

Mountain-top and valley-bottom experiences: the stress axis as an integrator of environmental variability in arctic ground squirrel populations

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Keywords

climate variation; fecal glucocorticoid metabolites; environmental stressors; population limitation; enzyme immunoassay validation.

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Editor: Nigel Bennett

Received 22 August 2011; revised 20 November 2011; accepted 29 November 2011

doi:10.1111/j.1469-7998.2011.00888.x

Abstract

Environmental variation in mountainous regions can impose major differences in demography and physiology on animal populations that occupy a large elevation range. This variation can be both predictable and unpredictable. In the south-western Yukon, arctic ground squirrel (AGS) populations occur all the way from the forested valley bottoms to the alpine meadows and, in so doing, experience a wide range of predation risk, forage quality and exposure to weather variables. To assess how AGS integrate these factors and respond to them, we measured fecal glucocorticoid metabolites (FCM) as an index of the stress axis on four sites at different elevations. We first validated the enzyme immunoassay with a hormonal challenge protocol and a simulated predator stressor. AGS show a strong diurnal pattern in FCM levels, with peaks at mid-day and a marked increase in response to adrenocorticotrophic hormone and the simulated predator and a decline in response to dexamethasone. The lag time between the challenge and its reflection in the feces was 4–12 h. Using this method in our field studies, we found that FCM levels decreased as the active season progressed; however, specific patterns differed among sites. We hypothesized that the early season peak in FCM levels followed by the general decline was due to brief, intense early season breeding, followed by the necessity of AGS to increase mass in preparation for hibernation. Although we found no clear, single explanation for the different FCM patterns among sites, we hypothesized that differences in seasonal climate and adverse weather may be major factors affecting FCM levels. The environment was markedly different between years, with 2008 being colder and wetter than 2009 and this was associated with AGS in 2008 having much higher FCM levels in general than 2009. We found that population density and visibility may also contribute. In conclusion, AGS live in a mosaic of habitats and each population is faced with a variety of environmental stressors; how they cope and respond to these stressors may not depend on a single factor but the complete aggregate of these stressors.

Introduction

Wildlife populations exist in a habitat mosaic that produces variation in many local environmental factors. How animals respond to and cope with habitat differences within this mosaic can affect their fitness. Local changes in one population may have far reaching consequences throughout this patchwork and could determine large-scale population trends (Brown, 1984; Krebs, 1985; Pulliam, 2000; Breininger *et al.*,

2009). One way in which animals respond to and cope with environmental stressors is through the activation of the stress axis and the resultant release of glucocorticoids (GC; Wingfield *et al.*, 1998; Boonstra, 2004). In poor habitats (where animals encounter more adverse environmental stressors) animals may increase GC secretion, which could ultimately lead to a decrease in fitness. Many factors can affect the stress axis, including unpredictable events such as predation risk, nutrition, competition with conspecifics, and inclement

weather and predictable events such as changes in reproductive status, and seasonal changes in climate. Thus, the interpretation that high GC levels equate to high stress levels and lower fitness may be incorrect and deviations from the normal pattern of predictable, seasonal changes in GC levels may be more indicative of lower fitness.

The stress axis is composed of the limbic system (dentate gyrus and hippocampus) and the hypothalamic–pituitary–adrenal axis. It is involved in normal day-to-day activities associated with the diurnal cycle such as increased locomotion, exploratory behaviour, increased appetite and food-seeking behaviour (McEwen, Brinton & Sapolsky, 1988; Wingfield & Romero, 2001). As normal daily events undergo seasonal changes (e.g. end of the reproductive season), so too may the stress axis. Under normal daily activities, the stress axis plays a central role in maximizing lifetime reproductive success; however, if an animal lives in a habitat with chronic environmental stressors, the stress axis can deleteriously affect fitness (Wingfield *et al.*, 1998; Boonstra, 2005).

Arctic ground squirrels (AGS; *Urocitellus parryii*) in the south-western Yukon are an excellent species in which to examine the relationship between habitat and stress physiology as they are found in four distinct habitats: boreal forest, low elevation meadows, high elevation shrub stands and alpine meadows. Mammal populations living along an elevation gradient provide a unique opportunity to investigate how changes in local stressors affect patterns of stress physiology because elevation changes result in a rapid change in environmental variables (e.g. temperature, snow depth and length of growing season) and vegetation type over a relatively small spatial scale (Darrow & Bowers, 1997; Richardson, 2004). On average, temperature drops 6°C for each 1000 m gain in elevation, which is equivalent to a 15° gain in latitude (Körner, 1999). These rapid changes in environmental variables can result in sub-populations that are fragmented and thus may act as meta-populations.

In the boreal forest, AGS populations fluctuate with the snowshoe hare cycle and decline in tandem with the hare population (Karels *et al.*, 2000). As the hare population declines, predators switch to AGS as an alternative food source and only squirrels in open meadow habitats with high visibility survive (Karels & Boonstra, 1999). In the alpine and tundra, AGS populations appear to be more stable (Carl, 1971; Green, 1977; Gillis *et al.*, 2005). The alpine populations may be limited by food, spacing behaviour and burrow availability and quality, all of which translate into lower winter survival; predation appears less important. In contrast, in the boreal forest, habitat-related factors such as predation translates into lower summer, but higher winter, survival (Karels & Boonstra, 2000; Gillis *et al.*, 2005). There are potential source-sink dynamics among boreal forest, low elevation meadow and alpine populations. Demographic rates indicate that forest populations are sinks, whereas meadow and alpine populations are sources (Gillis *et al.*, 2005; Donker & Krebs, 2011). From 2003 to 2008, AGS became extirpated in a number of boreal forest sites in the

south-western Yukon and were not re-colonized prior to the hare peak of 2006 (Donker & Krebs, 2011; Sheriff, Krebs & Boonstra, 2011c). Although a greater predator-induced chronic stress may have contributed to lower fitness of boreal forest populations (Hik, McColl & Boonstra, 2001), little is known about the relationship between local stressors and AGS in meadow, shrub and alpine populations. How AGS in these habitats cope with and respond to local stressors may be critical to both their local survival and their landscape level population dynamics.

In this study, we first validated a robust method for measuring GC levels in AGS through fecal cortisol metabolites (FCM – cortisol is the major GC in AGS; Boonstra *et al.*, 2001b). Fecal sampling offers a non-invasive alternative to blood sampling and provides an integrated measure of both baseline and stress response levels of GCs (Palme *et al.*, 2005; Sheriff *et al.*, 2009). Although this method is becoming increasingly popular, a proper validation is not only necessary but crucial (see review by Sheriff *et al.*, 2011a). We then used this method to measure FCM levels of free-ranging AGS from May to August in low-, mid-, and alpine meadows, and in a mid-elevation shrub habitat. At each site, we measured AGS density and an index of visibility (the distance a squirrel could see before vegetation obstructed the view). Climate and weather data for the area where the four sites are located were compiled from Climate Western North America and Environmental Canada. We made the following predictions:

- 1 FCM levels should decrease as the active season progresses, being highest during breeding and lowest in August prior to hibernation. During the early part of the active season (breeding), the intense competition for mating (among males), mating itself, and the resultant pregnancy and lactation can all increase GC concentrations (Bradley, 1987; McDonald *et al.*, 1988; Handa *et al.* 1994; Delehanty & Boonstra, 2011). Because of the catabolic nature of GCs, GC concentrations of AGS should decline over the active season as their focus shifts to over-winter survival. In late summer, AGS direct their efforts to anabolism as they begin to increase mass in preparation for hibernation (Fridinger, 2011).

- 2 Adverse climate or weather patterns should increase FCM levels. Because AGS populations in this study live at different elevations, seasonal climate patterns may be highly localized (e.g. snow melt happens earlier in low elevation habitats than high elevation ones). Adverse weather and different spring and autumn conditions can also affect behaviour and GC concentrations (Wingfield, Moore & Farner, 1983; Sheriff *et al.*, 2011b).

- 3 High population density should increase FCM levels. High population densities can intensify spacing behaviour and increase agonistic interactions and competition, leading to an increase in stress (Christian, 1980).

- 4 Lower visibility should increase FCM levels. AGS living in areas with poor visibility should be less able to detect predators and thus experience higher predation. In a number of studies, wild animals exposed to greater predation risk increase GC concentrations (Boonstra *et al.*, 1998; Clinchy *et al.*, 2004, 2011; Sheriff *et al.*, 2011c).

Methods

Laboratory validation study

Housing

Three male and four female AGS were used for the validation. Squirrels were live-trapped in late July 2009 in the Shakwak Trench east of Kluane Lake, Yukon Territory (61°N, 138°W) with Tomahawk traps (Tomahawk Live Trap Co., Tomahawk, Wisconsin, USA) and transported to housing facilities at Kluane Lake Research Station. All were non-reproductive and adult. AGS were placed in individual 60 × 60 × 120 cm wire cages. Each cage included a nest box with cotton nesting material. Cages were visually separated with a plywood wall and partially covered on the top and front by a burlap cloth. The floor of each cage was made with 1.27 cm reinforced wire mesh that allowed both urine and feces to pass freely. Feces were then caught on a finer wire mesh placed on an angle below the cage. This allowed feces to roll to the collection tray and not be contaminated by urine. Nest boxes were bottomless, and thus, feces could fall through the cage floor; no feces were found in nest boxes at the end of the experimental period.

AGS were fed *ad libitum* with standard rabbit pellets (18% protein, crude fat 2%, crude fibre 18%; Unifeed Ltd, Okotoks, Alberta, Canada) supplemented daily with carrots; water was provided *ad libitum*. All AGS were released at the site of capture at the end of the experiment. The University of British Columbia Animal Care Committee approved all procedures in accordance with the guidelines of the Canadian Council for Animal Care.

Diurnal rhythm

After a 4-day habituation period, during which AGS were visited every 2 h from 06:00 h until 20:00 h and feces cleared from the collection tray but not collected, fecal samples were collected every 2 h from 06:00 h until 20:00 h for 2 days. All feces were cleared from the trays at each time point. During the collection, AGS would enter their nest box, sit on top of it, or stand on the wire mesh watching the collection take place below them.

Adrenocorticotrophic hormone (ACTH) and dexamethason (DEX) injections

To demonstrate that changes in adrenal activity are reflected by changes in FCM concentrations, we conducted two pharmacological manipulations over 4 days; an ACTH stimulation test and a DEX suppression test. AGS were injected with either 40 µg kg⁻¹ of synthetic ACTH (Synacthen Depot, CIBA, Ontario, Canada) into the thigh muscle or 0.4 mg kg⁻¹ DEX (Sabex, Montreal, Canada) intraperitoneally. Injections occurred between 08:00 and 08:30 h. Feces were collected every 2 h from 06:00 to 20:00 h on the day of the treatment and every 4 h from 08:00 to 20:00 h on day 2. To control for possible habituation effects, on day 1, three AGS were ran-

domly assigned to receive ACTH and four to receive DEX; on day 3, after the 2-day collection period, animals were given the other injection.

Predation

To demonstrate that changes in plasma cortisol levels caused by a realistic biological stressor are reflected in FCM concentrations, we exposed AGS to a trained dog as a simulated predator. Exposure occurred at 08:00 h and the dog focused on each squirrel for 2–3 min; the dog was in the room for a total of 25 min and all AGS reacted to its presence, whether or not the squirrel was the focal animal. Feces were collected for 2 days as in the ACTH and DEX treatments. FCM levels were compared with those from the diurnal rhythm study.

Sample handling and FCM analysis

Upon collection, feces were immediately frozen at –80°C. Dry ice and freezer packs were used to transport samples from Yukon to Toronto. In the laboratory, fecal samples were freeze-dried using a lyophilizer (LabConco, Missouri, USA) for 14–18 h to control for water content (Wasser *et al.*, 1993) and homogenized with a mortar and pestle. We then extracted 0.050 ± 0.003 g of the feces with 1 mL of 80% aqueous methanol (v/v) for 30 min at 1450 r.p.m. on a multi-vortexer. After centrifugation (15 min at 2500×g), an aliquot of the supernatant was stored at –80°C until analysis.

In the further diluted (1:50) extracts, FCM levels were assessed with an 11-oxoetiocholanolone EIA (Möstl *et al.*, 2002) and a 5 α -pregnane-3 β , 11 β , 21-triol-20-one enzyme-immunoassay (EIA) (Touma *et al.*, 2003). As the former EIA was better suited to quantify FCM levels in AGS (based on early results of precision and detectability; data not shown), the validation study was performed with this EIA. Antibody cross-reactivity can be found in Möstl *et al.* (2002) and details of the assay procedure can be found in Palme and Möstl (1997) and Möstl *et al.* (2002). Intra-assay coefficient of variation (CV) was 9.68% and the inter-assay CV for pooled high and low fecal extracts was 19.16 and 14.55%, respectively ($n = 10$ plates).

Field study

Study areas

AGS populations were studied in Kluane National Park (KNP) and 20 km away in the Ruby Range (RR), Kluane region, Yukon Territory. In KNP, AGS were studied on two 9 ha sites; one low elevation meadow (~600 m a.s.l.), which we will refer to as LOW, and one mid-elevation meadow MID (~1600 m a.s.l.; Donker & Krebs, 2011). LOW was surrounded by boreal forest on three sides with a large cliff dropping away on the fourth side and MID was above tree line in the alpine. In RR, sites were located above tree line in the same high alpine 4 km² valley running east-west. AGS were studied on four 4 ha shrub sites (~1450 m a.s.l.) collectively called SHRUB and four 4 ha alpine meadow sites called

ALPINE (~1950 m a.s.l.). Vegetation on SHRUB was highly heterogeneous, ranging from ground-level forbs and grasses to approximately 2 m high shrubs (willow and birch). Data for monthly averages of temperature and precipitation are from the Burwash Landing Climatological Station (Climate Western North America and Environmental Canada) located approximately 20 km from the KNP and RR sites.

Trapping

In KNP, trapping occurred in June and August 2008 and in May, June and August 2009; in RR, trapping occurred in July and August in both 2008 and 2009. AGS were trapped on all sites using Tomahawk Live Traps (14 × 14 × 40 cm, Tomahawk Live Trap Co.) baited with peanut butter. Upon capture, AGS were tagged with monel no. 1 tags (National Band and Tag Co., Newport, Kentucky, USA) in both ears (first capture only), weighed with a Pesola spring scale (± 5 g), sexed and measurements of skull width (± 0.5 mm) were taken. Traps were set between 08:00 and 09:00 h and checked 1–2 h later. We calculated minimum number alive (MNA, the number of squirrels caught at time t plus those not caught but known to be present because of captures before and after time t) as a proxy of AGS population density on all sites (Boonstra, McColl & Karels, 2001a).

Fresh feces were collected between 10:00 and 12:00 h (LOW $n = 21, 29$; SHRUB $n = 11, 13$; MID $n = 35, 36$; ALPINE $n = 18, 59$ in 2008 and 2009, respectively). Feces were not collected from any AGS that had been trapped in the previous 48 h to avoid carry over stress effects from a prior capture. Upon collection, fecal samples were immediately placed in an ice filled thermos and 4–6 h later fecal samples were frozen at -20°C until transport to the University of Toronto for analysis. Dry ice and freezer packs were used throughout the transport to ensure samples remained frozen. Fecal samples were analyzed as described earlier.

Visibility

Visibility was estimated by observing the mean per cent visible of a 1 × 1 m board (split in to 25 squares) 20 cm above the ground. Measurements were taken at each of the four cardinal directions at 5, 10, 15, 25 and 50 m from the four corners and the center of each site.

Statistical analysis

For the validation study, repeated measures analysis of variance (ANOVA) and t -tests were performed to analyze changes over time and to compare treatments. The assumption of normality was tested with Shapiro-Wilk test and of homogeneity of variances with Levene's test. If these assumptions were not met, data were log-transformed and re-tested. Sexes were pooled as there was no difference between these non-breeding animals (ANOVA comparing sex at each treatment, $P > 0.05$).

For the field study, a mixed effects model (year × month with animal ID as a random effect) was used to test for differences in FCM concentrations over time at each site. Animal ID had no

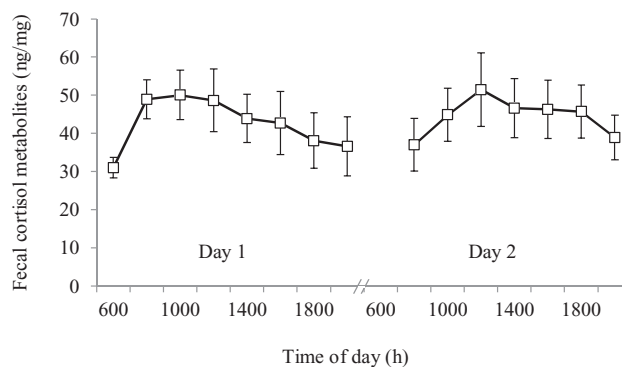


Figure 1 Fecal cortisol metabolite (FCM) concentration (mean \pm SE) of arctic ground squirrels over the first 2 days of collection during the validation study. FCM concentrations show a clear diurnal rhythm.

effect and was not included in the final model. At KNP, because fecal samples were collected only in May 2009 (but not 2008), we used a t -test to compare FCM levels between May and June 2009. At RR, grids within each habitat type were grouped (SHRUB or ALPINE) because of the low sample size. Within each location (KNP or RR), FCM levels between sites were compared on a month-by-month basis using a t -test with a Bonferroni correction for multiple comparisons; adjusted alpha values are $P = 0.02$ for the KNP comparisons and $P = 0.03$ for the RR comparisons. Among all four sites, August FCM levels (the only time we had FCM values for sites at both KNP and RR) were compared using a two-way ANOVA (site × year). As we found no difference between the sexes (possibly because of the small sample size), samples were pooled. However, because the proportion of each sex at each sample period and site was similar (i.e. in May, we had the same proportion of males to females as we had in August), any bias that may have been caused by one sex (i.e. a greater FCM level because of a greater proportion of males) did not occur.

A single monthly average from April to August for temperature and precipitation was calculated. AGS density is reported as MNA. Visibility was compared using a repeated measures ANOVA using increased distance at each point as the repeated measure. A one-way ANOVA with a Tukey's honestly significant difference post-hoc was then used to identify site specific differences at each distance.

All results are reported as mean \pm SE. Statistical tests were performed using the software package STATISTICA 10. (StatSoft, Inc., Tulsa, Oklahoma, USA) Although comparisons of means are considered significant if $P < 0.05$, P values between 0.10 and 0.05 may be biologically relevant and are reported here (Yoccoz, 1991).

Results

Laboratory validation study

AGS exhibited a strong diurnal rhythm in the concentrations of their FCM ($F_{14,70} = 2.58$, $P = 0.005$; Fig. 1). FCM levels were

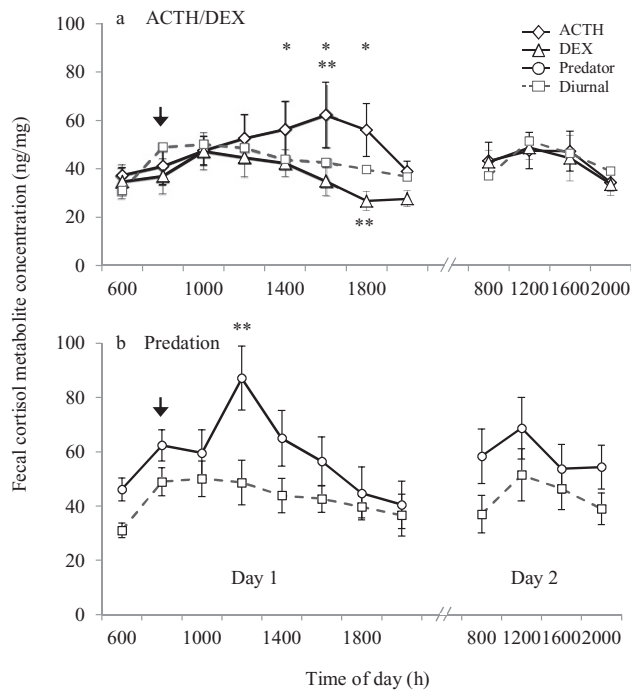


Figure 2 Fecal cortisol metabolite (FCM) concentration (mean \pm SE) of arctic ground squirrels during the experimental procedures of the validation study. The diurnal rhythm is shown as a comparison. (a) The change in FCM concentration after the adrenocorticotrophic hormone (ACTH) or dexamethason (DEX) injections. (b) The change in FCM concentration after exposure to the simulated predator. Arrows indicate when the injections (ACTH, DEX) or exposure (dog) occurred. An asterisk (*) denotes a significant difference between ACTH and DEX and (**) denotes a significant difference between the treatment and the diurnal rhythm. FCM concentrations were not different among treatments (or the diurnal rhythm) on day 2.

lowest at 06:00 h, increased rapidly to a peak at 12:00 h and slowly declined throughout the rest of the day.

ACTH and DEX injections significantly altered AGS's FCM concentrations when compared with each other and when compared with levels found during the diurnal rhythm baseline. At 16:00 h, 8 h after the ACTH injection, FCM concentrations were 78% higher than the DEX injections ($t_{10} = 2.65$, $P = 0.02$; Fig. 2a) and 46% higher than baseline levels ($t_{11} = -2.05$, $P = 0.06$). At 18:00 h, 10 h after the DEX injections, FCM concentrations were 33% lower than baseline levels ($t_{11} = 2.28$, $P = 0.04$; Fig. 2a). At 20:00 h, 12 h after the injections, FCM concentrations returned to diurnal, baseline levels.

The simulated predator (dog) increased FCM levels by 179% compared with diurnal, baseline levels 4 h post exposure (i.e. at 12:00 h; $t_{12} = -2.68$, $P = 0.02$; Fig. 2b). Levels remained 48% higher at 14:00 h ($t_{12} = -1.75$, $P = 0.10$) and by 18:00 h, 10 h after exposure, levels declined to baseline concentrations. Thus, FCM provides a robust measure of the stress axis state of AGS.

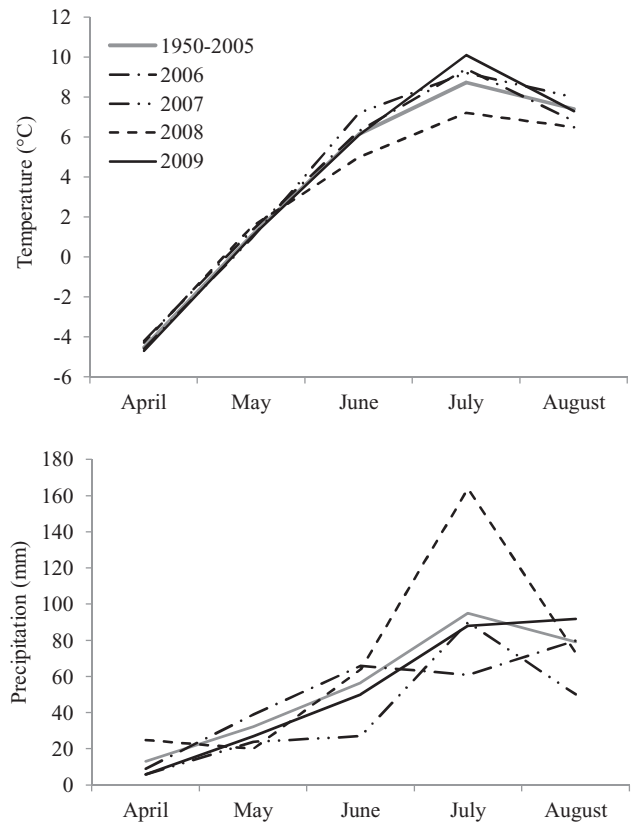


Figure 3 Monthly average temperature and precipitation from the Burwash Climatological Station, Yukon Canada approximately 20 km from the LOW, SHRUB, MID and ALPINE sites. Data are presented for the average over 1950–2005, for the 2 years prior to the study (2006 and 2007), and for the 2 years of the study (2008 and 2009).

Field study

Climate differences among years

The typical average monthly temperature in the region is below freezing in April, increasing steadily to a peak in July and declining slightly in August (Fig. 3). In 2008, temperatures were 1–2°C colder in June, July and August than historical averages dating back to 1950. In 2009, temperatures were similar to historical averages. Average monthly precipitation is highly variable but had an increasing trend from April to August (with April precipitation falling mostly as snow; Fig. 3). In 2008, precipitation was four times greater in April (an extra 12 mm) and two times greater in July (an extra 70 mm) than historical averages. In 2009, precipitation was slightly lower in April but was similar in all other months compared with historical averages. Overall, these climatic differences meant 2008 had delayed spring conditions (and plant phenology; Myers-Smith, 2011) and a cooler, wetter growing season compared with 2007, 2009 and the historical averages.

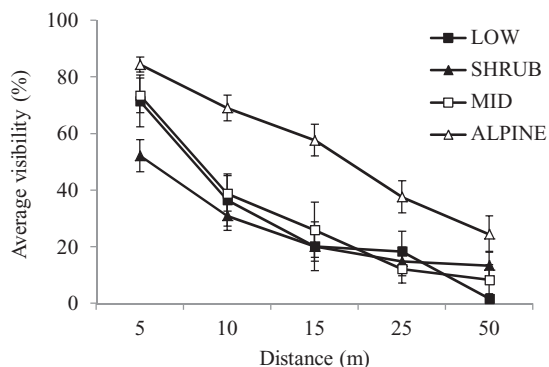


Figure 4 Average visibility (percent of 1 m² test board visible) over an increasing distance at each of the four study sites in Kluane National Park and Ruby Range, Yukon.

Habitat differences among sites

At all sites, visibility decreased significantly with distance ($F_{4,172} = 107.36$, $P < 0.0001$) and there was an interaction between site and distance ($F_{12,172} = 3.48$, $P = 0.0001$; Fig. 4). At 5 m from the sighting board, visibility ranged from 85% at ALPINE to 52% at SHRUB. Visibility progressively declined at ALPINE but markedly fell at all other sites by 10 m (70% visibility at ALPINE, <40% at all other sites). Overall, AGS living at ALPINE had the greatest visibility with all other sites having a similar, lower visibility.

Density differences among sites

AGS population densities were lowest in LOW (1.4 ha⁻¹ in 2008; 1.6 ha⁻¹ in 2009), intermediate in SHRUB (2.8 ± 0.4 ha⁻¹ in 2008; 2.0 ± 0.2 ha⁻¹ in 2009) and highest in MID (5.0 ha⁻¹ in 2008; 6.1 ha⁻¹ in 2009) and ALPINE (4.6 ± 1.1 ha⁻¹ in 2008; 5.1 ± 0.5 ha⁻¹ in 2009; Fig. 5).

Within site seasonal and yearly FCM changes

As the active season progressed, FCM levels decreased at all sites. At LOW, AGS FCM levels decreased 23% from May 2009 to June 2009 ($t_{16} = 3.71$, $P = 0.002$) and then were similar in June and August of 2008 and 2009 ($F_{1,37} = 0.67$, $P = 0.42$; Fig. 6a). At MID, AGS had similar FCM levels in May 2009 and June 2009 ($t_{25} = 0.05$, $P = 0.96$) and then levels declined by an average of 41% from June to August of 2008 and 2009 ($F_{1,55} = 26.85$, $P < 0.0001$; Fig. 6a). At SHRUB and ALPINE, AGS FCM levels decreased by an average of 45% and 21%, respectively, from July to August (SHRUB $F_{1,20} = 20.25$, $P = 0.0002$; ALPINE $F_{1,74} = 4.60$, $P = 0.04$; Fig. 6b). However, there was an interaction effect between year and month at these RR sites (SHRUB $F_{1,20} = 5.52$, $P = 0.03$; ALPINE $F_{1,74} = 6.47$, $P = 0.01$); FCM levels were similar in July and August of 2008.

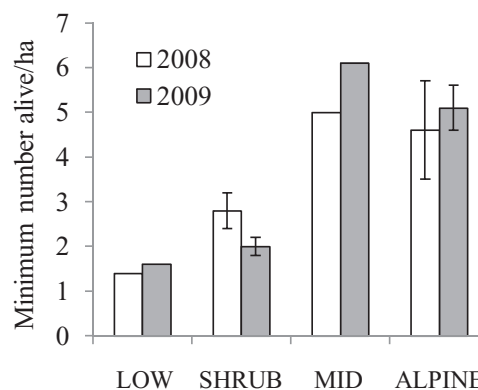


Figure 5 Arctic ground squirrel populations density (minimum number alive per ha) at the four study sites in Kluane National Park and Ruby Range, Kluane, Yukon. SE are provided for Ruby Range sites because each site was an aggregate of four sub-sites.

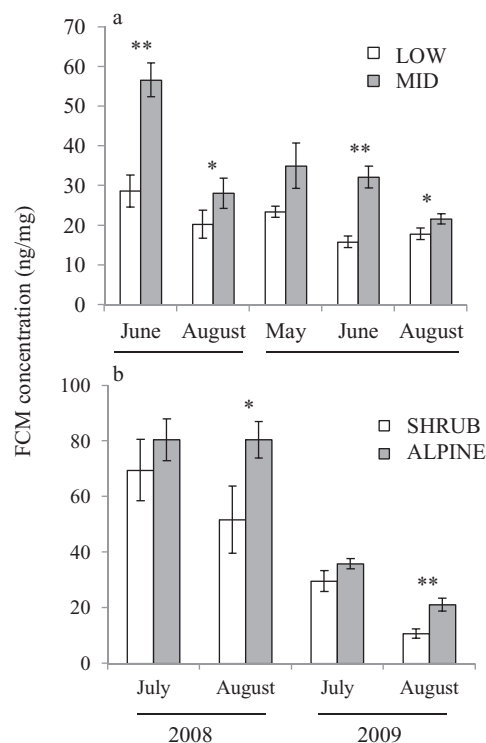


Figure 6 Fecal cortisol metabolite (FCM) concentrations (mean ± SE) of arctic ground squirrels living at (a) LOW and MID meadows in Kluane National Park and (b) SHRUB and ALPINE in Ruby Range, Kluane, Yukon in 2 years. Asterisk denotes differences between sites within the same location. *0.05 < $P < 0.01$; ** $P < 0.05$.

Within sites, AGS FCM levels were two to three times higher in 2008 than in 2009 at all four sites (LOW $F_{1,37} = 5.40$, $P = 0.03$; MID $F_{1,55} = 14.18$, $P = 0.0004$; SHRUB $F_{1,20} = 66.14$, $P < 0.0001$; ALPINE $F_{1,74} = 66.85$, $P < 0.0001$; Fig. 6).

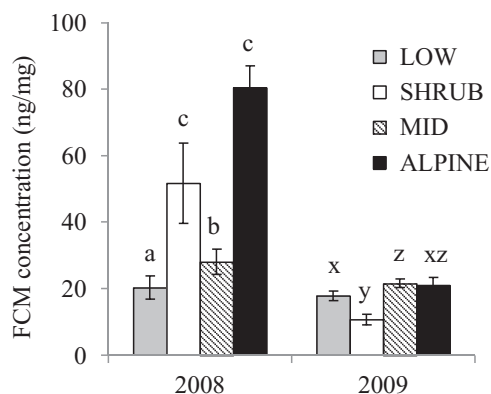


Figure 7 Fecal cortisol metabolite (FCM) concentrations of arctic ground squirrels in August at each of the four study sites in 2008 and 2009. FCM concentrations were significantly lower in 2009 than 2008 ($P < 0.0001$). Letters denote differences among sites within each year ($P < 0.1$).

Among site FCM differences

Although FCM levels decreased by the end of the active season at all sites, the pattern of this decline differed markedly among sites. In KNP, AGS living at LOW had FCM levels similar to those at MID in May (2009: $t_{18} = 1.45$, $P = 0.16$), but LOW averaged 54% lower levels in June (2008: $t_{36} = 4.84$, $P < 0.0001$; 2009: $t_{23} = 5.11$, $P = 0.0001$) and 24% lower levels in August (2008: $t_{16} = 1.81$, $P = 0.09$; 2009: $t_{17} = 1.95$, $P = 0.07$; Fig. 6a) than MID. In RR, AGS living at SHRUB had FCM levels similar to those at ALPINE in July (2008: $t_{18} = -0.81$, $P = 0.43$; 2009: $t_{37} = -1.43$, $P = 0.16$), but SHRUB averaged 39% lower levels in August than those at ALPINE (2008: $t_7 = -1.93$, $P = 0.09$; 2009: $t_{32} = -2.04$, $P = 0.04$; Fig. 6b).

In a comparison of all sites in August between 2 years, we found a year effect ($F_{1,72} = 47.36$, $P < 0.0001$), a site effect ($F_{3,72} = 7.41$, $P = 0.0002$) and an interaction effect ($F_{3,72} = 11.31$, $P < 0.0001$; Fig. 7). Overall, FCM levels in 2008 (37.31 ± 4.98) were two times higher than those in 2009 (19.35 ± 1.35). However, this effect was largely driven by sites in RR. LOW and MID had 2009 FCM values that were 87 and 77% of those in 2008, respectively, while SHRUB and ALPINE had 2009 values that were 21 and 26% of those in 2008, respectively. In 2008, FCM levels were lowest at LOW, and compared with levels at LOW, 138, 254 and 396% higher at MID, SHRUB and ALPINE, respectively. In 2009, FCM levels were lowest at SHRUB, and 170, 210 and 210% higher at LOW, MID and ALPINE, respectively.

Discussion

Validation

Using the 11-oxoetiocholanolone EIA (Möstl *et al.*, 2002), we found that FCM levels show a clear diurnal rhythm (Fig. 1). Concentrations were lowest at 06:00 h, peaked at 12:00 h and slowly decreased throughout the remainder of the

day. This pattern is expected because AGS are diurnal animals and peak plasma GC levels would occur very early in the morning when they wake. The delay in peak FCM levels is due to the metabolism and excretion of plasma GCs and depends mainly on gut passage time (Touma *et al.*, 2003; Palme *et al.*, 2005). Diurnal rhythms in FCM have been shown for other small mammals (e.g. Touma *et al.*, 2003; Lepschy *et al.*, 2007) including Columbian ground squirrels (Bosson, Palme & Boonstra, 2009).

The ACTH/DEX injections and the dog stressor all significantly altered FCM levels compared with AGS' baseline diurnal patterns within 4–12 h (Fig. 2). Boonstra *et al.* (2001a) and Boonstra, Bradley & Delehanty (2011) showed that in AGS both ACTH and DEX injections resulted in rapid changes in plasma cortisol concentrations. A dog has been used as a stressor in other FCM validation studies (Sheriff *et al.*, 2009) and, because it mimics a mammalian predator, it is a better biological validator than handling, which is often used even though it is not encountered by animals in the wild (e.g. Teskey-Gerstl *et al.*, 2000). Based on FCM values, the predator stress resulted in a very pronounced, immediate release of glucocorticoids, while the effect of the ACTH injection was delayed by 4 h. This difference may be due to other physiological changes in AGS when confronted with a real predator (e.g. gut passage time). Several studies have shown that colonic function can be increased through stress-induced changes and function may also be altered with exposure to different stressors (Stam, Akkermans & Wiegant, 1997; Okano, Nagaya & Inatomi, 2005). In other small mammals, where feces were collected every 2–4 h, changes in adrenocortical activity have also been detected in FCM concentrations within 12 h [e.g. 4–10 h in mice (Touma *et al.*, 2003); 6–8 h Columbian ground squirrels (Bosson *et al.*, 2009); 6–10 h in North American red squirrels (Dantzer *et al.*, 2010)]. Differences in the time lag between hormonal changes in the blood and their reflection in the feces are due to the variation in gut passage time between species and among individual animals (Palme *et al.*, 2005; Touma & Palme, 2005). Thus, in terms of obtaining FCM levels for small rodents, which have not been affected by the stress of trapping and handling, one should collect feces within 4 h of capture.

Field study

Seasonal and yearly changes

We found that AGS's FCM levels significantly decreased during the active season, reaching the lowest level in August (Fig. 6), supporting our first hypothesis. Our results are consistent with the findings of Boonstra *et al.* (2001a,b) and Delehanty & Boonstra (2011), and support the latter's 'basal stress hypothesis'. This hypothesis proposes that AGS have a high basal, free cortisol concentration during breeding that falls in the non-breeding, late summer. Breeding can have a major impact on GC levels in mammals. Pregnancy and lactation, under the influence of oestrogens, can cause major increases in GC concentrations (Handa *et al.* 1994; McDonald *et al.*, 1988; Cavigelli, 1999) and the intense intrasexual com-

petition for mates can have severe deleterious effects resulting in high GC concentrations, particularly in males (Bradley, 1987; Delehanty & Boonstra, 2011). However, in breeding male lab mice, Handa *et al.* (1994) found that high testosterone leads to lower GC concentrations.

Under the basal stress hypothesis, the high basal, free cortisol concentrations in spring are largely the result of low corticosteroid binding globulin (CBG) levels, which in AGS are lowest during the pre-breeding season and increase four-fold by mid-summer (Boonstra *et al.*, 2001a,b; Delehanty & Boonstra, 2011). Because high levels of GCs, such as cortisol, are catabolic and stimulate hepatic gluconeogenesis and inhibit glucose uptake by peripheral tissues (Munck, Guyre & Holbrook, 1984), high levels of CBG in late summer limit the amount of free GC and thus the latter's catabolic effects. AGS may increase CBG levels in mid-late summer to protect against the effects of high cortisol as they attempt to increase lean body mass and fat stores in preparation for hibernation. Buck & Barnes (1999) found that AGS reached peak body mass in late July (females) and August (males), and both sexes significantly increased percent body fat in August. In addition, both sexes significantly increase their lean body mass over summer (Fridinger, 2011). Thus, we suggest that AGS may increase CBG concentrations to focus on tissue anabolism as they prepare for hibernation.

Food availability and diet (i.e. abundance and composition) may also contribute to the seasonal changes in FCM levels (Sheriff *et al.*, 2011c). During the early part of the active season, food availability is low (AGS feed mostly on roots and seeds prior to green-up) and spring snow storms may further restrict access to food sources (AGS will dig through only 2–3 in. of snowpack to locate food and usually only feed on snow-free ground) and this may have major impacts on reproduction and survival (Morton & Sherman, 1978). The low food availability may result in high FCM levels in AGS in spring, while greater food availability in the later season may contribute to the decrease in AGS's FCM levels. Reduced food intake has been found to cause an increase in GC concentration in other mammals and birds (Harris *et al.*, 1994; Kitaysky *et al.*, 1999; Ortiz, Wade & Ortiz, 2001). AGS diet also changes throughout the active season and this shift in diet type may also contribute to the decrease in FCM levels (McLean, 1985).

At all four sites, AGS had greater FCM levels in 2008 than in 2009 (Figs. 6 and 7). This may have been caused by the cooler temperatures and increased precipitation in 2008 (Fig. 3). The increase in precipitation in April (when temperatures were still below freezing) would have increased snow depth, decreasing the ability of AGS to access food upon emergence. The increased precipitation in July would have also restricted AGS foraging (AGS decrease above ground behaviour on cold, wet days and rarely leave the burrow on days when it is raining heavily) at a critical time when they need to fatten in preparation for hibernation. The timing of plant green-up was also delayed in 2008 (Myers-Smith, 2011), likely due to the increase in April snow. Other studies have shown that brief storms can increase GC concentrations (Wingfield *et al.*, 1983; Astheimer, Buttemer & Wingfield,

1995; Romero, Reed & Wingfield, 2000), and we suggest that AGS's FCM concentrations are elevated in 2008 due to overall poor seasonal conditions.

Although the general seasonal and yearly trends are similar for all AGS studied, we found that the pattern of seasonal changes varied at our different sites (Fig. 6). AGS living in the LOW had a significant decrease in their FCM levels from May to June, while the decrease in FCM levels occurred later in AGS living at higher elevations. The prolonged, elevated FCM levels of AGS living at higher elevations may be due to the delay in spring events in these habitats. The LOW meadow was 800–1300 m lower than the other habitats. Elevation differences of 1000 m are associated with a 6°C change in temperature, similar to a 15° change in latitude (Körner, 1999). Thus, higher elevation habitats have a later onset of spring conditions including snowmelt and green-up. The timing of spring conditions has been shown to significantly affect AGS; Sheriff *et al.* (2011b) found that in habitats with an earlier spring, AGS advanced their emergence from hibernation and breeding. In habitats with delayed snow cover in autumn, AGS delayed entrance into hibernation. Thus, although all AGS may decrease FCM levels throughout the active season in preparation for hibernation, local seasonal changes in climate and weather may affect the specific patterns of the FCM decrease.

Population density

We found that the AGS living at sites with the greatest population density (MID and ALPINE) also had the highest FCM levels within each respective location (Figs. 5 and 7), supporting our hypothesis that greater population densities should result in greater FCM levels. High population densities can disrupt spacing behaviour and increase agonistic interactions and competition, leading to an increase in stress (Christian, 1980; Raouf *et al.*, 2006). In the alpine and tundra, Carl (1971) found that the number of burrows can limit AGS populations and the competition for burrows may also affect AGS stress levels. However, within our study sites the greater FCM concentrations seemed to be well within the tolerance limits of AGS. A concurrent study on our LOW and MID sites found that populations in both habitats increased from 2008 to 2009 (Donker & Krebs, 2011), but we found that FCM levels decreased (Figs. 6 and 7). Furthermore, local recruitment and population growth were higher at MID, where the population density was greater, than the LOW meadow (Donker & Krebs, 2011). Thus, higher population density may increase FCM levels, but in this study did not raise levels to the point where GC concentrations have deleterious effects on fitness.

Visibility

Visibility was similar on three of our four sites, with ALPINE being the outlier and having the greatest visibility (Fig. 4). However, AGS living on ALPINE had greater August FCM levels compared with the other sites. Although ALPINE also had greater density than the SHRUB and LOW site, which may, in part, explain the greater FCM levels, it had a slightly

lower density than MID. Because AGS are line-of-site animals and engage in vigilance behaviour to reduce the risk of predation (Hubbs, Karels & Byrom, 1996), we expected AGS living in habitats with the greatest visibility to have the lowest FCM levels. Our results suggest that being able to better detect predators does not necessarily decrease GC levels, and we tentatively reject our hypothesis that increased visibility lowers FCM levels. Mateo (2007) also found that Belding's ground squirrels living in open habitats had greater FCM levels than those living in a closed habitat. Blumstein, Patton & Saltzman (2006) found that, within a colony, yellow-bellied marmots that emitted an alarm call had greater FCM levels than those that did not emit an alarm call. Thus, although AGS living in open habitats may have higher survival because they can better detect predators (Karels & Boonstra, 1999), they may have a greater perceived risk of predation resulting in higher FCM levels.

Alternatively, the number of predators may have been greater at our ALPINE site compared with our MID site. The risk of predation greatly affects many populations, and animals exposed to higher predation risk are often more stressed (e.g. tropical stonechats – Scheuerlein, Van't Hof & Gwinner 2001; song sparrows – Clinchy *et al.*, 2004; European rabbits – Monclús *et al.*, 2009; snowshoe hares – Sheriff *et al.*, 2011c). Further studies are needed to better assess the impact of predation risk on AGS stress physiology in meadow and alpine habitats.

This is the first study validating the use of an EIA to measure FCMs in AGS. Using this new method, we found that AGS undergo a general decline in FCM levels as the season progresses; however, seasonal changes in climate and weather produce specific local patterns. Population density and visibility may also contribute to local differences in FCM levels of AGS living in different habitats; however, further studies on these subjects are required. Together, these factors caused a mosaic of FCM levels specific to each habitat type, which may contribute to different dynamics within each population.

Acknowledgements

We thank BJ Delehanty for comments on the paper. In addition, we thank the Kluane Research Base, Arctic Institute of North America for their support. Research support from the Natural Sciences and Engineering Research Council, the Northern Student Training Program, the Leverhulme Trust and Alberta Ingenuity are gratefully acknowledged.

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