Sexually mature and immature yearling male European ground squirrels: A comparison of behavioral and physiological parameters

Anna Strauss [a,*], Elvira Mascher [a], Rupert Palme [b], Eva Millesi [a]

[a] *Department of Behavioural Biology, University of Vienna, Althanstr, 14, A-1090 Vienna, Austria*
[b] *Institute of Biochemistry, Department of Natural Sciences, University of Veterinary Medicine, Vienna, Austria*

Abstract

Early puberty may increase lifetime reproductive success, but may also entail diverse costs. In male European ground squirrels (*Spermophilus citellus*), age at sexual maturity varies among individuals. We compared sexually active and inactive yearling males under standardized conditions in outdoor enclosures. Non-reproductive yearling males hibernated significantly longer than mature males and emerged when the mating period had already started. Testosterone concentrations peaked in the precocious males during premating and mating, and were depressed in the non-reproductive individuals throughout the study period. Emergence body mass was similar in both groups, although sexually immature males were significantly heavier at the end of the active season. Additionally, non-reproductive yearlings grew faster than precocious individuals. Male–male aggression peaked during mating and the following 4 weeks. Intense aggressive interactions like chases and fights were mostly initiated by reproductive males and were directed towards mature competitors. Aggressive behavior in immature males was mainly expressed as spatial displacements. Non-agonistic interactions with females were only observed in sexually active males. Concentrations of fecal cortisol metabolites did not differ between the two male groups and were elevated in the second half of the active season. Leucocyte numbers were reduced during mating in mature males, whereas in immature animals, cell numbers did not change significantly. Our results indicate that early puberty in European ground squirrels involves lower growth rates, high intrasexual aggression, lower prehibernatory body mass, and probably worse immunological condition during mating.
© 2007 Elsevier Inc. All rights reserved.

Introduction

Age at puberty is an important life-history trait (Daan and Tinbergen, 1997). Precociousness shortens the latency period for reproduction and thereby can prolong the overall time span for reproduction. Early puberty, however, may create costs for the males in terms of reduced immunocompetence (Boonstra et al., 2001) and impeded structural growth (Kenagy et al., 1989). Delayed maturity, on the other hand, allows yearling males to allocate more energy to growth and improve body condition (Kenagy et al., 1989), potentially resulting in increased competitive ability in intrasexual contests and higher mating success later in life (Schwagmeyer and Brown, 1983).

Variation in the timing of sexual maturity has been described among males in a number of *Spermophilus* species, and in most cases, body mass and the amount of energy stored as fat prior to hibernation were proposed to affect the potential of yearlings to breed in spring (Barnes, 1984; Buck and Barnes, 2003; Bushberg and Holmes, 1985; Dark et al., 1992; Holmes, 1988; Kenagy et al., 1989; McLean and Towns, 1981; Schwagmeyer and Brown, 1983; Slade and Balph, 1974).

Free-living male European ground squirrels have a maximum life span of 4 years (Hoffmann et al., 2003), and the lifetime reproductive success could be increased by early puberty. However, the mating period is a challenging time for mature males, with decreased foraging activity, high intrasexual conflicts, and body mass loss of about 4 g/day (Millesi et al., 1998, 2004). Additionally, molt, prehibernatory fattening, and the onset of hibernation can be delayed in individuals with high mating effort (Millesi et al., 1998). In *Spermophilus citellus*, yearling females

usually reproduce, whereas males could become sexually active either as yearlings or as 2-year olds (Millesi et al., 1999). Therefore, two different reproductive strategies occurred in males: either starting reproduction early in life as yearling or waiting an additional season and becoming mature as 2-year old. The timing of puberty was related to population density in that, at high densities with strong intrasexual competition, most yearling males were immature. In low-density years with higher female availability, however, all yearling males in the same area were reproductively active (Hoffmann et al., 2003; Millesi et al., 2004).

Male ground squirrels with an early or a delayed puberty may differ in physiological and behavioral parameters. In this study, we compared sexually active and inactive yearling males in terms of body mass, growth rates, social interactions, stress levels, testosterone patterns, and leucocyte numbers throughout the active season under standardized conditions in outdoor enclosures.

Materials and methods

Animals, study area, and capture techniques

The European ground squirrels (*S. citellus*) used in this study lived in three outdoor enclosures (125–185 m^2) located north of Vienna (Austria) within the distribution range of the species. The animals inhabited natural, self-made burrows. All animal procedures were approved by the Austrian Committee for Animal Research of the Federal Ministry of Science. Sexually active yearling males ($n=9$) were compared to sexually inactive ones ($n=5$) throughout the active season (late March–late September). Yearlings were the juveniles of the preceding year. The 14 experimental animals originated from 13 different litters, and only 2 of them were brothers (both sexually active, allocated to different enclosures). Reproductive status of individuals was determined at first capture in spring. Males were classified as sexually active when their testes were descended into a darkly pigmented scrotum. Sexually inactive males had abdominal testes, and their scrota remained unpigmented. Testis development is a discrete phenomenon in this species: in sexually inactive yearling males no subsequent testicular development has been found (Millesi et al., 1998). The rate of sexual activity among all emerged yearling males was similarly high in all three enclosures (enclosure 1: 81.8%, enclosure 2: 75.0%, enclosure 3: 66.7%). All animals were permanently marked with transponder chips injected subcutaneously in the neck region (RFID-tag, Datamars Comp.). For distant recognition the fur was painted with commercial hair dye in individual patterns. Males were captured in weekly intervals with Tomahawk live traps. At each capture, animals were weighed (±1 g), and head length was measured using a caliper (±0.1 mm, condylo-basal length). The vegetation, the soil conditions, and the initial population density (enclosure 1: 0.09 animals/m^2, enclosure 2: 0.07 animals/m^2, enclosure 3: 0.09 animals/m^2) of the three enclosures were similar. Individual density varied with season in a similar pattern in all enclosures depending on the emergence of the juveniles from the natal burrows and the onset of hibernation in females in early August. In all enclosures, sexually active (experimental and non-experimental males) as well as inactive yearling males (all of them experimental males) and reproductive females were present. All females weaned litters, whereas litter size did not differ significantly between the enclosures (enclosure 1: 3.8±2.1 juveniles/litter, $n=4$, enclosure 2: 6.5±0.7 juveniles/litter, $n=2$, enclosure 3: 6.0±1.7 juveniles/litter, $n=5$, means±SD; Kruskal–Wallis *H*-test: $p>0.05$). Additional food (standard rodent laboratory chow, dried bread, carrots, apples, dandelion leaves) and water were provided daily.

Although the fences of the enclosures extended about 1.5 m below the ground, this was insufficient to prevent some of the ground squirrels from escaping. Accordingly, group size during the study decreased from 9 to 7 in the sexually active yearlings and from 5 to 4 in the sexually inactive group.

Seasonal phases

Emergence and immergence dates were defined as first and last trapping or sighting of an individual during daily observations in spring and fall, respectively.

Seasonal phases were defined per enclosure according to Millesi et al. (1998). Female reproductive state and emergence of the juveniles were relevant to male behavior. Based on the duration of different seasonal processes like mating, molt, and preparation for hibernation, seven phases were defined as follows (mean onset and end±SD are given in parentheses).

Premating (PREM; March 27±5.5 days–April 4±5.6 days) lasted from the emergence of the first male until the first female was observed.

Mating (M; April 5±5.6 days–April 23±5.8 days) started with the emergence of the first female and ended 1 week after the emergence of the last, as by this time all females showed signs of gestation.

Thereafter, the data were analyzed on a 4-week basis.

Postmating 1 (PM1; April 24±5.8 days–May 23±5.8 days) included the main period of testis regression.

Postmating 2 (PM2; May 24±5.8 days–June 23±5.8 days) encompassed males' first molt and ended with the emergence of the juveniles from the natal burrows.

Postmating 3 (PM3; June 24±5.8 days–July 23±5.8 days) was the phase of litter weaning.

Prehibernation 1 (PH1; July 24±5.8 days–August 23±5.8 days) included males' second molt.

Prehibernation 2 (PH2; August 24±5.8 days–September 29±3.2 days) lasted from the end of PH1 until all experimental males had entered hibernation.

Behavioral observations

Behavioral observations were carried out throughout the active season 2–6 times per week in each enclosure, depending on weather conditions, because ground squirrels were not active on rainy days. Behavior was monitored alternatively to trapping during the main activity period of the animals (10:30–16:30). Observations were done with binoculars always from the same spot per enclosure (at a distance of about 3 m from the enclosure fence), where the whole enclosure could be overlooked. The animals were used to the presence of an observer, and their behavior remained unaffected. Using event-sampling techniques, agonistic (displacement, chase, fight) and non-agonistic interactions (nose-to-nose contact, anogenital inspection, spatial cohesion) were recorded whenever they occurred during 1-h observation periods. Initiator and recipient (identity, sex) were noted. For further analyses, chases and fights were classified as high-aggressive interactions and spatial displacements as low-aggressive interactions. We calculated the interaction rate per seasonal phase for each individual and class of interactions as the number of interactions per observation protocol corrected for the presence of potential interaction partners and the number of protocols in the respective phase. Mating occurred underground and was defined as the common and synchronous use of the female's burrow by a male for at least 30 min (Millesi et al., 1998).

Hormone assays

The animals were sampled for blood and feces during their main activity period within a time window of 6 h (10:30–16:30). Intra-individual comparisons showed that hormone concentrations of samples collected in the morning (before 12:30) did not differ from samples collected in the afternoon (after 14:30; Wilcoxon signed rank tests: $p>0.05$). The ground squirrels were removed from the traps immediately after capturing.

Blood samples (150–200 μl) were taken from the femoral vein in weekly intervals. The animals were put into a black, cone-shaped cotton bag, where they remained calm and were easy to handle. The manipulations lasted less than 5 min. The blood was collected in heparinized capillary tubes and centrifuged in the field. Plasma was stored at −20 °C until analysis. Testosterone levels were analyzed after diethylether extraction (85% recovery rates) with a biotin–streptavidin enzyme immunoassay according to Palme and Möstl (1994). Using testosterone as standard, the assay showed cross-reactions with testosterone (100%), 5α-androstane-17β-ol-3-one (23%), and 5β-androstane-17β-ol-3-one

Table 1
Timing of hibernation and interaction rates in sexually active and inactive male European ground squirrels (means±SE)

Timing of hibernation		Sexually active	Sexually inactive	p	z	n
Onset of first hibernation (month/day)		10/05 (±3.4)	09/25 (±3.2)	n.s.		5/3
Spring emergence (month/day)		03/27 (±1.3)	04/12 (±2.7)	0.04	−2.3	5/3
Duration of first hibernation (days)		172.8 (±4.3)	199.7 (±2.0)	0.04	−2.2	5/3
Onset of second hibernation (month/day)		09/14 (±7.2)	09/01 (±4.9)	n.s.		5/3
Interaction rates						
M	High-aggressive—males	0.65 (±0.30)	0.08 (±0.08)	n.s.		9/5
M	Low-aggressive—males	0.49 (±0.22)	1.09 (±0.14)	0.03	−2.2	9/5
M	Non-agonistic—females	0.78 (±0.41)	0.00 (±0.00)	0.04	−2.2	9/5
PM1	High-aggressive—males	0.60 (±0.23)	0.03 (±0.02)	0.04	−2.1	9/5
PM1	Low-aggressive—males	0.59 (±0.16)	0.56 (±0.28)	n.s.		9/5
PM1	Non-agonistic—females	0.19 (±0.07)	0.00 (±0.00)	0.02	−2.5	9/5

Reliable hibernation data were available for 5 sexually active and 3 sexually inactive males. Rates of initiated interactions were analyzed in the seasonal phases mating (M) and postmating 1 (PM1). High-aggressive (chase, fight) and low-aggressive interactions (spatial displacement) with consexuals as well as non-agonistic interactions (nose-to-nose contact, anogenital inspection, spatial cohesion) with females are presented. In case of significant group differences statistical values are included.

(12%). Intra- and inter-assay coefficients of variation were 13.5% and 14.3%, respectively.

Fecal cortisol metabolites (FCM) were analyzed from weekly samples, as the animals usually defecate at capture. We stored fresh feces at −20 °C. Before analysis, fecal samples were dried (60 °C for 24 h) and powdered. After suspending 0.2 g of each sample in 80% methanol, FCM were measured using an 11-oxoetiocholanolone enzyme immunoassay. Details of this group specific assay (measuring FCM with a 3α,11-oxo structure) are described in Möstl et al. (2002). Intra- and inter-assay coefficients of variation were 13.3% and 14.4%, respectively.

To demonstrate the biological relevance of the measured FCM in European ground squirrels, a validation experiment was performed (Palme, 2005; Touma and Palme, 2005). Male ground squirrels ($n=4$) were exposed to a stressful situation by putting them into traps without cover for 4 h and confronting them briefly (5 min) with a potential predator (dog). Afterwards the animals were kept in a cage containing a wooden nest box for shelter for an additional 22 h. In 2-h intervals, the traps and the cages were checked for feces, and all fecal samples were collected. The experimental situation resulted in a 2- to 3-fold (2.3±0.6, mean±SD) increase in FCM concentrations. Peak levels were reached after 7.5 h (±2.5, mean±SD).

Hematology

To approximate immune function, blood samples (200 μl, heparinized Eppendorf tubes) were collected and analyzed in a veterinary laboratory (Invitro GmbH, Vienna). Hemograms and differential white blood cell counts were done. Leucocyte counts are an indirect assessment of immunocompetence and have been used previously as a proxy for immune function in sciurids (Bachman, 2000, 2003; Boonstra and McColl, 2000; Boonstra et al., 2001). Leucocyte numbers were assessed once in mating, once in PM1, as this is the phase of the onset of testis regression with potential carryover effects from mating, and once after testis regression was definitely completed in PM3. Leucocytes other than lymphocytes and segmented neutrophils were rare; analyses were therefore confined to these cell types.

Statistical analyses

For hormonal (testosterone, FCM) and conditional parameters (body mass, head length), individual means per phase were used in the analyses. Shapiro–Wilk tests revealed that most data were not distributed normally; therefore, non-parametric tests were applied. Male groups were tested pairwise with Mann–Whitney U-tests. Friedman tests were performed to test for differences between the seasonal phases. In case of significant results, post hoc pairwise comparison with Wilcoxon signed rank tests were carried out for intra-individual comparisons. Statistical significance was set at $p<0.05$. In the event of multiple pairwise comparisons, we modified the significance level with a

Bonferroni correction. Unless stated otherwise, means±SE are shown in all data sets.

Results

The timing of hibernation differed significantly between sexually active and inactive animals (Table 1). All males had
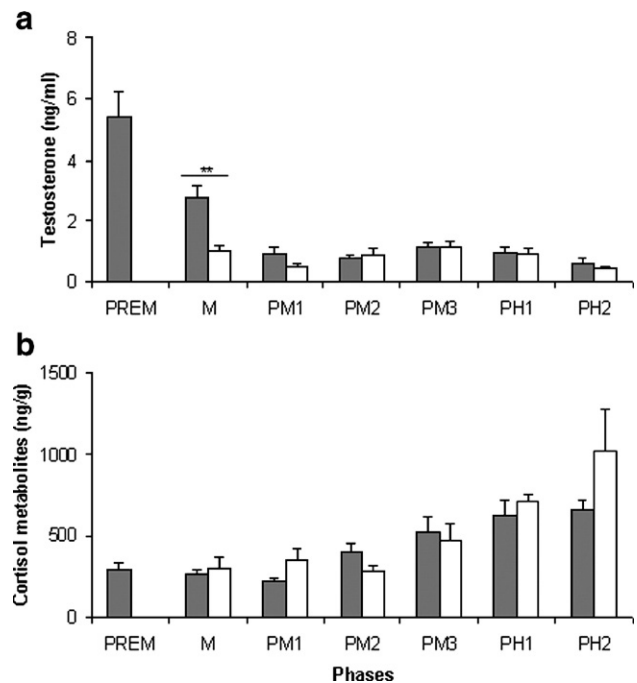

Fig. 1. (a) Plasma testosterone levels (ng/ml) during the seasonal phases (PREM—premating, M—mating, PM1—postmating 1, PM2—postmating 2, PM3—postmating 3, PH1—prehibernation 1, PH2—prehibernation 2) in sexually active (filled bars) and inactive (open bars) male European ground squirrels (means+SE). (b) Fecal cortisol metabolites (ng/g) during the seasonal phases in sexually active (filled bars) and inactive (open bars) male European ground squirrels (means+SE). Number of individuals per phase: sexually active/inactive: 9/–, 9/5, 9/5, 7/5, 7/4, 7/4, 7/3. One sexually inactive male had already started hibernation in PH2. Significant group difference is marked with asterisks: **$p<0.01$.

started hibernation as juveniles in late September or early October, but the non-reproductive individuals hibernated for about 4 weeks longer than the reproductive ones. The immature yearlings emerged significantly later in spring when the mating period had already started. In the subsequent fall, no differences in immergence dates were found between the two yearling groups.

Testosterone concentrations remained baseline in sexually inactive males throughout the active season ($p > 0.05$; Fig. 1a). In contrast, reproductive males exhibited peak levels during premating and mating ($p < 0.001$, $\chi^2 = 30.2$, $df = 6$, $n = 7$; post hoc: PREM vs. M, and M vs. PM1: $p < 0.008$ Bonferroni corrected), and titres were significantly higher than in non-reproductive individuals during mating ($p = 0.007$, $z = -2.6$, $n = 9/5$). Thereafter, testosterone concentrations dropped off and were indistinguishable from the immature animals.

We compared body mass changes in sexually active and immature yearling males in the course of the active season. Emergence body mass did not differ between the groups (sexually active: 215.6 ± 15.0 g, $n = 5$, sexually inactive: 197.0 ± 15.7 g, $n = 3$; $p > 0.05$). We used only data of individuals that were captured within 2 days after emergence from hibernation. From the onset of mating until the end of the mating phase, sexually active males stagnated in body weight (0.6 ± 0.4 g/day), and some individuals even lost mass. In contrast, immature

males gained body mass during the mating period (4.0 ± 0.8 g/day; $p = 0.001$, $z = -3.0$, $n = 5/9$). In all males, body weight increased after mating in PM1, and then values continuously increased between PM2 and PH1 (sexually active: $p < 0.001$, $\chi^2 = 37.9$, $df = 6$, $n = 7$, sexually inactive: $p = 0.01$, $\chi^2 = 14.6$, $df = 5$, $n = 3$; post hoc, both groups pooled: M vs. PM1, PM2 vs. PM3, and PM3 vs. PH1: $p < 0.008$ Bonferroni corrected; Fig. 2a). Shortly before hibernation in PH2, the sexually inactive yearlings were significantly heavier than the sexually active individuals ($p = 0.03$, $z = -2.2$, $n = 7/3$).

Head length did not differ between the two male groups in any of the phases ($p > 0.05$) and increased in all yearling males from PM1 to PM2 and from PM2 to PM3 (sexually active: $p < 0.001$, $\chi^2 = 36.1$, $df = 6$, $n = 7$, sexually inactive: $p = 0.01$, $\chi^2 = 14.6$, $df = 5$, $n = 3$; post hoc, both groups pooled: PM1 vs. PM2, and PM2 vs. PM3: $p < 0.008$ Bonferroni corrected; Fig. 2b). When we examined the daily head growth from the beginning (March/April) until the end (August/September) of the active season, the non-reproductive males showed a significantly higher daily increase rate (0.016 ± 0.003 mm/day) than the reproductive males (0.009 ± 0.002 mm/day; $p = 0.04$, $z = -2.1$, $n = 6/4$).

The rate of aggressive interactions with males and non-agonistic interactions with females differed between precocious and non-reproductive males. In general, 77.6% of all observed male–male encounters and 83.5% of all non-agonistic interactions with females occurred during mating and PM1. The remaining interactions spread over the period from PM2 to PH2. Therefore, we confined our analyses to mating and PM1. Precocious males directed the majority (82.0%) of agonistic interactions towards sexually mature competitors and only 18.0% towards sexually inactive yearlings. All experimental males were very rarely observed to initiate intense aggression against females (sexually active: 3 chases and 3 fights, sexually inactive: 2 chases and 1 fight, over the whole active period). In the reproductive animals, the rates of initiated vs. received male–male aggression were balanced (initiated/received: M: 1.1 ± 0.5/1.6 ± 0.5, $n = 9$, $p > 0.05$; PM1: 1.2 ± 0.4/1.0 ± 0.3, $n = 9$, $p > 0.05$), while the immature individuals received more agonistic encounters than they initiated during PM1 (initiated/received: M: 1.2 ± 0.2/1.3 ± 0.4, $n = 5$, $p > 0.05$; PM1: 0.6 ± 0.3/1.4 ± 0.3, $p = 0.04$, $z = 2.0$, $n = 5$). Low-aggressive displacements of males were more frequent in the sexually inactive yearlings than in the mature ones during the mating period (Table 1). Sexually active animals launched significantly more chases and fights against consexuals than the immature males in PM1. Solely sexually active males initiated non-agonistic interactions with females. Most male–female interactions (86.7%) were nose-to-nose contacts, 9.1% were spatial cohesion, and 4.2% anogenital inspections. Although all females in the enclosures became pregnant, we were not able to observe synchronous burrow use by a male and a female indicating mating. In non-reproductive animals, no non-agonistic interactions with females were observed.

In 44.4% of the sexually active yearling males, fresh wounds were found during mating and PM1, while none of the immature males got hurt. Half of the injured males had received
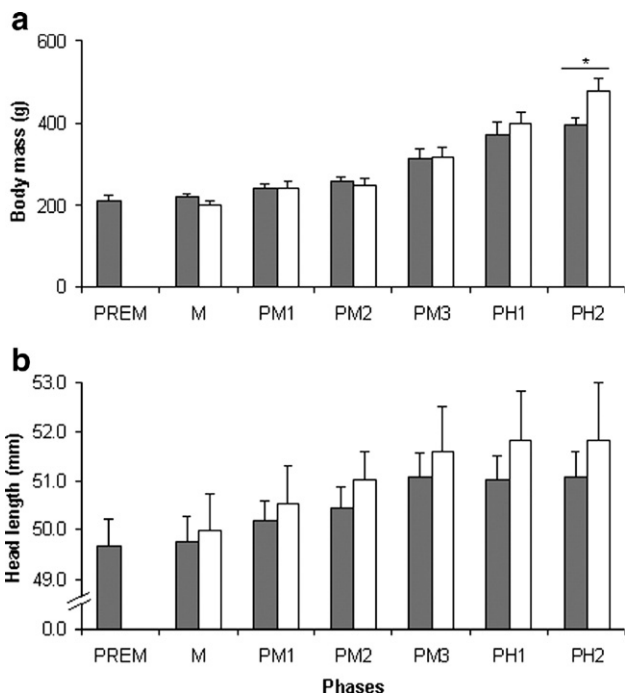

Fig. 2. (a) Body mass (g) during the seasonal phases (PREM—premating, M—mating, PM1—postmating 1, PM2—postmating 2, PM3—postmating 3, PH1—prehibernation 1, PH2—prehibernation 2) in sexually active (filled bars) and inactive (open bars) male European ground squirrels (means + SE). (b) Head length (mm) during the seasonal phases in sexually active (filled bars) and inactive (open bars) male European ground squirrels (means + SE). Number of individuals per phase: sexually active/inactive: 9/–, 9/5, 9/5, 7/5, 7/4, 7/4, 7/3. One sexually inactive male had already started hibernation in PH2. Significant group difference is marked with asterisk: *$p < 0.05$.

Table 2
Numbers of leucocytes in the blood (G/l) during the three seasonal phases mating (M), postmating 1 (PM1), and postmating 3 (PM3) in sexually active and inactive male European ground squirrels (means±SE)

Group	Cell type	M	PM1	PM3	Post hoc tests
Sexually active	Leucocytes	3.8±0.3	4.9±0.6	6.4±0.8	M vs. PM3: $p=0.018$, $z=-2.4$
	Lymphocytes	2.5±0.3	4.0±0.5	3.6±0.5	M vs. PM1: $p=0.012$, $z=-2.5$
	Segmented neutrophils	1.2±0.1	0.4±0.1	1.8±0.7	M vs. PM1: $p=0.011$, $z=-2.5$
					PM1 vs. PM3: $p=0.018$, $z=-2.4$
Sexually inactive	Leucocytes	3.7±0.4	4.5±0.7	5.2±0.2	n.s.
	Lymphocytes	2.7±0.3	3.8±0.8	3.5±0.3	n.s.
	Segmented neutrophils	0.9±0.2	0.3±0.1	1.0±0.2	n.s.

In the sexually active males, cell numbers changed significantly between the phases, while in the sexually inactive males, no significant changes occurred. The statistical values of the significant post hoc tests are included. Number of individuals per phase: sexually active/inactive: 8/4, 9/5, 7/4.

small, superficial wounds at all parts of the skin, whereas the other half had sustained more severe, suppurating lesions at the hindfeet. All injuries were presumably bites from competitors received during fights and chases.

The two male groups did not differ in fecal cortisol metabolites (FCM) levels ($p>0.05$; Fig. 1b). In both groups, FCM concentrations changed over the study period, with significantly elevated levels in the second half of the active season starting in PM3 (sexually active: $p<0.001$, $\chi^2=29.5$, $df=6$, $n=7$, sexually inactive: $p=0.02$, $\chi^2=13.1$, $df=5$, $n=3$; post hoc, both groups pooled: PM2 vs. PM3: $p<0.008$ Bonferroni corrected).

The numbers of circulating leucocytes were counted during mating, at the onset of testis regression in PM1, and after completion of testis regression in PM3. In the sexually active males, cell numbers changed significantly (Table 2). Overall leucocytes and lymphocytes were lowest during mating and increased thereafter (leucocytes: $p=0.03$, $\chi^2=7.0$, $df=2$, $n=6$, lymphocytes: $p=0.04$, $\chi^2=6.3$, $df=2$, $n=6$; post hoc, leucocytes: M vs. PM1: $p>0.025$, M vs. PM3: $p<0.025$; lymphocytes: M vs. PM1: $p<0.025$, M vs. PM3: $p>0.025$ Bonferroni corrected). The segmented neutrophils were elevated during mating, decreased significantly in PM1, and increased again in PM3 ($p=0.01$, $\chi^2=9.0$, $df=2$, $n=6$; post hoc: M vs. PM1, and PM1 vs. PM3: $p<0.025$ Bonferroni corrected). In the sexually inactive males, cell changes showed a similar pattern, but no significant changes in the numbers of leucocytes, lymphocytes, and segmented neutrophils occurred between the phases ($p>0.05$).

Discussion

In our study, sexually active males hibernated for a shorter period and, accordingly, started the active period earlier than the non-reproductive males. This is a usual pattern in ground squirrels and has been reported for *S. lateralis* (Barnes, 1984), *S. tridecemlineatus* (Schwagmeyer and Brown, 1983), and free-living *S. citellus* (Millesi et al., 1998). Reproductively active males appeared above ground before female emergence. An early emergence from hibernation could increase the reproductive potential because males have time to recuperate from hibernation and increase their chances of encountering sexually receptive females.

Unsurprisingly, testosterone levels were high in mature males at the beginning of surface activity and during mating, and decreased significantly thereafter. In non-reproductive yearling males, androgen levels remained baseline throughout the active season. Similar results were found in free-ranging animals (Millesi et al., 1998).

Emergence body mass did not differ significantly between sexually active and inactive yearling males. Additionally, immergence mass as juveniles was similar in future reproductive and non-reproductive animals (Strauss, unpublished data). The timing of puberty was therefore not mass-dependent in the studied individuals. At high population densities of free-living European ground squirrels, yearling males had to attain a threshold body mass of 220 g to become sexually mature, while in the low-density situation, even yearlings with relatively low body mass were reproductively active (Millesi et al., 1999, 2004). In our study animals, males of both groups were below as well as above this threshold value. In the mating period, immature males were able to gain body mass, whereas reproductive animals stagnated or even lost mass. In the field, body mass loss of about 4 g/day has been reported in mature males during mating (Millesi et al., 2004). Participating in mating has proved to be costly in terms of increased locomotory activity, high rates of intrasexual aggression, and reduced time for foraging, resulting in body mass loss (Millesi et al., 1998). Non-reproductive males could forgo these energetic costs and improve body condition during the mating period.

Even under optimal conditions in enclosures, where animals were provided with additional food, sexual maturation processes affected growth patterns. Absolute head lengths did not differ between the two groups, but daily growth rates were higher in the non-reproductive yearlings. Structural growth is limited in the European ground squirrel: optimal diet composition resulted in faster growth but not in absolutely larger individuals at the end of the active season (Strauss et al., in press). Accelerated growth may save valuable time for other physiological processes like prehibernatory fattening. In free-living European ground squirrels, non-reproductive males had significantly larger head lengths at the end of the season than the precocious individuals (Millesi et al., 2004). Immature male Golden-mantled ground squirrels can allocate four times as much energy to growth as mature individuals, and their total annual energy turnover is only 91% that of reproductive males (Kenagy et al., 1989). Delayed

puberty and the greater energy allocation to growth might represent an investment in future reproductive potential. Prehibernatory body mass was higher in the immature vs. reproductive males. In the field as well, sexually inactive yearlings entered hibernation with a significantly higher body mass (Millesi et al., 2004). In our study, structural size did not differ between the two groups at the end of the active season, although immature males showed higher daily growth rates; therefore, non-reproductive males were probably able to invest the saved time in the accumulation of body fat. As overwinter mass loss is known to be relatively constant (Millesi et al., 1999), delayed puberty might yield a better body condition upon emergence from the second hibernation and participation in breeding. Additionally, Murie and Boag (1984) found that heavier Columbian ground squirrels had higher overwinter survival rates.

We were able to recapture 6 of the former sexually active yearlings and 2 of the inactive group at emergence from their second hibernation. We have indications that also some of the remaining experimental animals have survived hibernation, but emerged from the hibernacula outside of the enclosure and dispersed immediately after emergence. As expected, all recaptured males had developed testes as 2-year olds and participated in mating. Mean emergence mass was higher in the former sexually inactive males (302.5 ± 75.5 g, $n=2$) than in the precocious group (279.8 ± 17.3 g, $n=6$), however, sample size was too small for statistical comparisons. In the field, intrasexual competition increased with increasing population density (Millesi et al., 2004). Therefore, an appropriate body condition may be a prerequisite to acquire a mate at high population densities, and males that delay puberty and instead invest more energy in growth and prehibernatory fattening may have an elevated reproductive fitness. In low-density situations, however, male survival was decreased (Hoffmann et al., 2003), and selection should favor precocious males, as the probability to survive to the next spring might be low.

Consistent with field data (Millesi et al., 1998), male–male aggression peaked during mating and the following 4 weeks and dropped off thereafter. Sexually active males directed most attacks towards mature competitors and only a few against non-reproductive yearlings. Reproductive males initiated more fights and chases than immature yearlings. The strong intensity of aggression in reproductive yearlings was reflected by the occurrence of injuries. In 44.4% of precocious yearlings, fresh wounds were found during mating and the 4 weeks thereafter, while none of the immature males received injuries. The high testosterone levels in sexually active males may play a role in the intensity of aggression. Intraspecific aggressiveness is known to be enhanced by elevated testosterone concentrations (Giammanco et al., 2005). In European ground squirrels, male behavior changed and intrasexual conflict rates increased in testosterone-primed males in the presence of female stimuli (Millesi et al., 2002). In non-reproductive males, the observed agonistic interactions were mainly spatial displacements. Immature males received more aggression than they initiated and seemed to refrain from severe intrasexual conflicts.

Apart from intrasexual aggression, mating effort involved locating and checking as many estrous females as possible. In free-ranging males, locomotory activity and home-range size increased significantly in the mating period (Millesi et al., 1998). Additionally, non-agonistic interactions between males and females preceded the mating event (Millesi et al., 1998). In our study, only males with an activated reproductive system reacted to female stimuli. Non-agonistic interactions with females were frequent in the precocious males during mating and the 4 weeks thereafter. Immature males did not appear to be interested in females.

One may suppose that mating effort including intense intrasexual aggression leads to stress, indicated by increased adrenocortical activity and glucocorticoid secretion. However, FCM concentrations did not differ between sexually active and immature yearlings and did not increase in either group during mating. In all males, levels were elevated during summer. Interestingly, the increase coincided with the emergence of the juveniles. Litter emergence might affect male adrenocortical activity, as individual density in the enclosures increased abruptly. It has been reported previously in S. citellus (Shivatcheva et al., 1988), S. lateralis (Nunes et al., 2006), S. saturatus, and S. beldingi (Boswell et al., 1994) that glucocorticoid levels increased prior to prehibernatory fattening and were elevated at the end of the season. This seasonal pattern supports a role of cortisol metabolites in regulating body mass changes. In rats, glucocorticoids appeared to mediate weight gain by modulating hypothalamic control of food intake (Green et al., 1992).

In reproductive yearling males, the numbers of circulating leucocytes and lymphocytes were relatively low during mating and increased significantly thereafter, while in immature males, no significant cell changes occurred. Similar results have been found in Arctic ground squirrels: breeding males were in worse immunological condition than non-reproductive males (Boonstra et al., 2001). Immune function might be depressed by elevated testosterone titres during breeding. In response to such elevated levels, leucocytes probably exit the peripheral blood circulation and enter lymph nodes, skin, and other tissues, where they are well positioned to combat challenges from new injuries (Braude et al., 1999). In the field, injuries were common during mating in mature male European ground squirrels (Millesi et al., 1998); our study also revealed wounds in about half the precocious yearlings. The numbers of segmented neutrophils were elevated in the precocious males during mating and after emergence of the juveniles. Neutrophils form a "first line of defence" against infections and are released in response to stress and social conflicts (Gryazeva et al., 2001). Mating is a stressful period for sexually active males, with intense aggression and potential injuries. At litter emergence, intrasexual conflicts had fallen off, but rising individual density may also represent a stressor because FCM concentrations increased after juvenile emergence.

In conclusion, early puberty in male European ground squirrels appeared to lead to intense intrasexual aggression involving injuries, decelerated growth rates, lower prehibernatory body mass, and probably worse immunological condition during mating. Non-reproductive yearling males were able to dedicate

one complete active season to growth and preparation for hibernation, which may result in improved body condition at spring emergence and higher survival rates.

Acknowledgments

References

Bachman, G.C., 2000. Changes in leukocyte counts in hibernators from spring through summer. Am. Zool. 40, 933.

Bachman, G.C., 2003. Food supplements modulate changes in leucocyte numbers in breeding male ground squirrels. J. Exp. Biol. 206, 2373–2380.

Barnes, B.M., 1984. Influence of energy stores on activation of reproductive function in male Golden-mantled ground squirrels. J. Comp. Physiol., B 154, 421–425.

Boonstra, R., McColl, C.J., 2000. Contrasting stress response of male Arctic ground squirrels and red squirrels. J. Exp. Zool. 286, 390–404.

Boonstra, R., McColl, C.J., Karels, T.J., 2001. Reproduction at all costs: the adaptive stress response of male Arctic ground squirrels. Ecology 82, 1930–1946.

Boswell, T., Woods, S.C., Kenagy, G.J., 1994. Seasonal changes in body mass, insulin and glucocorticoids of free-living Golden-mantled ground squirrels. Gen. Comp. Endocrinol. 96, 339–346.

Braude, S., Tang-Martinez, Z., Taylor, G.T., 1999. Stress, testosterone, and the immunoredistribution hypothesis. Behav. Ecol. 10, 345–350.

Buck, C.L., Barnes, B.M., 2003. Androgen in free-living Arctic ground squirrels: seasonal changes and influence of staged male–male aggressive encounters. Horm. Behav. 43, 318–326.

Bushberg, D.M., Holmes, W.G., 1985. Sexual maturation in male Belding's ground squirrels: influence of body weight. Biol. Reprod. 33, 302–308.

Daan, S., Tinbergen, J.M., 1997. Adaptation of life histories. In: Krebs, J.R., Davies, N.B. (Eds.), Behavioural Ecology: An Evolutionary Approach. Blackwell Science Ltd., Oxford, pp. 311–333.

Dark, J., Ruby, N.F., Wade, G.N., Licht, P., Zucker, I., 1992. Accelerated reproductive development in juvenile male ground squirrels fed a high-fat diet. Am. J. Physiol. 262, R644–R650.

Giammanco, M., Tabacchi, G., Giammanco, S., DiMajo, D., LaGuardia, M., 2005. Testosterone and aggressiveness. Med. Sci. Monit. 11, RA136–RA145.

Green, P.K., Wilkinson, C.W., Woods, S.C., 1992. Intraventricular corticosterone increases the rate of body weight gain in underweight adrenalectomized rats. Endocrinology 130, 269–275.

Gryazeva, N.I., Shurlygina, A.V., Verbitskaya, L.V., Mel'nikova, E.V., Kudryavtseva, N.N., Trufakin, V.A., 2001. Changes in various measures of immune status in mice subject to chronic social conflict. Neurosci. Behav. Physiol. 31, 75–81.

Hoffmann, I.E., Millesi, E., Everts, L.G., Huber, S., Dittami, J.P., 2003. Population dynamics of European ground squirrels (*Spermophilus citellus*) in a suburban area. J. Mammal. 84, 615–626.

Holmes, W.G., 1988. Body fat influences sexual maturation in captive male Belding's ground squirrels. Can. J. Zool. 66, 1620–1625.

Kenagy, G.J., Sharbaugh, S.M., Nagy, K.A., 1989. Annual cycle of energy and time expenditure in a Golden-mantled ground squirrel population. Oecologia 78, 269–282.

McLean, I.G., Towns, A.J., 1981. Differences in weight changes and the annual cycle of male and female Arctic ground squirrels. Arctic 34, 249–254.

Millesi, E., Huber, S., Dittami, J.P., Hoffmann, I.E., Daan, S., 1998. Parameters of mating effort and success in male European ground squirrels, *Spermophilus citellus*. Ethology 104, 298–313.

Millesi, E., Strijkstra, A.M., Hoffmann, I.E., Dittami, J.P., Daan, S., 1999. Sex and age differences in mass, morphology, and annual cycle in European ground squirrels, *Spermophilus citellus*. J. Mammal. 80, 218–231.

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. Horm. Behav. 41, 51–58.

Millesi, E., Hoffmann, I.E., Huber, S., 2004. Reproductive strategies of male European sousliks at high and low population density. Lutra 47, 75–84.

Möstl, E., Maggs, J.L., Schrötter, G., Besenfelder, U., Palme, R., 2002. Measurement of cortisol metabolites in faeces of ruminants. Vet. Res. Comm. 26, 127–139.

Murie, J.O., Boag, D.A., 1984. The relationship of body weight to overwinter survival in Columbian ground squirrels. J. Mammal. 65, 688–690.

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. Gen. Comp. Endocrinol. 146, 136–143.

Palme, R., 2005. Measuring fecal steroids: guidelines for practical application. Ann. N.Y. Acad. Sci. 1046, 75–80.

Palme, R., Möstl, E., 1994. Biotin-streptavidin enzyme immunoassay for the determination of oestrogens and androgens in boar faeces. In: Görög, S. (Ed.), Advances in Steroid Analysis '93. Akadémiai Kiadó, Budapest, pp. 111–117.

Schwagmeyer, P.L., Brown, C.H., 1983. Factors affecting male–male competition in thirteen-lined ground squirrels. Behav. Ecol. Sociobiol. 13, 1–6.

Shivatcheva, T.M., Ankov, V.K., Hadjioloff, A.I., 1988. Circannual fluctuations of the serum cortisol in the European ground squirrel, *Citellus citellus* L. Comp. Biochem. Physiol., A 90, 515–518.

Slade, N.A., Balph, D.F., 1974. Population ecology of Uinta ground squirrels. Ecology 55, 989–1003.

Strauss, A., Hoffmann, I.E., Millesi E., in press. Effects of nutritional factors on juvenile development in male European ground squirrels (*Spermophilus citellus*). Mamm. Biol. doi:10.1016/j.mambio.2006.09.003.

Touma, C., Palme, R., 2005. Measuring fecal glucocorticoid metabolites in mammals and birds: the importance of validation. Ann. N.Y. Acad. Sci. 1046, 54–74.