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Research Article

Stress load in European ground squirrels living in habitats with high and low human impact

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Abstract

Anthropogenic land use and its after-effects are potential sources of stress for European ground squirrel (*Spermophilus citellus*) populations, which increasingly have to cope with human impact throughout the species' range. To determine whether habitat alteration impacts the stress load of free-ranging populations in Austria, we live-trapped and faecal-sampled individuals both in a nearly unaltered steppe habitat (TD) and in a strongly altered alfalfa meadow (FB). Overall and seasonal faecal cortisol metabolite (FCM) concentrations were analysed and compared between the two study sites. FCM levels of adult males and of juveniles of both sexes were higher at FB than at TD. Adult females showed no such differences, but exhibited increased faecal progesterone metabolites (FPM) levels at both sites during June. Our results indicate that human activities affected stress load in adult males and juveniles. The altered vegetation led to highly abundant food at FB and, together with the isolation of the habitat, caused a high population density. This apparently intensified social stress in certain periods of the active season. Elevated FCM levels in both adult males and juveniles at FB coincided with the period of highest population density, when all juveniles had emerged from the natal burrows, and hibernation had not started yet. At the same time, predation pressure and human recreational activities also peaked. The highest FCM levels were found in juveniles at FB shortly after natal emergence, suggesting that this age class is most vulnerable to social stress, predation and human disturbance. The lack of a measurable stress response in adult females may be due to increased progesterone concentrations attenuating the stress-induced elevation of glucocorticoids.

Keywords: *Spermophilus citellus*, behavioural ecology, anthropogenic influence, faecal samples, corticosteroids, progesterone.

Introduction

Human land use and the resulting alteration and fragmentation of natural habitat belong to the greatest threats to wildlife (e.g., Wilson 1985, Ehrlich and Wilson 1991, Soulé 1991, Dirzo *et al.* 2014). One example of a species increasingly under pressure from human impact is the European ground squirrel (*Spermophilus citellus*), a small diurnal ground-dwelling sciurid endemic to central and south-eastern Europe (Ružic 1978, Kryštufek 1999). Since the mid-20th century, the species has declined throughout its geographic range due to habitat alteration, fragmentation, and destruction caused by the spread of intensive agriculture and urbanisation (Smit and van Wijngaarden 1981). Meanwhile, *S. citellus* is listed as "Vulnerable" by the International Union for Conservation of Nsature and Natural Resources (Coroiu *et al.* 2008). In Austria, population relics occur both in the species' primary habitat (short-grass steppe and dry grassland; Kryštufek 1999, Spitzenberger and Bauer 2001) and in human-shaped landscapes (recreational sites, sports fields, runways, and other frequently mown lawns; Spitzenberger

and Bauer 2001). Details on the species' life history appear elsewhere (Millesi et al. 1998, Huber *et al.* 1999, Millesi *et al.* 1999a, 1999b, Huber *et al.* 2001, Millesi *et al.* 2008a, 2008b, Strauss *et al.* 2009). Previous research has shown that European ground squirrels in human-altered habitats may achieve exceptionally high population densities, often exceeding those in unaltered habitats (Hoffmann *et al.* 2003a, 2003b). Anthropogenic land use seems to affect population dynamics, life-history traits, and movement behaviour of *S. citellus* (Hoffmann *et al.* 2003b, Hoffmann et al. 2008, Turrini *et al.* 2008).

To develop efficient conservation strategies, it is crucial to gain a better understanding of the physiological effects of human land use (Wikelski and Cooke 2006). Steroid hormones including glucocorticoids and gestagens are essential in regulating an animal's health and reproductive success, and glucocorticoid concentrations are considered to be reliable indicators of stress (Möstl and Palme 2002). High glucocorticoid levels triggered by prolonged stress affect fitness by decreasing memory and learning capacity as well as fertility and immune system (reviewed in Sapolsky *et al*. 2000). Thus, an elevated stress load of animals living in altered habitats may affect population survival. Progesterone, on the other hand, plays a key role in female reproduction by regulating ovulation, implantation, gestation, parturition, and lactation (Gellersen *et al.* 2009).

To determine possible effects on stress load, we compared glucocorticoid levels of two free-ranging *S. citellus* populations facing antipodal degrees of anthropogenic influence over the course of the active season. In addition, we analysed progesterone levels of adult and juvenile females, and discuss potential interactions with glucocorticoid levels, habitat alteration, and phases of the annual cycle.

Material and methods

Study area

This study was conducted during the active season from late March to mid-August 2008 on two free-living *S. citellus* populations in eastern Austria in frame of a research project. The four-year project (2006-2009) focused on five locally distinct ground squirrel habitats exposed to varying degrees of anthropogenic influence. Our criteria for a high degree of human impact included a strongly altered vegetation, substantial isolation, and frequent direct disturbance through human recreational or management activities. In contrast, low human impact was defined by a nearly natural vegetation, little isolation of the respective habitat patch, and hardly any direct human disturbance. For the purpose of this study, we selected the two habitats with the apparently highest and lowest human impact.

The strongly altered habitat patch, 'Falkenbergwiese' (hereafter FB), was an isolated 5-ha meadow in the north of Vienna (48°18'N, 16°22'E; elevation 318 m). The native dry grassland vegetation had been altered when alfalfa (*Medicago sativa*) was sowed several years before the study. The landscape adjacent to the study area consisted of a mixed oak forest (N), a huge conventionally cultivated vineyard (E), a transmitting station including buildings and transmission masts (S), and an intensively managed arable field (W). The study population was isolated from the next nearest ground-squirrel colony by the vineyard in the east. The study site belongs to an area of excursions, with daily presence of people running their dogs, hikers, bikers, and picnickers. Ground squirrels were sampled on a focal area of about 1 ha size.

The near-natural study site, 'Trausdorfer Hutweide' (hereafter TD) was a continuous steppe-like habitat measuring almost 100 ha, located west of lake Neusiedl (Burgenland; 47°48'N, 16°33'E; elevation 164m). In the decades preceding the study, the area had been used for grazing and had also served as a grassy airport. Since 1997, the site has been a protected area, with human activities limited to occasional sheep grazing and mowing it once

annually. The surroundings of the study site consisted of vineyards and small fields with dust roads, constituting small-scale agricultural land use that provided connectivity to adjacent ground-squirrel populations. Except for occasional cars, equestrians and walkers, the area was undisturbed by humans. Ground squirrels were sampled on a focal area of approximately 7 ha.

Live-trapping

Ground squirrels were captured by placing baited Tomahawk live-traps near burrows or by inserting tube traps into burrow entrances. Trapping was restricted to the animals' main activity period (10:00-15:30). Traps were observed continuously with binoculars, and a captured ground squirrel was immediately released into a funnel-shaped handling bag. Detailed descriptions of capture technique and protocol appear in Huber *et al.* (1999), Millesi *et al.* (1999b) and Hoffmann *et al.* (2008).

The following age classes were established based on weight and data from previous years: juveniles (born during the study period) and adults, comprising yearlings (born in the year before the study) and older individuals (born at least 2 years before the study). Reproductive condition of adult individuals was noted at each capture (males: testis position and scrotal pigmentation, females: condition of vulva and nipples). Body weight was measured to the nearest 1 g with a kitchen scale. Extent of moult was determined by plucking hairs. Animals were released at the locations of their capture immediately after the data recording procedure.

Seasonal phases

Based on the seasonal activities of the different age and sex classes, we defined the following phases for each individual (means ±SE are given in parentheses).

Adult males (Fig. 1a)

Five seasonal phases were identified by documenting testis position, scrotal pigmentation, moult, weight and behaviour (Millesi *et al.* 1998, Strauss *et al.* 2007): (i) Mating (M; March 27 ± 0.7 d – April 17 ± 2.8 d) began with the vernal emergence of the first receptive females. Males' testes were descended and large, and the scrotum was darkly pigmented. One captured male had abdominal testes during this phase and hence was considered non-reproductive. The mating period lasted until all females were pregnant. (ii) Postmating 1 (PM1; April 18 ± 2.8 d – April 30 ± 0.7 d) was the period between the onset of testes regression until it was completed. (iii) Postmating 2 (PM2; May 01 ± 0.7 d – May 29 ± 9.9 d) included the first moult of the year and ended with the emergence of the first juveniles from the natal burrows. Although males' testes were abdominal, but they still had a pigmented scrotum. (iv) Postmating 3 (PM3; May 30 ± 9.9 d – June 25 ± 2.8 d) started when all litters had emerged aboveground and ended when the males' scrotum lost its pigmentation. (v) Prehibernation (PH; June 26 ± 2.8 d – August 13 ± 1.4 d) included the second moult and prehibernatory fattening.

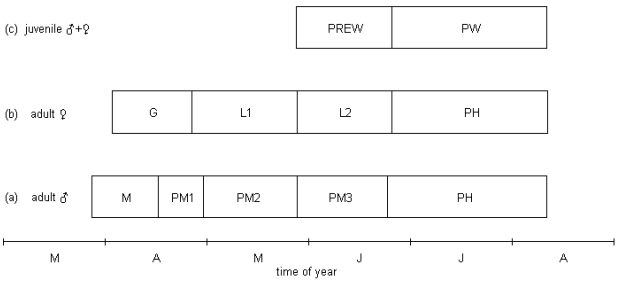

Figure 1. Timing of the seasonal phases in (a) adult males, (b) adult females, and (c) juveniles. Vertical lines indicate mean onset of each phase. See main text for phase definitions.

Adult females (Fig. 1b)

The season was divided into four phases identified by vulval and teat development as well as weight changes (Huber *et al.* 1999): (i) Gestation (G; April 2 ± 7.8 d – April 27 ± 3.5 d) was the phase in which females had a swollen and open vulva as well as darkly pigmented nipples. Females rapidly gained weight until a sudden loss of body weight, indicating parturition. (ii) Lactation 1 (L1; April 28 ± 3.5 d – May 29 ± 9.9 d) lasted from parturition until the emergence of a female's

litter from the natal burrow. Lactating females showed enlarged and light-coloured teats. (iii) Lactation 2 (L2; May 30 ± 9.9 d – June 26 ± 2.8 d) started with the emergence of a female's litter from the natal burrow and lasted until weaning. (iv) Prehibernation (PH; June 27 ± 2.8 d – August 13 ± 1.4 d) was the phase after weaning until hibernation. Teats were regressed and became dark. Prehibernation fattening occurred as reflected in a pronounced mass increase.

Juveniles (Fig. 1c)

Two phases (before and after weaning) were defined. As juveniles attain a threshold weight (females: 140 g, males: 146 g) before they are weaned (Hoffmann unpublished data), phases were distinguished based on body weight: (i) Preweaning (PREW; May 30 ± 1.4 d – June 26 ± 9.9 d) lasted from natal emergence when juveniles began to forage aboveground, but were still dependent on their mother. (ii) Postweaning (PW; June 27 ± 9.9 d – August 13 ± 1.4 d) was the phase after the juveniles had been weaned until the end of the study in mid-August. During this period the juveniles dispersed from their natal burrow to establish their own burrows for hibernation.

Faecal sampling

Analyses of faecal cortisol metabolites (FCM) were used to assess glucocorticoid levels. Ground squirrels usually defecate at capture, thus, collection of faeces is a non-invasive sampling method avoiding effects of handling-related stress. Furthermore, cortisol metabolites in faeces are good indicators of circulating plasma cortisol concentrations (Mateo and Cavigelli 2005, Sheriff *et al.* 2010), and also represent pooled amounts of plasma concentrations over a certain period of time, providing an integrated measure of adrenocortical activity (Goymann *et al.* 1999). Ovarian activity in females was monitored by measuring faecal progesterone metabolites (FPM).

Fresh faeces were collected directly after excretion, immediately put in a cool box and subsequently stored at -20°C. Before analysis, samples were dried (60°C for 24 h), then pulverized. To extract metabolites from faeces, 0.1g of dry faeces was suspended in 80% methanol and then centrifuged. Hereafter, all faecal hormone metabolite concentrations are expressed in ng per g of dry weight, which has been shown to be the most robust measure with respect to dietary effects on excretion (Wasser *et al.* 1993).

Cortisol assay

FCM levels were determined by an 11-oxoetiocholanolone-enzyme immunoassay (EIA), measuring 3alpha,11-oxo-cortisol metabolites. This EIA had been experimentally validated for *S. citellus*, revealing a delay time between hormone secretion and faecal excretion of 7.5 ± 2.5 h (mean ±SD; Strauss *et al.* 2007). FCM concentrations were assayed in duplicate. Intra- and inter-assay coefficients of variation were 14.79% and 16.73%, respectively.

Progesterone assay

Progesterone metabolites were measured using a biotin-streptavidin EIA (Palme and Möstl 1994). The intra-assay coefficient of variation was 11.70%, the inter-assay coefficient 15.54%. For validation, we analysed progesterone concentrations in plasma and faecal samples taken from individual European ground squirrels at the same day and time in previous studies. Faecal progesterone concentrations in faeces and plasma were highly correlated ($r = 0.87$, $n = 65$, $p < 0.0001$; Strauss *et al.* unpublished data).

Statistical analysis

Four data sets were established for statistical analyses: (1) adult males (response variable: FCM), (2) adult females (FCM, FPM), (3) juveniles (FCM), and (4) juvenile females (FPM). As Shapiro-Wilk tests revealed that data were not normally distributed, FCM and FPM values were transformed using logarithms and square roots, respectively. In each data set, variation of faecal steroid metabolites was

analysed with linear mixed effects (LME) models with site, phase, and age (dataset 1 and 2), site, phase, and sex (dataset 3), and site and phase (dataset 4) as fixed main effects in the model. ID was entered as a random effect because we had repeated measures on the same individuals.

Akaike's information criterion (AIC) was used to choose the best models, starting with all main effects and all 2-way and 3-way interactions between main effects. Only the main effects and the 2-way interaction site × phase were included in the final models. Components were estimated with the restricted maximum likelihood model procedure. ANOVAs from LME models were computed using marginal (Type III) sums of squares.

In case of significant main effects with more than two levels, post-hoc pairwise comparisons were performed, using Bonferroni correction to make adjustments to the confidence interval. For paired comparisons for a significant site × phase interaction, a single factor was created from the interaction. This factor was tested by running a single factor ANOVA, using a LME model and Bonferroni post-hoc tests.

Hereafter, all data are expressed as back-transformed estimated marginal means +SE unless stated otherwise. All p-values are two tailed. The level of statistical significance was set at $\alpha = 0.05$. Data were analysed using SPSS software (SPSS for Windows, release 17.0, SPSS, Chicago, IL).

Results

A total of 220 fecal samples from 109 individuals was collected throughout the study period (Table 1). Sex ratio was balanced among age classes at both study sites. At TD, more individuals were captured in total, but fewer were recaptured than at FB (Table 1).

Adult males

Faecal cortisol metabolite levels were similar among yearling and older males, which hence where pooled for analyses. Adult males had significantly higher FCM levels at FB than at TD (Table 2, Fig. 2), and their FCM levels changed significantly in the course of the active season (Table 2, Fig. 3).

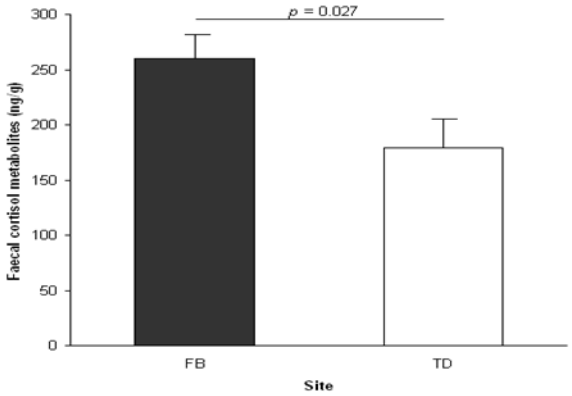

Figure 2. Levels of faecal cortisol metabolites in adult male *S. citellus* at the two study sites (FB: n = 15, TD: n = 18). Bars represent back-transformed estimated marginal means +SE; analysis was based on square-root transformed data.

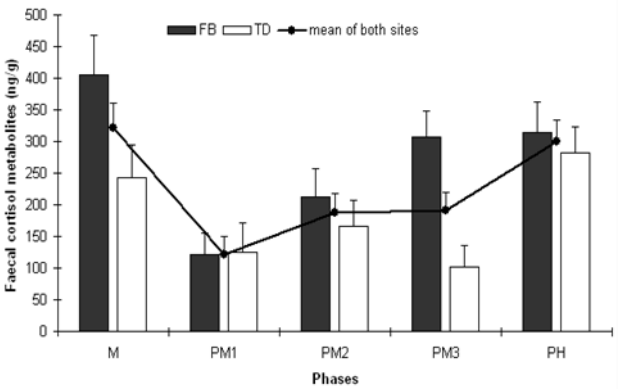

Figure 3. Faecal cortisol metabolite concentrations during the seasonal phases in adult male *S. citellus* at the two study sites (M: n = 4/6, PM1: n = 7/3, PM2: n = 6/6, PM3: n = 8/6, PH: n = 7/7; see main text for phase definitions). Filled circles indicate mean of both sites in the respective phase. Results are given as back-transformed estimated marginal means +SE; analysis was based on square-root transformed data. See Table 2 for ANOVA results and main text for post-hoc results.

Post-hoc tests revealed that FCM levels peaked in the mating period (320.66 + 40.87 ng/g), followed by a significant decrease in PM1 ($p<0.001$). Compared to mating, FCM levels remained low in PM2 ($p = 0.056$) and PM3 ($p = 0.042$) until PH, when they were significantly higher than in PM1 ($p < 0.001$), almost reaching values during mating.

Furthermore, there was a significant interaction between site and phase (Table 2, Fig. 3), indicating site-dependent seasonal patterns in FCM levels. Post-hoc analyses showed that within sites, compared with PM1 adult FB males exhibited elevated FCM concentrations in M ($p < 0.001$), in PM3 ($p = 0.011$), and in PH ($p = 0.024$), whereas FCM levels of adult TD males significantly increased from PM3 to PH ($p = 0.025$). Within phases, PM3 values of FB males were three times higher than those of TD males ($p = 0.012$).

Adult females

Table 1. Cumulative number of *S. citellus* trapped and sampled at the two study sites (numbers of recaptured individuals in parentheses). Recapture rate refers to percentage of individuals recaptured at least once.

	Adult		Juvenile		Total	Recapture rate (%)
	Male	Female	Male	Female		
FB (~1ha)	15 (12)	13 (8)	8 (7)	10 (8)	46 (35)	76
TD (~7ha)	18 (9)	19 (7)	14 (9)	12 (6)	63 (31)	49

Table 2. Results of ANOVAs (Type III) from LME models examining effects of different predictor variables on variation in faecal cortisol metabolites in *S. citellus*, with ID as random variable for each data set.

Data set	Fixed effects		ANOVA
Adult males	Main effects	Site	$F_{1,29} = 5.41$, $p = 0.027$
		Phase	$F_{4,69} = 7.30$, $p < 0.001$
		Age	$F_{1,33} = 0.11$, $p = 0.743$
	Interaction effect	Site × Phase	$F_{4,69} = 2.73$, $p = 0.036$
Adult females	Main effects	Site	$F_{1,25} = 0.07$, $p = 0.793$
		Phase	$F_{3,48} = 0.18$, $p = 0.907$
		Age	$F_{1,22} = 0.07$, $p = 0.798$
	Interaction effect	Site × Phase	$F_{3,48} = 0.32$, $p = 0.809$
Juveniles	Main effects	Site	$F_{1,34} = 8.14$, $p = 0.007$
		Phase	$F_{1,74} = 4.25$, $p = 0.043$
		Sex	$F_{1,31} = 1.52$, $p = 0.227$
	Interaction effect	Site × Phase	$F_{1,74} = 10.98$, $p = 0.001$

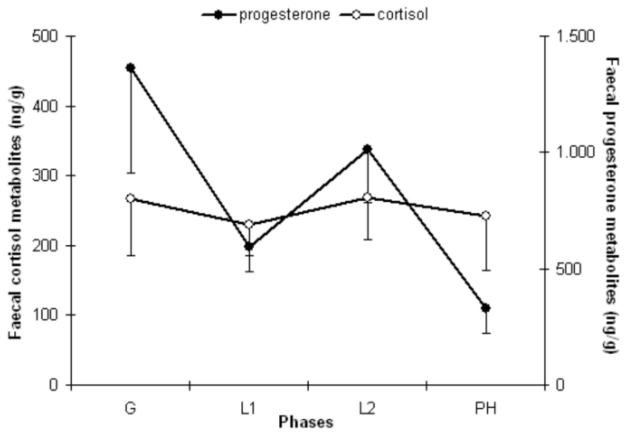

Figure 4. Faecal cortisol (open circles) and progesterone (closed circles) metabolite concentrations during gestation (G, n = 8), lactation 1 (L1, n = 16), lactation 2 (L2, n = 11), and prehibernation (PH, n = 7) in adult female *S. citellus*. Each data point represents the back-transformed estimated marginal mean -SE of the respective phase; analyses were based on log-transformed data. See Tables 2 and 3 for ANOVA results and main text for post-hoc results.

There was no difference in faecal cortisol metabolite levels between yearling and older females (Table 2). Faecal progesterone metabolite levels were only marginally, but not significantly higher in yearlings than in older individuals (Table 3), which hence were pooled for analyses. Neither site nor seasonal phases had significant effects on FCM levels (Table 2). While FPM levels were also similar between sites, they differed significantly among phases (Table 3, Fig. 4). Post-hoc tests showed elevated FPM concentrations both during G and L2 with significantly lower values during PH (Bonferroni pairwise comparisons: G vs. PH: p = 0.006; L2 vs. PH: p = 0.015).

Juveniles

While there was no sex-related difference in faecal cortisol metabolite concentrations, juvenile FCM levels differed significantly between sites (Table 2) throughout the study period, with juveniles at FB having higher FCM concentrations than at TD. Furthermore, FCM values differed significantly among phases (Table 2), with elevated levels in preweaning compared to postweaning. The LME model also revealed a significant site × phase interaction (Table 2). Post-hoc tests performed on this interaction showed that FCM levels of FB juveniles in the preweaning phase were significantly higher than those of all other site × phase-categories (Bonferroni pairwise comparisons: FB PREW vs. TD PREW: p = 0.001; FB PREW vs. FB PW: p = 0.002; FB PREW vs. TD PW: p = 0.024; Fig. 5). Hence, the significant main effects of site and phase reflected elevated FCM levels of FB juveniles after natal emergence, whereas the levels after weaning were similar at both sites.

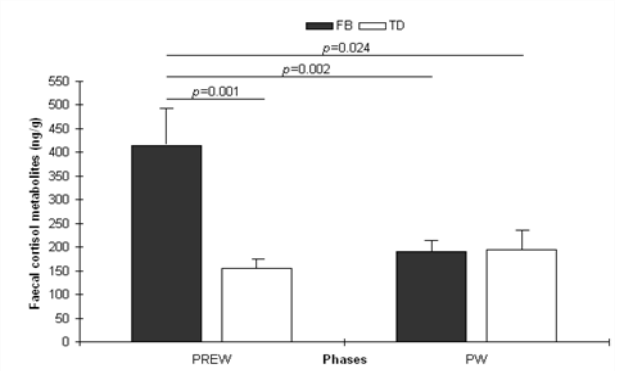

Figure 5. Faecal cortisol metabolites during preweaning (n = 8/15) and postweaning (n = 16/13) in juvenile *S. citellus* at the two study sites. Results are given as back-transformed estimated marginal means +SE; analysis was based on log-transformed data. P-values of post-hoc pairwise comparisons are given for each site × phase-category as compared to the FCM levels of FB juveniles during the preweaning phase.

Table 3. Results of ANOVAs (Type III) from LME models examining effects of different predictor variables on variation in faecal progesterone metabolites in female *S. citellus*, with ID used as random variable for each data set.

Data set	Fixed effects		ANOVA
Adult females	Main effects	Site	$F_{1,24}$ =1.03, p= 0.321
		Phase	$F_{3,48}$ = 5.49, p= 0.003
		Age	$F_{1,17}$ = 3.19, p= 0.092
	Interaction effect	Site × Phase	$F_{3,48}$ = 1.00, p= 0.400
Juvenile females	Main effects	Site	$F_{1,16}$ = 4.95, p= 0.041

		Phase	$F_{1,24} = 1.04$, $p = 0.317$
	Interaction effect	Site × Phase	$F_{1,24} = 0.74$, $p = 0.400$

Juvenile females

Faecal progesterone metabolites levels of juvenile females showed a site effect, with significantly higher levels at TD than at FB, but neither a phase nor an interaction effect (Table 3, Fig. 6). FPM levels at TD (144.54 + 35.11 ng/g) were almost twice as high as those at FB (75.34 + 16.71 ng/g).

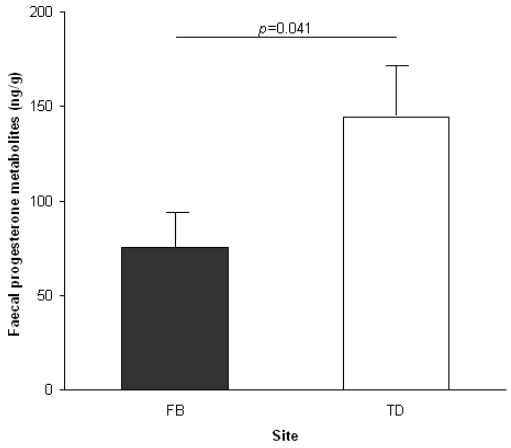

Figure 6. Levels of faecal progesterone metabolites in juvenile female European ground squirrels at the two study sites (FB: n = 10; TD: n = 11). Bars represent back-transformed estimated marginal means + SE; analysis was based on log-transformed data.

Discussion

Significant site differences in faecal cortisol metabolites levels were found in adult males and in juveniles of both sexes, but not in adult females. Adult males and juveniles dwelling in the altered alfalfa meadow (FB) exhibited higher overall FCM concentrations and showed a more pronounced seasonal variation than those in the nearly natural steppe habitat (TD). In contrast, adult females had similar levels at both sites and throughout the season. Past field studies examining potential stress factors responsible for population differences in glucocorticoid levels in mammals focused on food availability (e.g., Foley *et al.* 2001, Behie *et al.* 2010), population density (e.g., Boonstra and Boag 1992, Rogovin *et al.* 2003), predation risk (e.g., Boonstra *et al.* 1998, Hik *et al.* 2001, Mateo 2007, Sheriff *et al.* 2011), and direct human disturbance (e.g., Creel *et al.* 2002, Barja *et al.* 2007). Given the high availability and abundance of alfalfa at FB throughout the active season, a lack of food (i.e., nutritional stress) was unlikely responsible for elevated FCM levels. Furthermore, another study on the same focal populations (Hoffmann *et al.* 2008) showed that both adult males and females at FB were heavier than those at TD, demonstrating that alfalfa is a nutritional food for ground squirrels (Yensen and Sherman 2003, Johnson-Nistler *et al.* 2005).

In contrast to their consexuals at TD, FB adult males showed maximum FCM concentrations during mating and a significant decrease in the subsequent PM1 phase. Mating is an energy-demanding and stressful period for reproductive males, associated with increased aggression and locomotion, resulting in a larger home range and overlap with other males, and body-mass loss (Millesi *et al.* 1998).

However, significantly elevated male glucocorticoid levels during this period have been reported only once so far, namely for *S. citellus* males at high population density (Millesi *et al.* 2004). Adult population density at FB was fivefold that of TD (43 vs. 9 individuals/ha; Hoffmann *et al.* 2008). The frequency of interactions increases with population density (Feldhamer *et al.* 1999), resulting in high glucocorticoid levels, as has been reported for several rodent species (e.g., great gerbils, Rhombomys opimus: Rogovin *et al.* 2003; meadow voles, Microtus pennsylvanicus: Boonstra and Boag 1992).

In *S. citellus*, high density during mating has been shown to increase male-male aggression and body-mass loss due to decreased foraging activity (Millesi *et al.* 2004). Thus, the higher population density at FB might have caused social stress and contributed to the increased FCM values during mating at this site. This interpretation is supported by observations of

both more chases between adult males at FB than at TD and more injuries and scars in FB vs. TD males (personal observation). The high population density at FB could additionally have caused elevated FCM levels by intensifying locomotor activity in males while searching for receptive females. Indeed, despite the small habitat size of FB relative to TD, adult males in the former had home-range sizes similar to those in the latter (Turrini *et al.* 2008). As a consequence, male home ranges at FB overlapped extensively during the mating period, whereas they were more or less discrete at TD (Brenner *et al.* 2008).

At both sites, FCM levels of adult males were elevated (FB) or even peaked (TD) during prehibernation, as previously reported for *S. citellus* (Strauss *et al.* 2007). This is common in hibernating sciurids relying on fat stores (e.g., Cascade golden-mantled ground squirrel, *Callopermophilus saturatus*: Boswell et al. 1994, Golden-mantled ground squirrel, *C. lateralis*, and Belding's ground squirrel, *Urocitellus beldingi*: Nunes et al. 2006). Elevated glucocorticoid levels might reflect an endogenous seasonal change of adrenocortical activity in obligate hibernators preparing for hibernation. This elevation coincides with the onset of fattening in male *S. citellus* (Millesi et al. 1998), indicating that glucocorticoids might be crucial for accumulating lipid energy stores (King 1988). In adrenalectomised rats, fat intake and fat stores were diminished, but could be restored with corticosterone replacement (Castonguay et al. 1986); in adrenalectomised mice, corticosterone treatment even overcame inhibitory effects of leptin on food intake, body mass, and body fat (Solano and Jacobson 1999). An earlier onset of fattening of adult males at FB might have explained that their FCM values exceeded TD values threefold during PM3 in June, however, body mass data (Hoffmann unpublished) indicated no different timing. Rather, the peaking site difference during PM3 was attributable to litter emergence during this period, entailing an abrupt increase of population density.

Another cause for elevated FCM levels at FB during PM3 could have been an increased predation pressure. We noticed both a peak of attacks by common kestrels, *Falco tinnunculus* and an increasing frequency of human activities during this phase at FB, while no such changes were evident at TD (personal observations). Predation risk has frequently been linked to glucocorticoid levels of small mammals: Several studies have shown increased stress load in response to predator abundance, e.g, in snowshoe hares (*Lepus americanu*s, Boonstra *et al.* 1998, Sheriff *et al.* 2011) and Arctic ground squirrels (*Urocitellus parryii*, Hik *et al.* 2001). Nevertheless, this is not always the case (e.g., Belding's ground squirrels, *U. beldingi*: Mateo 2007). Human presence can be considered "equivalent to a form of predation risk" (Hofer and East 1998) and may therefore have a similar effect as predator abundance. During the study, FB proved to be a disturbed recreational area with frequent human presence whereas at TD, walkers passed by only occasionally (personal observations). The more the season progressed, the more human activities occurred at FB.

Adult males attack juveniles, and these interactions lead to elevated juvenile FCM levels (Strauss *et al.* unpublished). Vice versa, adult males probably also show increased adrenocortical activity in response to agonistic interactions with juveniles. Frequent juvenile-adult male encounters may have been inevitable at FB because of spatial constraints resulting from the small habitat size, whereas they were able to avoid each other at TD. In conclusion, the seasonal FCM peak in adult males at both sites seems mainly attributable to changes of adrenocortical activity related to fattening and peaking population densities. Social stress at the already densely populated FB due to juvenile emergence, together with increased predation risk and human disturbance, may have contributed to the site difference in FCM concentrations of adult males.

FCM levels were similar in male and female juveniles, indicating that sex had no influence

on their glucocorticoid levels. Juveniles at FB exhibited peak FCM concentrations after natal emergence, i.e., the same timespan as adult males, exceeding the level of TD juveniles almost threefold. Besides the density-dependent social stress outlined above, the increased predation risk at FB in June most likely caused high juvenile FCM levels at this time. At FB, kestrels preyed upon small juveniles almost every day, whereas not a single kill by raptors (mainly Marsh harriers, *Circus aeruginosus*) was observed in TD (personal observations). Although the individual risk of being attacked is low in dense populations, predation attempts on nearby conspecifics are stressful events potentially increasing adrenocortical activity. Social stress, together with frequent human and dog approaches, may modify behaviour, which is in line with the fact that juvenile home ranges at FB were smaller than those at TD (Turrini *et al.* 2008). This might reflect reduced foraging distances to avoid both predator attacks and encounters with adult males and humans. The significant drop of juvenile FCM levels at FB after weaning may be explained by reduced population density (juvenile dispersal/mortality and onset of hibernation in adult females) together with diminished predation risk (sufficient juvenile size to preclude kestrel predation).

Since fattening in adult males seems to be triggered by high cortisol levels (see above), juvenile fat deposition may also depend on elevated glucocorticoids. As data collection was completed in mid-August, it is not surprising that we found no rise in FCM levels: Juveniles still invested in structural growth and not in prehibernatory fattening.

Despite apparent seasonal and site differences in population density, predator abundance and human disturbance, FCM of adult females were virtually not affected by these factors. Hence, they either did not perceive these factors as stressful or were more resistant to external stressors than adult males and juveniles. However, it cannot be excluded that large individual variation may have concealed significant differences.

As adult females are rarely involved in agonistic interactions (Strauss *et al.* unpublished), they might be less stressed by social factors. A further, not mutually exclusive, explanation would be a resilience to environmental stressors that may have evolved to ensure reproductive success (Wingfield and Sapolsky 2003). All adult females in this study were reproductively active. *S. citellus* females reproduce only once a year and bear the costs of parental effort, the latter peaking during lactation. It is therefore possible that breeding females attenuate endocrine responses that could interfere with successfully producing and rearing a litter. Consistently, captive lactating female Columbian ground squirrels (*Urocitellus columbianus*) that were exposed to a dog showed glucocorticoid concentrations lower than non-lactating females and similar to those without a stressor (Hubbs *et al.* 2000). Also, nonbreeding female Belding's ground squirrels, *U. beldingi*, had higher glucocorticoid levels than their breeding consexuals throughout the season (Nunes *et al.* 2006). Contradictorily, elevated glucocorticoid levels have been reported for pregnant *U. beldingi* (Nunes *et al.* 2006) and lactating yellow-pined chipmunks (*Tamias amoenus*, Kenagy and Place 2000). Those studies, however, assessed total glucocorticoid concentrations in blood samples instead of FCM, and hence are of limited comparability: High total plasma concentrations may reflect high glucocorticoid-binding globulin (CBG) levels. During gestation and lactation, CBG of female rodents is usually elevated (Rosenthal *et al.* 1969a, 1969b, McDonald *et al.* 1988, Boonstra and Boag 1992, Boonstra *et al.* 2001), keeping free glucocorticoids low. As analyses from faecal samples mirror free glucocorticoid levels in plasma (Sheriff *et al.* 2010), the statistically insignificant variation of female FCM levels in our study might be due to increased CBG levels during breeding, buffering potential changes in cortisol secretion. Moreover, elevated CBG levels

could be the mechanism underlying the resilience of reproductive females to external stressors. Further research is needed to clarify the role of CBG in female *S. citellus*.

Given that in both focal populations all adult females were reproductive and had similar FCM levels, it was not surprising that their faecal progesterone metabolites levels did not differ between sites either.

We did find, however, a significant variation of FPM among phases, with high levels during gestation, temporarily declining thereafter, peaking again before weaning, and finally dropping to minimum values during prehibernation. A similar pattern was found in plasma progesterone concentrations of breeding *S. citellus* females in semi-natural enclosures (Millesi *et al.* 2008a). Previous research has revealed that the second progesterone peak in *S. citellus* is caused by a non-reproductive oestrus cycle; the active luteal phase during this cycle may play a role in the fattening process of females prior to hibernation (Millesi *et al.* 2008a, 2008b). Our study supports these results. As discussed above, glucocorticoids seem to be essential for fat storage in adult males, but not in adult females. In mature female rats, progesterone treatment likely triggers altered food intake (Wade and Gray 1979), and therewith causes body weight gain due to increased fat deposition (Galletti and Klopper 1964). Accordingly, the elevation of progesterone rather than cortisol may be the initial signal for prehibernatory fattening in adult female European ground squirrels. Furthermore, the second FPM peak of adult females (between natal emergence and weaning) coincided with elevated FCM levels in adult males and juveniles in June. As experimental studies on rats showed that progesterone and its metabolite allopregnanolone attenuate the stress-induced elevation of corticosterone (Patchev *et al.* 1996), high FPM values could also explain why adult females at FB did not exhibit a likewise significant increase of FCM levels during this obviously stressful period.

Interestingly, juvenile females at TD had significantly higher FPM levels than those at FB. Combined with the lower FCM levels of TD vs. FB juveniles, this suggests that progesterone secretion in juvenile females at FB may be negatively linked to their elevated stress hormone concentrations. In non-pregnant females, progesterone is produced mainly in the ovaries by the corpus luteum and by developing follicles, and to a lesser extent in the adrenal cortex (Goodman 2009). Juvenile female *S. citellus* lack active corpora lutea prior to hibernation, but their ovaries contain secondary and tertiary follicles; the latter are fewer, but similar in size to those of adult females (Millesi *et al.* 2008b). Due to an expression of LH-receptors, granulosa cells of this follicle stage can already secrete progesterone, which has an autocrine function in further follicular development (Goodman 2009). In vitro, granulosa cells of corticosterone-treated rats showed both decreased basal progesterone secretion and reduced response to administered LH compared to those of control rats (Valli *et al.* 2000). Hence, high FCM levels at FB might have reduced progesterone release by developing follicles, potentially delaying follicle maturation. Unfortunately, no data are available on future reproductive output of juvenile females, thus evidence for this hypothesis cannot be provided.

In summary, the results of the present study suggest that anthropogenic land use raises stress load of European ground squirrels, particularly in certain periods of the annual cycle. Human interventions probably influenced stress load directly by disturbing animals and indirectly by affecting food availability and population demography. The high population density at FB may have resulted from food abundance and inhibited emigration, which in turn were consequences of human activities. Social stress elicited by the high population density in the altered habitat clearly peaked after juvenile emergence, when predation risk and direct human disturbance were also high. Further studies are needed to disentangle the different

potential stressors for European ground squirrels and quantify their relative importance. Nevertheless, these stressors did not seem to affect FCM levels of adult females, either because they did not perceive them as stressors or because different physiological pathways were activated. This is supported by the fact that elevated progesterone levels in adult females coincided with elevated glucocorticoid levels in the other age and sex classes. Further investigations should assess whether the higher stress load in the human-altered habitat impacts fitness parameters such as survival by suppressing the immune system and reproductive success by interfering with reproductive function.

In conclusion, our results highlight that one way in which intensive human land use can affect small mammal populations is via mediating changes in their physiology. Therefore it is important to consider physiological mechanisms during decision-making processes in conservation.

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