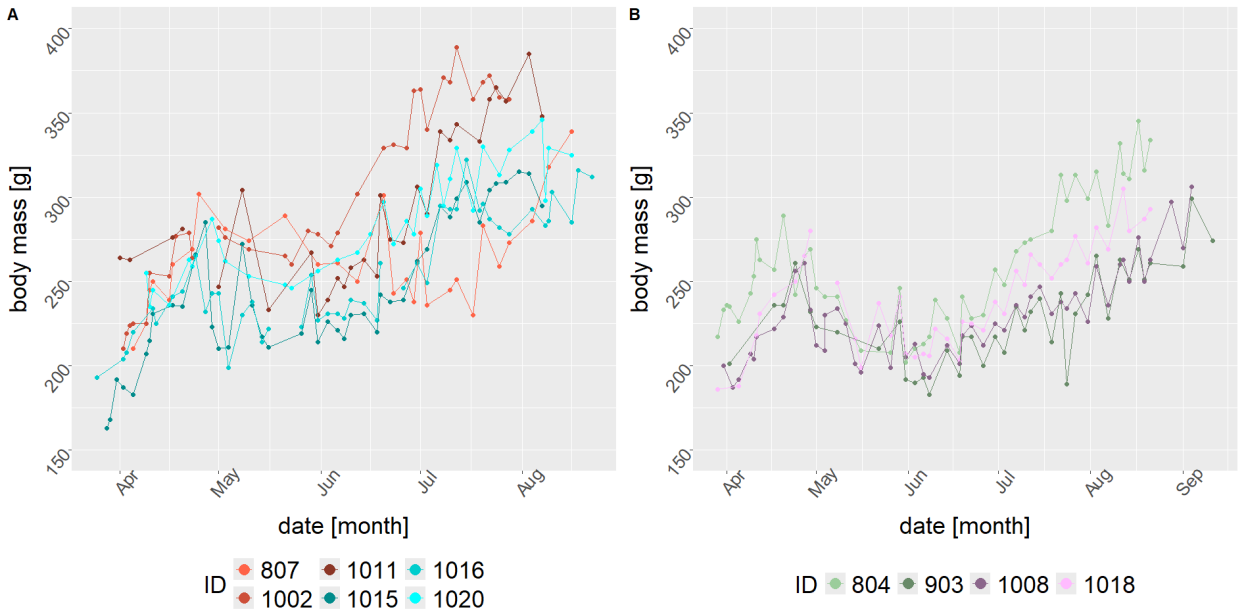

Figure 11 Individual course of body mass in grams [g] during the active season from March/April to September 2021 for six non-reproductive females (Plot A) and four reproductive females (Plot B).

After the initial rapid body mass gain in spring the six non-reproductive females reached a plateau phase in May and June and showed no pregnancy-related body mass drop. Onset of prehibernatory fattening was in the second half of June and they terminated the fattening phase at the end of July, during which their body mass increased until a plateau phase was reached shortly before onset of their next hibernation phase in August/September (Figure 11, Plot A). Looking at the averaged body mass curves, non-reproductive females reached a first plateau phase in May/June and second one in August, before the onset of hibernation, whereas body mass in reproductive females was declining in May and June, followed by a continuous increase until the females terminated above ground activity (Figure 12).

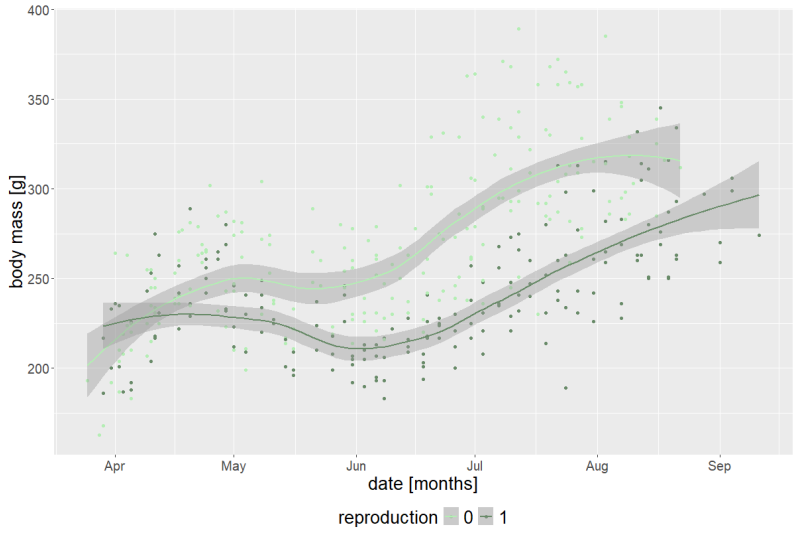

Figure 12 Body mass curves for the season of 2021 from April to September for four reproductive ("1") and six non-reproductive ("0") females with confidence interval (0.95).

The mean duration of the prehibernatory fattening phase was 25.9 days (± 6.24 d) for all ten females. Both groups began fattening in mid-June, reproductive females on 21.06 (± 10.75 d) and non-reproductive females on 22.06 (± 14.11 d). Reproductive females terminated their fattening phase on 20.07 (± 9.78 d) and non-reproductive females on 14.07 (± 16.83 d). The mean end date was 17.07 (± 14.13 d) for all ten females. The duration of the fattening phase, and total body mass gain (g) during fattening was similar in both groups (Figure 13, Table 5).

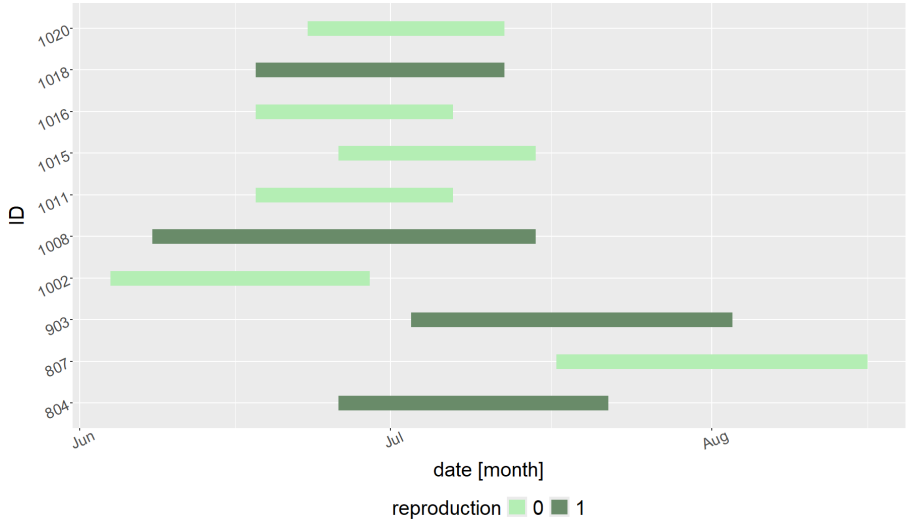

Figure 13 Duration of the fattening phase for the season of 2021. The plot shows four reproductive ("1") and six non-reproductive females ("0").

The animals gained 73.90 g (± 18.03) with a mean daily mass gain of 2.99 g/day (± 0.88) during the fattening phase. Reproductive females gained 2.2 g/day (± 0.74), and non-reproductive females gained 3.52 g/day (± 0.46). Non-reproductive females were heavier than reproductive ones, both at the onset and end of fattening (Table 5, Figure 14).

Table 5 Body mass gain in grams, duration of fattening in days, mean body mass gain per day, onset date of the fattening phase, and the body mass at the onset and end of the fattening phase in reproductive and non-reproductive females.

	reproductive mean (SD)	non-reproductive mean (SD)
body mass gain [g]	64.25 (13.05)	80.33 (18.94)
duration [days]	30.50 (5.80)	22.83 (4.67)
g/day body mass gain	2.2 (0.74)	3.52 (0.46)
fattening onset	21.06.2021 (10.75)	22.06.2021 (14.11)
body mass before fattening phase	208.5 (15.63)	248.67 (19.87)
body mass after fattening phase	272.75 (28.22)	329 (24.13)

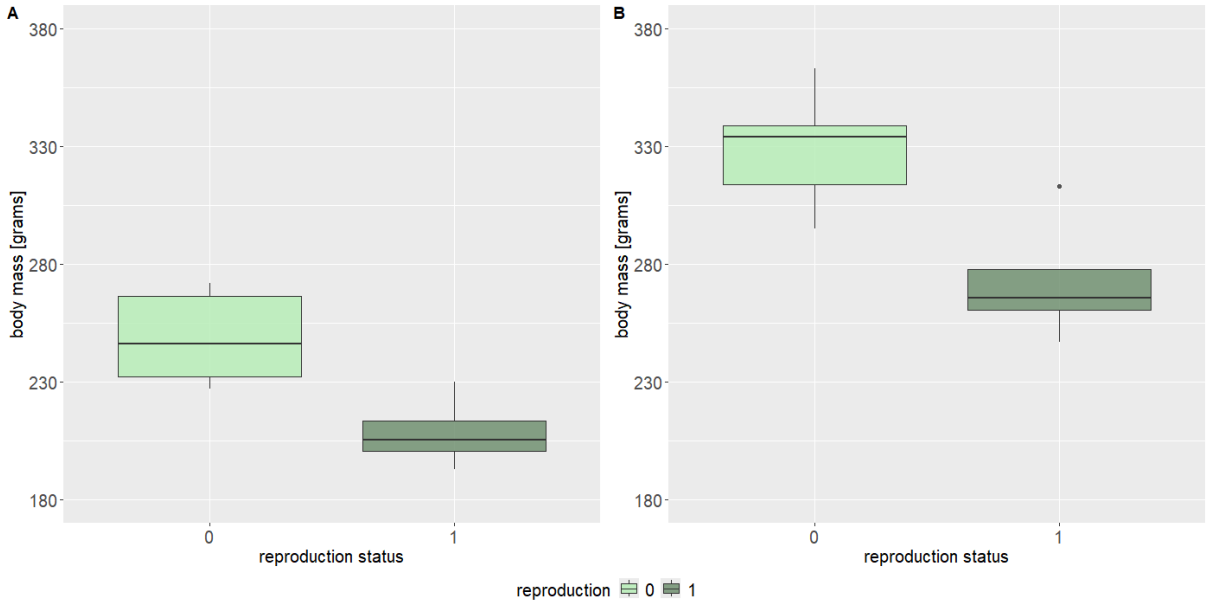

Figure 14 Body mass range before and after the fattening phase for reproductive and non-reproductive females. Plot A: Body mass at the onset of fattening. Plot B: Body mass at the end of fattening.

Body mass gain per day (g/day) was negatively correlated with the duration of the fattening phase (r = -0.72, p = 0.02) and positively correlated with total body mass gain of the fattening phase (r = 0.70, p = 0.02). Individuals who gained more mass per day had a shorter fattening phase and gained more mass in total (Figure 15, Plot A and B).

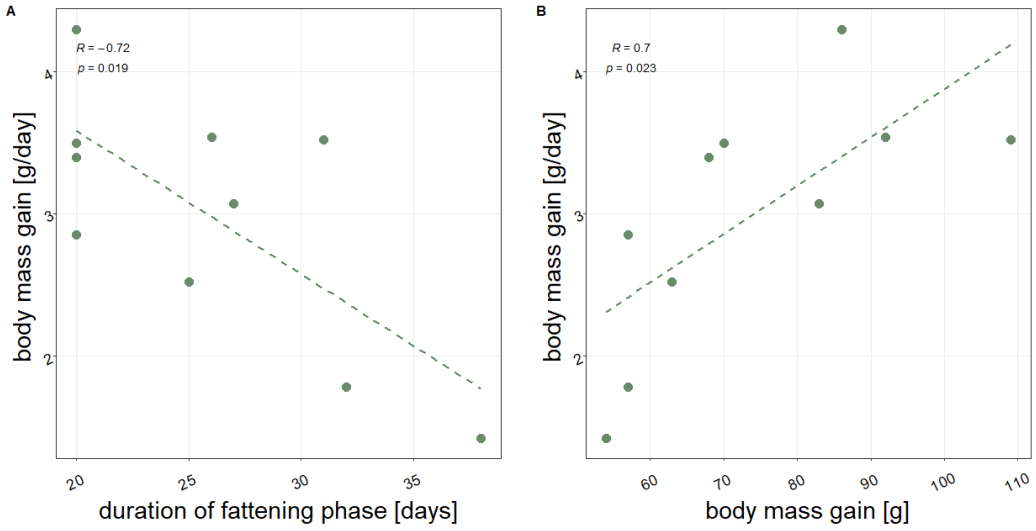

Figure 15 Correlations with Pearson´s correlation coefficient (R) and p-values. Plot A: body mass gain [g/day] during the fattening phase and duration of the fattening phase[days]. Plot B: body mass gain [g/day] and overall body mass gain [g] over the fattening phase.

Body mass at immergence was positively related to body mass gain per day during the fattening phase (r = 0.67, p = 0.03) and weakly negatively related to duration of the fattening phase (r = - 0.20, p = 0.57). Females with higher immergence mass showed a shorter duration of the fattening phase and gained more mass during fattening than individuals with less immergence mass at the onset of hibernation (Figure 16, Plot C and D).

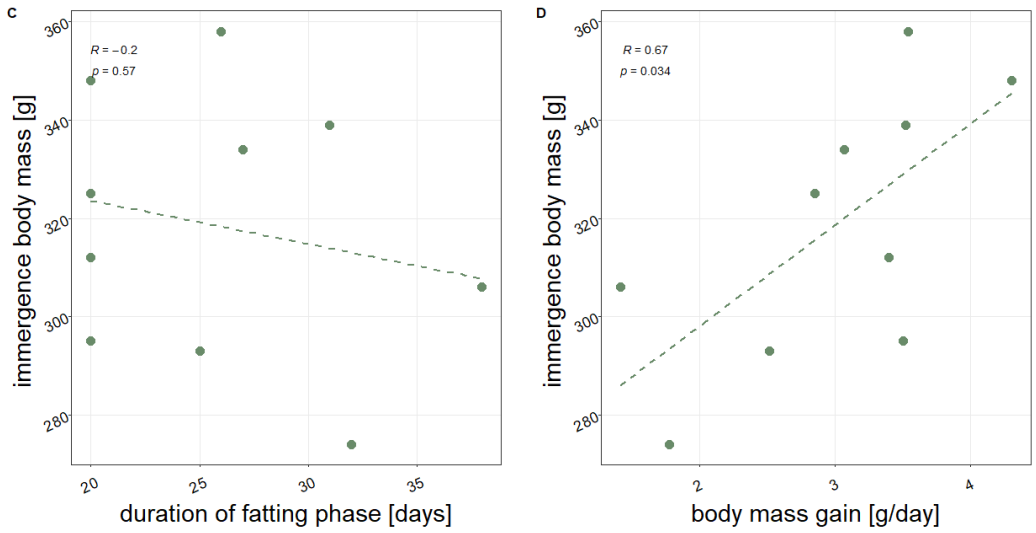

Figure 16 Correlations with Pearson´s correlation coefficient (R) and p-values. Plot C: immergence body mass [g] and duration of the fattening phase [days]. Plot D: immergence body mass [g] and body mass gain [g/day] during the fattening phase.

Females who had gained significantly more mass during the fattening phase showed higher immergence mass (r = 0.74, p = 0.01), (Figure 17, Plot E).

Duration of the fattening phase was marginally positively related to immergence date (r = 0.61, p = 0.06) and not related to overall body mass gain during the fattening phase (r = -0.06, p = 0.88). A longer fattening phase was associated with a later immergence date (Figure 17, Plot F) and did not influence the overall body mass gain.

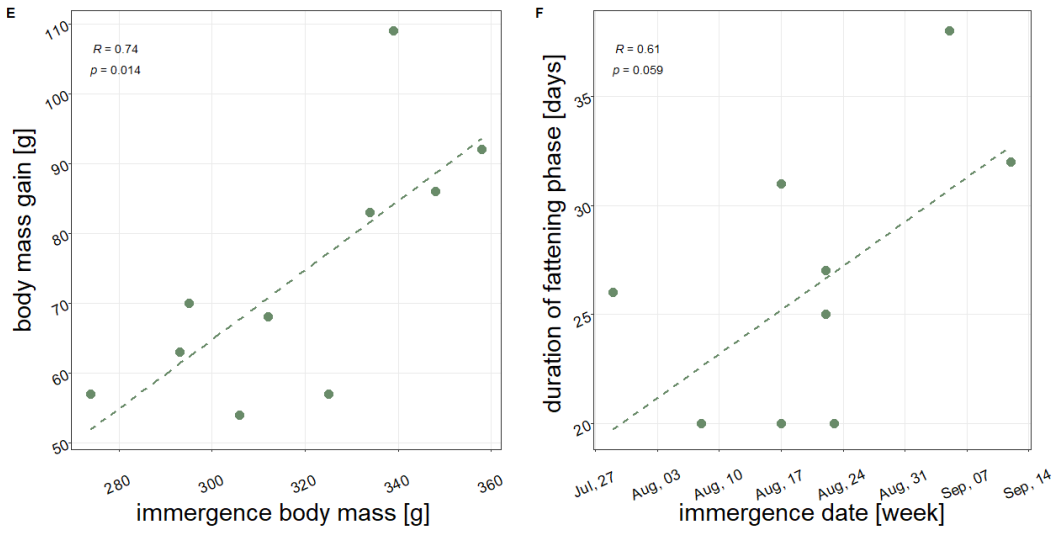

Figure 17 Correlations with Pearson´s correlation coefficient (R) and p-values. Plot E: immergence body mass [g] and overall body mass gain during the fattening phase [g]. Plot D: immergence date [week] and duration of the fattening phase [days].

A comparison of body mass changes and the course of FPM concentrations showed that in both reproductive and non-reproductive females the FPM concentration peaked at the beginning of June, before the onset of the fattening phase, which was initiated in the second half of June (Figure 18).

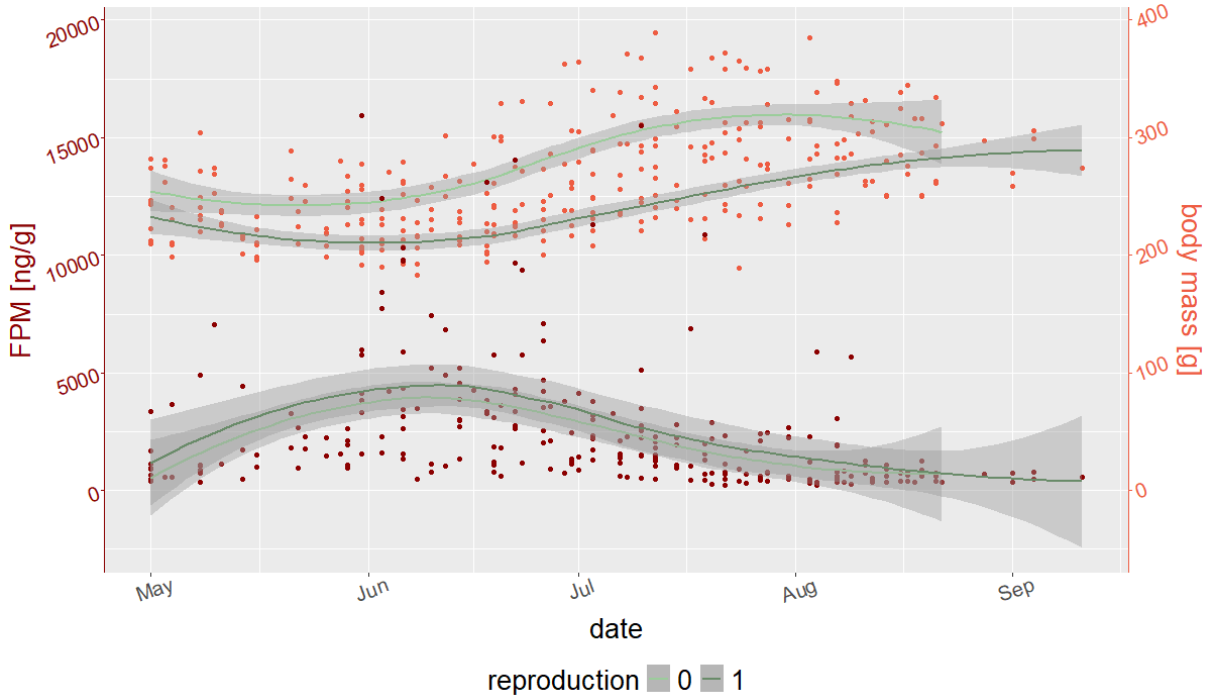

Figure 18 FPM concentration (left y-axis, dark red) and body mass (right y-axis, light red) for four reproductive ("1") and six non-reproductive ("0") females with confidence interval curves (0.95).

A comparison of body mass changes and the course of FCM concentrations showed that in non-reproductive females the FCM concentration was elevated at the beginning of May, at the time of parturition in reproductive females. During lactation FCM concentrations increased in reproductive females. FCM increase towards hibernation onset (Figure 19).

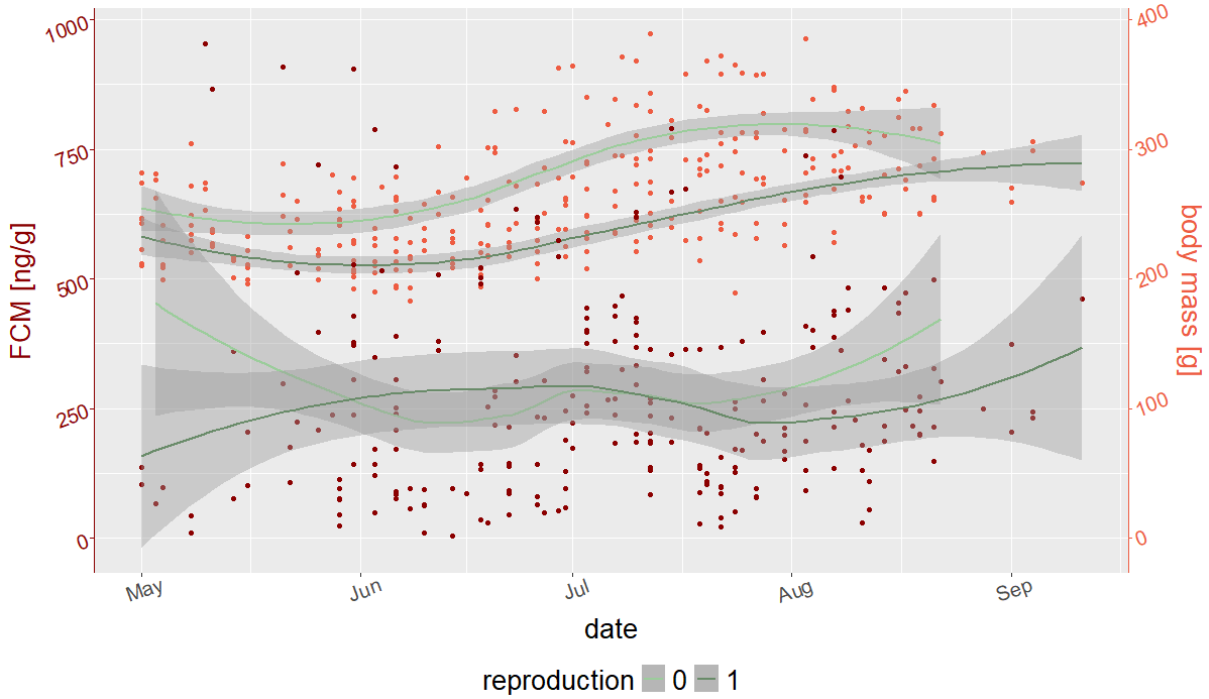

Figure 19 FCM concentration (left y-axis, dark red) and body mass (right y-axis, light red) for four reproductive ("1") and six non-reproductive ("0") females with confidence interval curves (0.95).

5 Discussion

5.1 Reproductive output

Although four out of ten females showed signs of pregnancy and/or parturition and were classified as reproductive, in total only three juveniles emerged from their mothers' burrows. The other six females were classified as non-reproductive. Pregnancy was assumed in females who gained weight quickly after the mating phase and then had an abrupt drop in body mass with successive loss of mass due to lactation (Millesi et al., 1999b; Strauss et al., 2009). In some females, bloody vulvas as indication of parturition could be observed concurrently with the body mass drop. Lactation in reproductive females was characterized by lightly coloured, greatly enlarged teats and milk secretion could be induced (Strauss et al., 2009). Usually a pronounced raise in progesterone levels is to be expected during pregnancy (Millesi et al., 2008a), which has been also demonstrated in a recent study conducted in captive thirteen-lined ground squirrels (*Ictidomys tridecemlineatus),* (Miller et al., 2021). Progesterone secretion during gestation could not be used to confirm pregnancy status in the current study, as the faecal samples collected in late-March and April, when gestation could be anticipated in reproductive females, were excluded from further analysis due to insufficient cooling of the samples.

Low reproduction rates in European ground squirrels at the study site have already been documented in preceding field work (Gaasch, 2016; Steinerberger, 2017), while in other years almost all females reproduced successfully (Millesi et al., 2008a; Strauss et al., 2009). Reproductive failure has also been documented in related species, as a study in free-living *U. richardsonii* found that 33% of breeding females failed to successfully rear their young until emergence from their natal burrows (Ryan et al., 2014).

The reasons for the low reproductive outcome during the active season of 2021 are largely unknown. During the mating season all non-juvenile females were kept in contact with males and theoretically had the chance to mate. Relocating the animals right after vernal emergence might have had a negative impact on the mating. In *S. citellus* males do not typically establish territories before the first receptive females are present. But rather appear to engage in scramble competition polygyny by keeping frequent contact with as many females as possible before copulation (Millesi et al., 2010). Females place strong time constraints on mating because reproductive males need to identify briefly receptive females and mating usually only occurs after a courtship period of a few days (Millesi et al., 2010). Removing some individuals from the enclosure they had hibernated in and putting them in the other enclosure could potentially have interfered with mating behaviour.

Additionally it might have been an unintentional stressor for the animals, but unavoidable because of space limitations (Vasilieva & Tchabovsky, 2014). Failed mating due to a temporarily highly female-biased operational sex ratio (OSR) caused by simultaneous oestrus of many females at once (Vasilieva & Tchabovsky, 2015) cannot entirely be ruled out for this study. However, the observed initial start of the vaginal oestrus extended over nine days which should have been sufficient to keep the OSR at a reasonable male-biased level and enable all females to mate.

Suboptimal weather conditions in spring could have had a negative impact on reproduction, as it has been shown in other species that harsh environmental conditions lead to a shift in seasonal timing and affected reproductive outcome (Morton & Sherman, 1978). Spring temperatures, particularly March, April, May, in 2021 were noticeably low compared to previous years and there was less precipitation. Millesi et al. (1999b) found spring emergence was negatively influenced by unfavourable weather conditions in males but not in females, who showed almost no fluctuations in emergence schedules. Female emergence and further reproductive timing seem to follow a strict timeline that is merely influenced by weather conditions (Millesi et al., 2000a).

Sperm was found in one vaginal smear of a non-reproductive female from enclosure Nr.2, indicating copulation, but ultimately no successful reproduction could be documented in each of the four females inhabiting this enclosure. Some non-reproductive females showed signs of an early gestation or parturition but were classified as non-reproductive because lactation was not documented in these individuals. Since teats usually remain small and dark for the most part of the gestation period and become lighter and larger after parturition it can be difficult to identify pregnant females and the onset of lactation based on teat development alone (Gaasch, 2016; Huber et al., 1999). Hence, resorption of the embryos or early abortions might have happened in those females (Vekhnik, 2020).

Gaasch (2016) suggested frequent use of pesticides in the vineyards around the study area as a possible negative influence on fertility. Many pesticides have been linked to have negative effects on fertility in males as well as fertility and pregnancy in females, such as spontaneous abortions or developmental defects causing stillbirths or resorption of the embryo (Mallozzi et al., 2016; Sharara et al., 1998). During the expected time of parturition and the onset of lactation in reproductive females higher FCM concentrations were documented in non-reproductive females than in reproductive females, as indications of higher stress levels, which has also been shown by Gaasch (2016). We noticed frequent injuries in both sexes, which can be used as indicators for antagonistic behaviour. While this is to be expected in males during the mating phase because of competition for access to females, it was quite unexpected in females and

often associated with territorial behaviour by females in the context of the offspring-defense hypothesis to defend their young against infanticide (Wolff & Peterson, 1998). Social stress, caused by high population densities and frequent territorial behaviour, including invasion of the natal burrows by other females, could also act on the neurohormonal system, and affect reproductive outcome by influencing secretion of prolactin, which stimulates progesterone secretion by the *corpora lutea* (Marchlewska-Koj, 1997; Steinerberger, 2017). Progesterone is needed to prepare the reproductive tract for enabling gestation and maintaining a pregnancy (Bachelot & Binart, 2005; Davis, 2002; Tomac et al., 2011). Thus, decreasing progesterone titres in early pregnancy caused by social stress could lead to pregnancy blocking and spontaneous abortions (Marchlewska-Koj, 1997).

Infanticide, often caused by lactating neighbouring females (Stevens, 1998) is another plausible explanation for low reproductive outcome but is, however, difficult to detect in a semi-natural enclosure before the time when juveniles first leave their natal burrows. In some ground squirrel species (e.g. *U. beldingi* (Sherman, 1982), *U. columbianus* (Stevens, 1998) and *O. beecheyi* (Trulio et al., 1986)) occasional infanticides have been documented, but have not been confirmed in *S. citellus* (Millesi et al., 1999a; Ramos-Lara et al., 2014). Considering all aspects above, social stressors and associated endocrine effects together with direct antagonistic interactions or infanticide could be responsible for the reproductive failure in some females.

5.2 Oestrus during the reproductive and non-reproductive period

In the current study ovarian activity during the reproductive period in spring ("mating phase") was compared with a second vaginal oestrus event during the non-reproductive period later in summer when no mating occurred. Timing and duration along with cell number and cell composition obtained from regularly taken vaginal smears was compared for ten females, of which four were reproductive. Cell numbers and cell compositions did not differ significantly between the oestrus phases within individuals and the two groups. In this study all females showed a vaginal oestrus in spring and 90% had a second oestrus in summer after the end of the mating season. This outcome is consistent with recent studies that observed spontaneous ovulation in populations of *S. citellus* without any contact to males during the mating phase (Aschauer, 2003; Divjak, 2009; Millesi et al., 2000a; Steinerberger, 2017).

Furthermore, no significant differences in duration of the two oestrus phases were found. This is particularly interesting, as they serve very different purposes. On the one hand a prolonged oestrus phase during the mating phase can be advantageous when population densities are low, if no sexually mature males are available, or in case extreme weather conditions prevent above ground activity. On the other hand, a lengthy second oestrus phase in summer including

ovulation and a luteal phase could be beneficial for accelerating prehibernatory fattening due to elevated progesterone levels (Millesi et al., 2008a; Steinerberger, 2017; Strauss et al., 2009). Another positive consequence of the second oestrus could be the priming effect of oestrogens, such as progesterone and oestradiol on follicular development before hibernation (Aschauer et al., 2006; Millesi et al., 2008b). Thus, explaining how females were able to become receptive within a few days after spring emergence.

The second oestrus in reproductive females occurred in late July or early August, while non-reproductive females had it mostly in May, except for one individual which also had the second oestrus in late July. Timing of the summer oestrus in non-reproductive females coincided with results found by Steinerberger (2017). Interestingly, reproductive females in the current study re-entered vaginal oestrus shortly before terminating above ground activity and long after weaning, while in other field studies the second oestrus was documented during or shortly after weaning, usually mid-June (Millesi et al., 2008a; Steinerberger, 2017).

Previous studies found that non-reproductive females without contact to males showed an extended vaginal oestrus phase compared to reproductive ones and that their second oestrus occurred earlier than in females who had offspring due to the lack of a gestation and lactation period (Divjak, 2009; Millesi et al., 2000a). The tactile stimuli of suckling offspring together with negative energy balance during lactation suppresses the normal oestrus cycle in many mammalian species, e.g rats (Tsukamura et al., 1991; Van der Schoot et al., 1978) and cattle (Montiel & Ahuja, 2005).

Advanced follicular maturation and ovulation are prevented because the pulsatile release and surge of gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH) are inhibited during lactation (Strauss et al., 2009; Tsukamura & Maeda, 2001). Thus, preventing the female from becoming receptive again until weaning has occurred. After this inhibitory period, which is also called “lactational anoestrus”, a spontaneous oestrus cycle often occurs (Tsukamura & Maeda, 2001). As expected, there was a longer latency for reproductive females to enter their second vaginal oestrus than for non-reproductive females in this study. It was observed that non-reproductive females had their second vaginal oestrus phase mostly in May, except for one individual, which had it in late July. Furthermore, reproductive females showed a second vaginal oestrus in late July or early August, after the juveniles had been weaned.

Most *Spermophilus* species mate within a few days after emergence as they are receptive to males only for a few hours (Richardson´s ground squirrels, *Urocitellus richardsonii*, (Michener, 1998), and become nonreceptive shortly after mating (Landau & Holmes, 1988). Females of the genus *Spermophilus* are receptive and show behavioural oestrus within 24 - 48 hours after emergence.

Some individuals show a period of persistent vaginal oestrus for up to three weeks (Thirteen-lined ground squirrel, *Ictidomys tridecemlineatus*) as reported by Landau & Holmes (1988). Specifically, *S. citellus* are reported to be receptive between two and thirteen days after vernal emergence (Millesi et al., 1999a, 2000a). But since not all individuals were captured each day, it is possible that we missed some stages of the vaginal oestrus cycle in some individuals. Therefore, the exact duration of the vaginal oestrus phases might be misinterpreted. This is partially due to the fact of the semi-natural conditions in this study, even though the animals were kept in an outdoor enclosure, not all individuals could be successfully captured on each day.

In one individual (ID 807) a possible third oestrus phase lasting for eight days was observed in August, after she had her summer oestrus in May (Table 2). In two other females, who had their second oestrus in July, indices for an oestrus in May were found, such as vaginal smears showing pro-oestrus and smears showing metoestrus which usually follows after an oestrus (Cora et al., 2015). It is thus conceivable that in some cases females had three oestrus phases. There is evidence in other species for females re-entering oestrus shortly after the initial mating phase is over (Landau & Holmes, 1988). A study in captive thirteen-lined ground squirrels (*S. tridecemlineatus*) found that even though females were receptive and showed vaginal oestrus within the first few days after emergence and mated within one week of emergence they can spend about three continuous weeks in initial oestrus, followed by shorter sporadic oestrus events, lasting less than one week (Landau & Holmes, 1988). Mated females transitioned from vaginal oestrus to metoestrus a few days after mating and remained in this state until after parturition, while unmated females stayed in a prolonged vaginal oestrus. The ability to extend the oestrus could give the female more time to find a suitable mate. This could be advantageous especially in case of low population densities or during extreme weather conditions in spring (Morton & Sherman, 1978).

5.3 Seasonal patterns of progesterone excretion

The overall course of the faecal progesterone metabolite (FPM) concentration did differ slightly between reproductive and non-reproductive females of this study. The main peak of the FPM concentration was shifted earlier in the season in females who did not rear offspring compared to those who did. Progesterone levels of non-reproductive females were elevated from mid-May to mid-June, representing the luteal phase following the second vaginal oestrus in summer. The second oestrus occurred in the first half of May in four out of six non-reproductive females. At this time of the year the reproductive females had not entered vaginal oestrus but were currently in the second half of lactation.

Previous studies showed inhibitory effects of lactation on ovarian activity (Tsukamura & Maeda, 2001). Instead, the reproductive females showed high FPM concentrations from the second half of May until mid-July, a time when no oestrus was documented.

Interestingly, reproductive females were in vaginal summer oestrus after their main FPM peak, concisely in late-July and early August. This second oestrus, indicated by a high abundance of cornified cells in the vaginal smears, was however not accompanied by a distinct increase in progesterone levels. After the main summer peak, the FPM levels of all females were constantly declining until the end of the season in August/September.

A similar phenomenon of vaginal oestrus occurring without a notable increase in progesterone has been documented in a study by Steinerberger (2017). Here the reproductive females showed elevated plasma progesterone levels during pregnancy that dropped with parturition in early May. Afterwards, prehibernatory fattening was not associated with a progesterone peak despite a vaginal summer oestrus was documented later in the season in those females (Steinerberger, 2017). In contrast, Divjak (2009) and Aschauer (2003) showed that the second vaginal oestrus in summer during the non-reproductive period was accompanied by increased progesterone levels which preceded the prehibernatory fattening phase. Usually, some kind of ovarian activity is to be expected if progesterone levels are noticeably elevated, which happens as a result of spontaneous ovulation along with the formation of active *corpora lutea* as the main source of progesterone (Bachelot & Binart, 2005; Davis, 2002). Histological analysis of ovaries collected shortly before and after hibernation showed that follicular development is initiated in summer during weaning and post-lactation and continued until immergence, thus enabling the females to become receptive within a few days after terminating hibernation (Aschauer et al., 2006; Millesi et al., 2000b)

5.4 Endocrine influence of the summer oestrus on prehibernatory fattening

After the summer peak the FPM levels of all females were constantly declining until the end of the season in August/September. The progesterone peak occurred before the onset of the fattening phase, which could indicate a priming effect of high progesterone titres for accumulating a lot of body mass in a short period of time. It has been hypothesized that prehibernatory fattening may be accelerated in female *S. citellus* due to the priming effect of an extended period of progesterone secretion induced by the summer oestrus (Millesi et al., 2008a; Strauss et al., 2009).

In the current study both reproductive and non-reproductive females initiated prehibernatory fattening in the second half of June and terminated the fattening phase at the end of July.

Body mass of the non-reproductives increased until a plateau phase was reached shortly before the onset of their next hibernation phase in August/September. No obvious plateau phase was observed for reproductive females.

Previous studies in *S. citellus* documented an earlier onset, usually late-May, of prehibernatory fattening in non-reproductive females (Divjak, 2009; Millesi et al., 2008a; Steinerberger, 2017). The study by Divjak (2009) found that non-reproductive females without contact to males were able to complete fattening about four weeks earlier compared to reproductive females kept in the same enclosure in previous years. Although starting simultaneously, non-reproductive females of this study terminated fattening about one week earlier than reproductive females and were able to gain more body mass. The rather small difference between reproductive and non-reproductive females of the current study could partially be explained by the superior food quality (e.g. additional protein pellets) the animals received during the lactation phase in June and July. Additional food supply in previous studies conducted at the study site consisted of less protein-rich food like apples, herbs (dandelion, clover, etc.), carrots, dried bread, grain mix for rodents and/or gras pellets (Divjak, 2009; Gaasch, 2016; Steinerberger, 2017). Therefore, the effect of progesterone on prehibernatory fattening might be less pronounced in the current study, explaining the small difference between the two groups.

5.5 Body mass

Individuals who emerged earlier in spring showed higher body mass than those emerging later and were subsequently able to precede the lighter females concerning the onset of hibernation. Early female emergence leading to early born litters together with overall good body condition of the mother are generally considered beneficial for offspring survival, as it gives both the juveniles and the mother sufficient time to physiologically prepare for hibernation (Huber et al., 1999; Millesi et al., 2004).

Females who showed higher daily body mass gains during fattening and who gained more mass in total, continued to show higher body mass until immergence and overall had a shorter fattening phase than individuals with less immergence mass at onset of hibernation. Although non-reproductive females gained significantly more g/day during prehibernatory fattening than reproductive females, there was no significant difference in the duration, the date of onset and end of the fattening phase when comparing both groups. This leads to the conclusion, that in general non-reproductive females were heavier than reproductive ones, both at the onset and at the end of the fattening phase. These differences might arise from the timing of the second oestrus and its accompanying endocrine influence on the acquisition of body mass during fattening.

Most non-reproductive females had an earlier summer oestrus compared to the reproductive females and accordingly, the rise in progesterone concentrations preceded those of reproductive females. This leads to the conclusion that having an early second oestrus can be beneficial for accelerated body mass increase and enable an early onset of hibernation. Considering all aspects above, changes in body condition and duration of the active season were mainly depending on individual parameters and apparently depended less on reproductive output.

Millesi et al. (2004) postulated that larger litters were significantly correlated with early emergence and reproduction, but not with emergence mass of the mothers. Furthermore higher reproductive output prolonged the active season and resulted in later emergence but not did not lead to lower body mass at the onset of hibernation (Millesi et al., 2004)

Body condition at hibernation onset is known to be an important parameter for over-winter survival and depends on maternal effort of the current active season. It has been shown that over-winter body mass loss is relatively constant in *S. citellus* (Millesi et al., 1999a). Individuals with lower body mass at immergence are usually also lighter at emergence in the subsequent spring. Poor body condition at emergence can then result in delayed oestrus and smaller, female-biased litters (Huber et al., 1999; Millesi et al., 1999b, 1999a). Thus, reaching a certain body fat content before hibernation can positively influence reproductive success in the next season. European ground squirrels will evidently terminate above ground activity as soon as they have reached a certain body mass or body fat content. In temperate climate food quality often declines in summer and high predation risks while foraging make early immergence more favourable, than prolonging the active season unnecessarily (Ruf & Bieber, 2023; Turbill et al., 2011; Turbill & Stojanovski, 2018). A study by Ruf and Bieber (2023) in edible dormice (*Glis glis*) proposed predator avoidance to be one of the main reasons for occasional early onset of prolonged hibernation periods in non-reproductive adults. Evidently, only individuals in good body condition with ample body fat deposits are able enter estivation in summer, which is then immediately followed by hibernation in autumn. This behaviour was observed in years when food availability was sufficient for fattening, but conditions for reproduction were still unfavourable due to low availability of high-caloric tree-seeds, which are needed for sustaining their fast-growing offspring born in August (Ruf & Bieber, 2023)

5.6 Cortisol excretion patterns

Shortly after emergence, the females showed basal faecal cortisol metabolite (FCM) concentrations. Non-reproductive females showed a peak in May after the second oestrus, that was marginally higher than the second peak in summer. Reproductive females showed high FCM values from May until June/July, concurring with the lactation phase.

Lactation poses significantly higher energetic costs for the mothers than pregnancy, which subsequently causes the mother´s body mass to plateau during lactation (Michener, 1998; Vasilieva & Tchabovsky, 2014). In various other rodent species, such as Yellow-Pine Chipmunks (*Tamias amoenus*, (Kenagy & Place, 2000)), post-partum glucocorticoid concentrations are elevated during lactation in association with energy metabolization. Cortisol reduces the ovary´s sensitivity to luteinizing-hormone (LH), thereby suppressing secretion of sexual steroid hormones and leading to delayed oestrus and ovulation (Aschauer et al., 2006; Baldwin, 1979).

The elevated cortisol titres could therefore possibly be responsible for the delayed second oestrus in reproductive females of the current study. The reproductive females showed a decrease of body mass during lactation, which could mean that the energetic costs of milk production exceeded the foraging possibilities, even though they received addition protein pellets at this time. Food shortage seems unlikely though, since all study subjects had access to additional food supplies throughout the active season to ensure sufficient food resources. Towards the end of the season there was an increase of cortisol levels in both non-reproductive and reproductive females. High glucocorticoid levels may stimulate foraging behaviour, which could allow females to accumulate sufficient body fat deposits during pregnancy and lactation, and prior to hibernation (Dallman et al., 2007; Dantzer et al., 2010).

These patterns of body mass and FCM levels roughly resemble those found by Nunes et al. (2006) in breeding female Belding´s ground squirrels (*U. beldingi*), who showed elevated baseline (non-stress) plasma corticosterone concentrations (PCC) during gestation with a decline throughout lactation as well as an annual peak of PCC prior to body mass increase during prehibernatory fattening. Corticosteroid secretion seems to be influenced by breeding activity, as non-breeding *U. beldingi* females had higher PCC levels than breeding females during the lactation phase of breeding individuals (Nunes et al., 2006). The same was true for the FCM levels of non-reproductive *S. citellus* females in the current study. Together these results suggest a conjunction of annual patterns in body mass, breeding activity and corticosteroid secretion (Dantzer et al., 2010).

5.7 Conclusion

Previous studies revealed the existence of a second non-reproductive vaginal oestrus phase in female European ground squirrels (S*permophilus citellus)* after weaning. Presumably it does not primarily serve reproduction purposes but seems to facilitate rapid body mass accumulation during prehibernatory fattening and stimulates spontaneous ovulation and follicular maturation shortly before the beginning of hibernation.

In the current study, reactivation of ovarian activity during the non-breeding season was observed either shortly after the first oestrus in May (four non-reproductive females) or delayed by gestation and lactation in July and August (one non-reproductive and four reproductive females). Durations and cell compositions were similar for both oestrus events and did not differ between groups.

Progesterone levels increased again after the second oestrus, because of the luteal phase of the oestrus cycle, but only in non-reproductive females. Reproductive females showed high levels of progesterone, indicating the presence of active *corpora lutea*, rising in the second half of lactation and up to post-lactation without a documented vaginal oestrus.

Later in summer, when they reactivated ovarian activity (July/August), indicated by high abundances of cornified epithelial cells, surprisingly progesterone levels did not increase but stayed baseline until hibernation. Interestingly, one non-reproductive female seemed to re-enter vaginal oestrus for a third time shortly before immergence. Together these findings can partially confirm the elevated progesterone levels post-breeding, but at the same time emphasize the need for further and more detailed research of this topic. In general, it can be assumed that the females benefit from the second oestrus, its endocrine influence on body mass accumulation and the associated stimulation of advanced follicular maturation in the following reproductive season.

Corticosteroid secretion seems to be related to reproductive activity, as reproductive females had lower levels of faecal cortisol metabolites during gestation and lactation than non-reproductive females at the same time. Cortisol levels reached an annual peak during prehibernatory fattening prior to immergence in both groups. Thus, glucocorticoids may have an influence on the accumulation of body fat or on some other aspect of the fattening phase, since they are known to stimulate foraging behaviour. Considering all aspects discussed above it can be concluded that the lack of reproductive effort can lead to a shift in seasonal timing of ovarian activity, secretion of sexual steroids and glucocorticoids and the course of prehibernatory fattening.

6 References

Ahmad, S. U. (2008). Reproductive biology of the ground-dwelling squirrels. *Proc. Pakistan Acad. Sci.*, *45*(4), 191–205.

Aschauer, A. (2003). *Ovarielle Aktivität außerhalb der Fortpflanzungsperiode beim Europäischen Ziesel (Spermophilus citellus).* [Diploma thesis]. University of Vienna.

Aschauer, A., Hoffmann, I. E., & Millesi, E. (2006). Endocrine profiles and reproductive output in European ground squirrels after unilateral ovariectomy. *Animal Reproduction Science*, *92*(3–4), 392–400. https://doi.org/10.1016/j.anireprosci.2005.06.004

Bachelot, A., & Binart, N. (2005). *Corpus Luteum* development: Lessons from genetic models in mice. In *Current Topics in Developmental Biology* (Vol. 68, pp. 49–84). Elsevier. https://doi.org/10.1016/S0070-2153(05)68003-9

Baldwin, D. M. (1979). The effect of glucocorticoids on estrogen-dependent luteinizing hormone release in the ovariectomized rat and on gonadotropin secretion in the intact female rat. *Endocrinology*, *105*(1), 120–128. https://doi.org/10.1210/endo-105-1-120

Barnes, B. M. (1996). Relationships between hibernation and reproduction in male ground squirrels. In F. Geiser, A. Hulbert, & S. Nicol (Eds), *Adaptations to the Cold: Tenth International Hibernation Symposium* (pp. 71–80). University of New England Press.

Barnes, B. M., Kretzmann, M., Licht, P., & Zucker, I. (1986). The influence of hibernation on testis growth and spermatogenesis in the Golden-mantled ground squirrel, *Spermophilus Lateralis*. *Biology of Reproduction*, *35*(5), 1289–1297. https://doi.org/10.1095/biolreprod35.5.1289

Carey, H. V., Andrews, M. T., & Martin, S. L. (2003). Mammalian hibernation: Cellular and molecular responses to depressed metabolism and low temperature. *Physiological Reviews*, *83*(4), 1153–1181. https://doi.org/10.1152/physrev.00008.2003

Cora, M. C., Kooistra, L., & Travlos, G. (2015). Vaginal cytology of the laboratory rat and mouse: Review and criteria for the staging of the estrous cycle using stained vaginal smears. *Toxicologic Pathology*, *43*(6), 776–793. https://doi.org/10.1177/0192623315570339

Dallman, M. F., Warne, J. P., Foster, M. T., & Pecoraro, N. C. (2007). Glucocorticoids and insulin both modulate caloric intake through actions on the brain. *The Journal of Physiology*, *583*(2), 431–436. https://doi.org/10.1113/jphysiol.2007.136051

Dantzer, B., McAdam, A. G., Palme, R., Fletcher, Q. E., Boutin, S., Humphries, M. M., & Boonstra, R. (2010). Fecal cortisol metabolite levels in free-ranging North American red squirrels: Assay validation and the effects of reproductive condition. *General and Comparative Endocrinology*, *167*(2), 279–286. https://doi.org/10.1016/j.ygcen.2010.03.024

Davis, J., S. (2002). The *corpus luteum*: An ovarian structure with maternal instincts and suicidal tendencies. *Frontiers in Bioscience*, *7*(1–3), d1949. https://doi.org/10.2741/davis1

Dewar, A. D. (1964). The nature of the weight gain induced, by progesterone in mice. *Quarterly Journal of Experimental Physiology and Cognate Medical Sciences*, *49*(2), 151–161. https://doi.org/10.1113/expphysiol.1964.sp001715

Divjak, A. (2009). *Verlauf der aktiven Saison und ovarielle Aktivität bei unverpaarten weiblichen Europäischen Zieseln (*Spermophilus citellus*)* [Master´s Thesis]. University of Vienna.

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

Geiser, F. (2004). Metabolic rate and body temperature reduction during hibernation and daily torpor. *Annual Review of Physiology*, *66*(1), 239–274. https://doi.org/10.1146/annurev.physiol.66.032102.115105

Hoffmann, I. E., Millesi, E., Huber, S., Everts, L. G., & Dittami, J. P. (2003). Population dynamics of European ground squirrels (*Spermophilus citellus*) in a suburban area. *Journal of Mammalogy*, *84*(2), 615–626. https://doi.org/10.1644/1545-1542(2003)084%253C0615:PDOEGS%253E2.0.CO;2

Huber, S., Hoffmann, I. E., Millesi, E., Dittami, J., & Arnold, W. (2001). Explaining the seasonal decline in litter size in European ground squirrels. *Ecography*, *24*(2), 205–211. https://doi.org/10.1034/j.1600-0587.2001.240211.x

Huber, S., Millesi, E., Walzl, M., Dittami, J., & Arnold, W. (1999). Reproductive effort and costs of reproduction in female European ground squirrels. *Oecologia*, *121*(1), 19–24. https://doi.org/10.1007/s004420050902

IUCN. (2019). *Spermophilus citellus: Hegyeli, Z.: The IUCN Red List of Threatened Species 2020: e.T20472A91282380* [Data set]. https://doi.org/10.2305/IUCN.UK.2020-2.RLTS.T20472A91282380.en

IUCN. (2023). *Spermophilus citellus: Ćosić, N., Ćirović, D., Fülöp, T., Gedeon, C., Hapl, E., Hoffmann, I.E., Kepel, A., Koshev, Y., Matějů, J., Nikolić Lugonja, T., Rammou, L.-D., Rusin, M., Váczi, O. & Hegyeli, Z.: The IUCN Red List of Threatened Species 2024: e.T20472A221789466* [Data set]. https://doi.org/10.2305/IUCN.UK.2024-2.RLTS.T20472A221789466.en

Kenagy, G. J., & Place, N. J. (2000). Seasonal changes in plasma glucocorticosteroids of free-living female Yellow-pine chipmunks: Effects of reproduction and capture and handling. *General and Comparative Endocrinology*, *117*(2), 189–199. https://doi.org/10.1006/gcen.1999.7397

Landau, I. T., & Holmes, W. G. (1988). Mating of captive thirteen-lined ground squirrels and the annual timing of estrus. *Hormones and Behavior*, *22*(4), 474–487. https://doi.org/10.1016/0018-506X(88)90052-9

Lobo, M. J., Remesar, X., & Alemany, M. (1993). Effect of chronic intravenous injection of steroid hormones on body weight and composition of female rats. *Biochemistry and Molecular Biology International*, *29*(2), 349–358.

Mallozzi, M., Bordi, G., Garo, C., & Caserta, D. (2016). The effect of maternal exposure to endocrine disrupting chemicals on fetal and neonatal development: A review on the major concerns. *Birth Defects Research Part C: Embryo Today: Reviews*, *108*(3), 224–242. https://doi.org/10.1002/bdrc.21137

Marchlewska-Koj, A. (1997). Sociogenic stress and rodent reproduction. *Neuroscience & Biobehavioral Reviews*, *21*(5), 699–703. https://doi.org/10.1016/S0149-7634(96)00021-8

Mascher, E. (2008). *Ontogenetische Entwicklung bei Europäischen Zieseln (Spermophilus citellus): Verhaltens- und physiologische Aspekte* [Diploma thesis]. University of Vienna.

Mendes, A.-M., Madon, R. J., & Flint, D. J. (1985). Effects of cortisol and progesterone on insulin binding and lipogenesis in adipocytes from normal and diabetic rats. *Journal of Endocrinology*, *106*(2), 225–231. https://doi.org/10.1677/joe.0.1060225

Michener, G. R. (1983). Spring emergence schedules and vernal behavior of Richardson's ground squirrels: Why do males emerge from hibernation before females? *Behavioral Ecology and Sociobiology*, *14*(1), 29–38. https://doi.org/10.1007/BF00366653

Michener, G. R. (1984). Age, sex and species differences in the annual cycles of ground-dwelling sciurids: Implications for sociality. In Murie, J. O. & G. R. Michener, *The Biology of Ground-dwelling Squirrels* (p. pp 81-107). Lincoln: University of Nebraska Press.

Michener, G. R. (1992). Sexual differences in over-winter torpor patterns of Richardson's ground squirrels in natural hibernacula. *Oecologia*, *89*(3), 397–406. https://doi.org/10.1007/BF00317418

Michener, G. R. (1998). Sexual differences in reproductive effort of Richardson's ground squirrels. *Journal of Mammalogy*, *79*(1), 1–19. https://doi.org/10.2307/1382838

Miller, A., Jentz, E., Duncan, C., & Merriman, D. (2021). Progestogen metabolites for use in pregnancy monitoring of 13-lined ground squirrels (*Ictidomys tridecemlineatus*). *Reproduction and Fertility*, *2*(2), 81–88. https://doi.org/10.1530/RAF-20-0071

Millesi, E., Divjak, A., & Strauss, A. (2008a). Seasonal timing and pre-hibernation fattening in breeding and non-breeding European ground squirrels. In *BG Lovegrove & A McKechnie (eds), Hypometabolism in animals: Topor, hibernation and cryobiology* (pp. 291–296). University of Kwazulu-Natal.

Millesi, E., & Hoffmann, I. E. (2008). Body mass and timing of the active season in European Ground Squirrels (*Spermophilus citellus*) at high and low population density. *Lynx, New Series*, *39(2)*, 305–315.

Millesi, E., Hoffmann, I. E., Aschauer, A., & Franceschini, C. (2004). Reproduction and hibernation in females: A comparison of two sympatric ground-dwelling rodents. In *Life in the Cold: Evolution, Mechanism, Adaptation, and Application. 12th International Hibernation Symposium, Biological Papers of the University of Alaska* (Vol. 27, pp. 127–135). Biological Papers of the University of Alaska.

Millesi, E., Hoffmann, I. E., & Huber, S. (2004). Reproductive strategies of male European sousliks (*Spermophilus citellus*) at high and low population density. *Lutra*, *47*(2), 75–84.

Millesi, E., Hoffmann, I. E., Steurer, S., Metwaly, M., & Dittami, J. P. (2002). Vernal changes in the behavioral and endocrine responses to GnRH application in male European ground squirrels. *Hormones and Behavior*, *41*(1), 51–58. https://doi.org/10.1006/hbeh.2001.1735

Millesi, E., Huber, S., Dittami, J., Hoffmann, I., & Daan, S. (2010). Parameters of Mating Effort and Success in Male European Ground Squirrels, Spermophilus citellus. *Ethology*, *104*(4), 298–313. https://doi.org/10.1111/j.1439-0310.1998.tb00070.x

Millesi, E., Huber, S., Everts, L. G., & Dittami, J. P. (1999a). Reproductive decisions in female European ground squirrels: Factors affecting reproductive output and maternal investment. *Ethology*, *105*(2), 163–175. https://doi.org/10.1046/j.1439-0310.1999.00379.x

Millesi, E., Huber, S., Pieta, K., Walzl, M., Arnold, W., & Dittami, J. P. (2000a). Estrus and estrogen changes in mated and unmated free-living European ground squirrels. *Hormones and Behavior*, *37*(3), 190–197. https://doi.org/10.1006/hbeh.2000.1574

Millesi, E., Huber, S., Walzl, M., & Dittami, J. P. (2000b). Follicular development and hibernation in European ground squirrels. In G. Heldmaier & M. Klingenspor (Eds), *Life in the Cold* (pp. 285–292). Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-662-04162-8_31

Millesi, E., Strauss, A., Burger, T., Hoffmann, I. E., & Walzl, M. (2008b). Follicular development in European ground squirrels (*Spermophilus citellus*) in different phases of the annual cycle. *REPRODUCTION*, *136*(2), 205–210. https://doi.org/10.1530/REP-08-0090

Millesi, E., Strijkstra, A. M., Hoffmann, I. E., Dittami, J. P., & Daan, S. (1999b). Sex and age differences in mass, morphology, and annual cycle in European ground squirrels, *Spermophilus citellus*. *Journal of Mammalogy*, *80*(1), 218–231. https://doi.org/10.2307/1383222

Montiel, F., & Ahuja, C. (2005). Body condition and suckling as factors influencing the duration of postpartum anestrus in cattle: A review. *Animal Reproduction Science*, *85*(1–2), 1–26. https://doi.org/10.1016/j.anireprosci.2003.11.001

Morton, M. L., & Sherman, P. W. (1978). Effects of a spring snowstorm on behavior, reproduction, and survival of Belding's ground squirrels. *Canadian Journal of Zoology*, *56*(12), 2578–2590. https://doi.org/10.1139/z78-346

Möstl, E., & Palme, R. (2019, October). *Measuring faecal steroid metabolites with enzyme immunoassays (EIA) on microtitre plates using biotinylated steroids as labels (Protocol)*. Department of Biomedical Sciences. Vienna: University of Veterinary Medicine.

Németh, I., Nyitrai, V., & Altbäcker, V. (2009). Ambient temperature and annual timing affect torpor bouts and euthermic phases of hibernating European ground squirrels (*Spermophilus citellus*). *Canadian Journal of Zoology*, *87*(3), 204–210. https://doi.org/10.1139/Z08-150

Nunes, S., Pelz, K. M., Muecke, E.-M., Holekamp, K. E., & Zucker, I. (2006). Plasma glucocorticoid concentrations and body mass in ground squirrels: Seasonal variation and circannual organization. *General and Comparative Endocrinology*, *146*(2), 136–143. https://doi.org/10.1016/j.ygcen.2005.10.013

Nuriel-Ohayon, M., Belogovski, A., Komissarov, S., Izhak, M. B., Shtossel, O., Neuman, H., Ziv, O., Turjeman, S., Bel, S., Louzoun, Y., & Koren, O. (2021). *Progesterone supplementation in mice*

leads to microbiome alterations and weight gain in a sex-specific manner. bioRxiv. https://doi.org/10.1101/2021.10.06.463337

Papanicolaou, G. N. (1942). A new procedure for staining vaginal smears. *Science (New York, N.Y.)*, *95*(2469), 438–439. https://doi.org/10.1126/science.95.2469.438

Papanicolaou, G. N., & Traut, H. F. (1944). Diagnosis of uterine cancer by the vaginal smear. *American Journal of Clinical Pathology*, *14*(2), 127.2-127. https://doi.org/10.1093/ajcp/14.2.127a

Pettitt, B. A., Wheaton, C. J., & Waterman, J. M. (2007). Effects of storage treatment on fecal steroid hormone concentrations of a rodent, the Cape ground squirrel (*Xerus inauris*). *General and Comparative Endocrinology*, *150*(1), 1–11. https://doi.org/10.1016/j.ygcen.2006.06.010

Ramos-Lara, N., Koprowski, J. L., Kryštufek, B., & Hoffmann, I. E. (2014). *Spermophilus citellus (Rodentia: Sciuridae)*. *Mammalian Species*, *46*(913), 71–87. https://doi.org/10.1644/913.1

Ruf, T., & Bieber, C. (2023). Why hibernate? Predator avoidance in the edible dormouse. *Mammal Research*, *68*(1), 1–11. https://doi.org/10.1007/s13364-022-00652-4

Ryan, C. P., Anderson, W. G., Berkvens, C. N., & Hare, J. F. (2014). Maternal gestational cortisol and testosterone are associated with trade-offs in offspring sex and number in a free-living rodent (*Urocitellus richardsonii*). *PLoS ONE*, *9*(10), e111052. https://doi.org/10.1371/journal.pone.0111052

Sharara, F. I., Seifer, D. B., & Flaws, J. A. (1998). Environmental toxicants and female reproduction. *Fertility and Sterility*, *70*(4), 613–622. https://doi.org/10.1016/S0015-0282(98)00253-2

Sherman, P. W. (1982). Infanticide in ground squirrels. *Animal Behaviour*, *30*(3), 938–939. https://doi.org/10.1016/S0003-3472(82)80174-7

Shirling, D., Ashby, J. P., & Baird, J. D. (1981). Effect of progesterone on lipid metabolism in the intact rat. *Journal of Endocrinology*, *90*(2), 285–294. https://doi.org/10.1677/joe.0.0900285

Slimen, H. B., Gedeon, C. I., Hoffmann, I. E., & Suchentrunk, F. (2012). Dwindling genetic diversity in European ground squirrels? *Mammalian Biology*, *77*(1), 13–21. https://doi.org/10.1016/j.mambio.2011.10.001

Steinerberger, S. (2017). *Prehibernation fattening in female European ground squirrels (*Spermophilus citellus*) with and without luteal activity during the non-breeding period* [Master´s Thesis, University of Vienna]. https://utheses.univie.ac.at/detail/40327

Stevens, S. D. (1998). High incidence of infanticide by lactating females in a population of Columbian ground squirrels (Spermophilus columbianus). *Canadian Journal of Zoology*, *76*(6), 1183–1187. https://doi.org/10.1139/z98-045

Strauss, A., Hoffmann, I. E., Vielgrader, H., & Millesi, E. (2008). Testis development and testosterone secretion in captive European ground squirrels before, during, and after hibernation. *Acta Theriologica*, *53*(1), 47–56. https://doi.org/10.1007/BF03194278

Strauss, A., Hoffmann, I. E., 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. https://doi.org/10.1016/j.anireprosci.2008.05.068

Tomac, J., Cekinović, Đ., & Arapović, J. (2011). Biology of the *corpus luteum*. *Periodicum Biologorum*, *113*(1), 43–49.

Traut, H. F., & Papanicolaou, G. N. (1943). Cancer of the uterus: The vaginal smear in its diagnosis. *California and Western Medicine*, *59*(2), 121–122.

Trulio, L. A., Loughry, W. J., Hennessy, D. F., & Owings, D. H. (1986). Infanticide in California ground squirrels. *Animal Behaviour*, *34*, 291–294. https://doi.org/10.1016/0003-3472(86)90037-0

Tsukamura, H., & Maeda, K. (2001). Chapter 13 Non-metabolic and metabolic factors causing lactational anestrus: Rat models uncovering the neuroendocrine mechanism underlying the suckling-induced changes in the mother. In *Progress in Brain Research* (Vol. 133, pp. 187–205). Elsevier. https://doi.org/10.1016/S0079-6123(01)33014-5

Tsukamura, H., Maeda, K.-I., Ohkura, S., Uchida, E., & Yokoyama, A. (1991). The suppressing effect of the sucking stimulus on the pulsatile luteinzing hormone release is not mediated by prolactin in the rat at mid-lactation. *The Japanese Journal of Animal Reproduction*, *37*(1), 59–63. https://doi.org/10.1262/jrd1977.37.59

Turbill, C., Bieber, C., & Ruf, T. (2011). Hibernation is associated with increased survival and the evolution of slow life histories among mammals. *Proceedings of the Royal Society B: Biological Sciences*, *278*(1723), 3355–3363. https://doi.org/10.1098/rspb.2011.0190

Turbill, C., & Stojanovski, L. (2018). Torpor reduces predation risk by compensating for the energetic cost of antipredator foraging behaviours. *Proceedings of the Royal Society B: Biological Sciences*, *285*(1893), 20182370. https://doi.org/10.1098/rspb.2018.2370

Van der Schoot, P., Lankhorst, R. R., De Roo, J. A., & De Greef, W. J. (1978). Suckling stimulus, lactation, and suppression of ovulation in the rat. *Endocrinology*, *103*(3), 949–956. https://doi.org/10.1210/endo-103-3-949

Vasilieva, N. A., & Tchabovsky, A. V. (2014). Timing is the only thing: Reproduction in female yellow ground squirrels (*Spermophilus fulvus*). *Canadian Journal of Zoology*, *92*(8), 737–747. http://dx.doi.org/10.1139/cjz-2014-0084. https://doi.org/10.1139/cjz-2014-0084

Vasilieva, N. A., & Tchabovsky, A. V. (2015). A shortage of males causes female reproductive failure in yellow ground squirrels. *Science Advances*, *1*(9), e1500401. https://doi.org/10.1126/sciadv.1500401

Vekhnik, V. A. (2020). Regulation of reproduction rate in terrestrial placental mammals. *IOP Conference Series: Earth and Environmental Science*, *607*(1), 012011. https://doi.org/10.1088/1755-1315/607/1/012011

Williams, C. T., Barnes, B. M., Kenagy, G. J., & Buck, C. L. (2014). Phenology of hibernation and reproduction in ground squirrels: Integration of environmental cues with endogenous programming. *Journal of Zoology*, *292*(2), 112–124. https://doi.org/10.1111/jzo.12103

Wolff, J. O., & Peterson, J. A. (1998). An offspring-defense hypothesis for territoriality in female mammals. *Ethology Ecology & Evolution*, *10*(3), 227–239. https://doi.org/10.1080/08927014.1998.9522854

7 Deutsche Zusammenfassung

Als obligate Winterschläfer zehren Europäische Ziesel (*Spermophilus citellus*) während des sechs- bis siebenmonatigen Winterschlafs ausschließlich von ihren Körperfettreserven, welche sie im Zuge einer mehrwöchigen Auffettungsphase nach dem Ende der Laktation anlegen. Somit verbleiben lediglich fünf bis sechs Monate für essenzielle saisonale Prozesse wie Paarung, Trächtigkeit, Laktation und adäquate Vorbereitung auf den Winterschlaf. Bedingt durch die kurze aktive Saison sind die Tiere strengen zeitlichen Abläufen unterworfen. Weibchen verpaaren sich üblicherweise bereits nach der ersten Winterschlafperiode, oft noch bevor sie das strukturelle Wachstum komplett abgeschlossen haben. Demzufolge ziehen Europäische Ziesel nur einen Wurf pro Saison groß, dessen Größe, Laktationsdauer und Überlebenschancen positiv vom körperlichen Zustand der Mutter und dem Zeitpunkt der Verpaarung abhängen. Innerhalb weniger Tage nach dem Aufwachen kommt es während der dreiwöchigen Paarungszeit im März/April zu einer spontanen Ovulation und steigender Ausschüttung von Progesteron und Östrogenen im Zuge der vierwöchigen Trächtigkeit. Gegen Ende der anschließenden vier- bis sechswöchigen Laktationsphase kam es in früheren Studien erneut zu einem Östruszyklus, welcher jedoch nicht unmittelbar der Fortpflanzung diente, da die Männchen zu dieser Jahreszeit nicht mehr sexuell aktiv sind. Es kommt zu einer spontanen Ovulation und Reifung neuer Gelbkörper, wodurch auch die Progesteron- und Östrogenkonzentrationen ansteigen, zeitgleich mit dem Beginn der Auffettungsphase.

Aufgrund bekannter Effekte von Progesteron nimmt man an, dass der zweite Östruszyklus sich positiv auf die Akkumulation von Körperfett vor dem Eintreten in den Winterschlaf auswirkt und es den Weibchen ermöglicht, trotz intensiver mütterlicher Investitionen, in kurzer Zeit ausreichend Fettreserven anzulegen.

Weibchen sind in der Regel innerhalb weniger Tage nach Beendigung des Winterschlafes empfängnisbereit, obwohl während des Winterschlafs keine weitere follikuläre Reifung im Ovar stattfindet. Histologische Untersuchungen von Ovarien kurz vor Antritt, während und nach dem Winterschlaf bestätigen die Existenz von fortgeschrittenen follikularen Reifestadien. Um die Zusammenhänge zwischen ovarieller Aktivität, endokriner Sekretion und saisonalen Veränderungen des Körpergewichts näher zu untersuchen wurden für diese Studie zehn weibliche Europäische Ziesel über die gesamte aktive Saison 2021 beobachtet. Die Tiere lebten in zwei naturnahen Außengehegen im Norden Wiens (Österreich) und wurden regelmäßig zugefüttert. Es wurden Vaginalabstriche entnommen, um die ovariellen Veränderungen während der Paarungszeit und der nicht-reproduktiven Phase im Sommer zu dokumentieren.

Der Zustand der Zitzen und Vulva diente zusammen mit dem Körpergewicht als Indikator für eine Schwangerschaft und anschließender Laktation. Metaboliten von Progesteron und Cortisol wurden mittels Kotproben erhoben. Obwohl alle Weibchen während der Paarungszeit Zugang zu Männchen hatten, war der Fortpflanzungserfolg gering und nur vier von zehn Weibchen wurden als reproduzierend klassifiziert.

In dieser Studie zeigte sich, dass nicht-reproduktive Weibchen größtenteils bereits im Mai einen zweiten Vaginalöstrus inklusive deutlich erhöhter Progesteronwerte hatten, während reproduzierende Weibchen teilweise erst sehr spät im Jahr (Juli/August) eine zweite Phase ovarieller Aktivität aufwiesen, welche jedoch ohne einen Anstieg des Progesterons vonstattenging. Die beiden Östrusphasen unterschieden sich weder in ihrer Dauer noch in der Zellzahl oder Zellkomposition voneinander. Der zweite Östrus trat bei nicht-reproduktiven Tieren bereits im Mai auf, und wurde von einem deutlichen Progesteronanstieg begleitet. Bei reproduktiven Weibchen konnten zu dieser Zeit ebenfalls erhöhte Progesteronwerte beobachtet werden, obwohl kein zweiter vaginaler Östrus festgestellt wurde. Dieser ereignete sich erst Ende Juli/Anfang August kurz vor dem Winterschlaf und es kam zu keinem Anstieg von Progesteron. Wie auch in vorherigen Studien, lag der Progesteron-Peak kurz vor dem Beginn der Auffettungsphase, was für einen Zusammenhang spricht. Verlauf und Ausmaß der Auffettungsphase vor dem Winterschlaf waren in beiden Gruppen ähnlich. Obgleich nicht-reproduktive Weibchen im Allgemeinen schwerer waren und die tägliche Gewichtszunahme signifikant höher war, gab es keinen erkennbaren Unterschied hinsichtlich der Dauer, des Beginns und des Enddatums der Auffettungsphase.

Der Cortisolspiegel stieg gegen Ende der Saison sowohl bei nicht-reproduktiven als auch bei reproduktiven Weibchen an. Es liegt die Vermutung nahe, dass erhöhte Progesteron- und Cortisolspiegel das Nahrungssuchverhalten fördern und es den Weibchen erleichtern, vor dem Winterschlaf ausreichend Körperfett anzulegen. Die Ergebnisse zeigen, dass verringerter Brutpflegeaufwand zu einem früheren Beginn der Ovarialaktivität im Sommer und einer verbesserten Körperkondition vor dem Winterschlaf führte, was sich positiv auf die nachfolgende Reproduktionsperiode auswirken könnte

Schlagwörter: *Spermophilus citellus*, Europäisches Ziesel, Progesteron, Winterschlaf, Östruszyklus, Auffettungsphase, Vaginalzytologie