Seasonal programming, not competition or testosterone, drives stress-axis changes in a partially-semelparous mammal

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ABSTRACT

Animals must make tradeoffs between reproduction and longevity. This is particularly pronounced in male arctic ground squirrels (Urocitellus parryii), that compete aggressively for territories and mates during a three-week breeding season. Breeding males have high rates of severe wounding, high mortality rates, and high free cortisol levels, along with downstream consequences of chronic stress (weight loss, reduced immune function) that appear to contribute to their early death. The elevated cortisol levels are thought to be a result of the intense intrasexual competition. An alternative hypothesis, however, is that the hormonal change is a seasonal adaptation facilitating the tradeoff of immediate competitive advantage at the expense of long-term survival. We tested a two-part hypothesis: first, that elevated free cortisol during the breeding period is a seasonal change that will still occur in the absence of actual competition, and second, that testosterone maintains this increase. We measured plasma cortisol, corticosteroid-binding globulin, and fecal glucocorticoid metabolites in three groups: wild male ground squirrels, captive males prevented from fighting, and captive castrated males. There were no differences amongst these three groups in free and total plasma cortisol, fecal glucocorticoids, or downstream measures of chronic stress. This suggests that high free cortisol and its effects on breeding males are not a consequence of contest competition during the breeding season, but rather a generalized seasonal change. We found no evidence that testosterone plays a role in maintaining elevated free cortisol in arctic ground squirrel males.

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1. Introduction

Male arctic ground squirrels have a competitive, high-stakes breeding season. They hibernate for an average of eight months a year, followed by an active season which lasts only four months (Buck and Barnes, 1999). Within this narrow timeframe, females produce one litter. Thus, a male's entire yearly (and often lifetime) reproductive success is determined by his breeding effort during a three-week window at the onset of the active season. Males acquire their mates by defending a territory and monopolizing females in that area. If they can successfully do this, they have a high likelihood of fathering the offspring of those females: 71% of females reproduce with the male whose territory they resided in (Lacey and Wieczorek, 2001). However, they must exclude competition by fighting off other males, resulting in high rates of wounding (severe wounding in 69%–100% of males; Gillis, 2003; Delehanty and Boonstra, 2011) and high rates of death (28%–48% of males die after breeding; Gillis, 2003; Boonstra et al., 2001b). The males who do survive the breeding season and hibernate again in the fall also have lower overwinter survival than females, perhaps due to the tolls of the breeding season, with 63% (Gillis, 2003) to a maximum of 83% (Hubbs and Boonstra, 1997) of those males dying before reaching

the next active season. Hence, the majority of the male breeding population dies annually.

During this period of intense competition, breeding males have higher free cortisol levels than non-breeding males, females, and juveniles (Boonstra et al., 2001a, 2001b). Cortisol, the primary glucocorticoid (GC) in arctic ground squirrels (Boonstra et al., 2001a), is involved in the stress response and energetic tradeoffs. GCs increase during stressful events, allowing animals to deal with the stressor by mobilizing energy reserves (lipolysis, raising blood glucose levels, and inhibiting storage of energy), enhancing cardiovascular response, preventing excessive vasoconstriction, and down-regulating immune function to prevent immune and inflammatory responses from overshooting (Sapolsky et al., 2000). When this is an acute stress response, it occurs on a time scale of minutes to hours. When GCs are elevated for a prolonged period of time (days or longer), along with the continuation of their downstream consequences, it is referred to as "chronic stress." It is often explained that while the acute stress response is adaptive, chronic stress is an emergent accident of prolonging the acute stress response, and so the effects are pathological and maladaptive (e.g. Sapolsky, 1987; Wingfield et al., 1998; McEwen, 2004; Chrousos, 2009). Male arctic ground squirrels do display these pathologies of chronic stress, with breeding males having significantly more mass loss (Gillis, 2003), lower white blood cell counts, and poorer ability to produce antibodies than non-breeding males (Boonstra et al., 2001b).

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The effects become progressively more severe as the breeding season continues (Delehanty and Boonstra, 2011).

On the other hand, the chronic stress and subsequent physiological changes in breeding males may not be a pathological consequence of their aggressive mating strategy, but rather an adaptive seasonal change. The view of chronic stress as a pathology has been challenged with the assertion that animals do experience chronic stress in nature under many circumstances, and so their physiological responses to it have undergone selection and are adaptive (Boonstra, 2013). Many species have elevated GC levels during breeding periods, which may have adaptive value in inducing energy mobilization, altering reactive behavior, or priming systems for future stressors (Romero, 2002; Moore and Jessop, 2003). In other species, elevated GCs suppress reproduction, and this difference is dependent on the ecological context in which breeding occurs (Wingfield and Sapolsky, 2003). For male arctic ground squirrels, the former strategy is adaptive: they have a very short reproductive window and a high reward for successful competitors. Hence, it is advantageous for them to put every bodily resource they have towards breeding and fighting other males, ultimately trading off other process involved in long-term survival, such as maintaining body mass or immune function. In this way, chronic elevation of GCs during breeding could be an "adaptive stress response" (Boonstra and Boag, 1992; Boonstra et al., 2001b) facilitating this distribution of bodily resources through glucocorticoids' permissive effects on fat catabolism and altering reactive behavior.

Similar tradeoffs during breeding involving the stress axis are seen in semelparous animals, those that breed only once before deterioration of the body and programmed death. Well-studied examples of this are Pacific Sockeye salmon (Donaldson and Fagerlund, 1968, 1970) and several species in the dasyurid family of Australian marsupials (Bradley et al., 1980; McDonald et al., 1981) which have elevated GCs during breeding. The increase in GCs in these species have been shown to be related to the increase in sex hormones and reproductive maturation, not only the challenges they experience during breeding. For example, landlocked Sockeye salmon (*Oncorhynchus nerka*) that only migrate short distances still display increases in GCs after sexual maturation, though they were not exposed to the environmental stressors of their migrating conspecifics (Carruth et al., 2000). In a population with both semelparous and iteroparous chinook salmon (*Oncorhynchus tshawytscha*), semelparous individuals were found to have significantly higher free cortisol than iteroparous ones (Barry et al., 2001). However, gonadectomy reduces GC levels in Pacific Sockeye salmon (Donaldson and Fagerlund, 1970), prevents interrenal (adrenocortical) hyperplasia, and can increase lifespan by years (Robertson, 1961). Both sex hormones and male conflicts affect GC levels in dasyruids. Isolation of *Antechinus stuartii* from other males reduces GC levels (Scott, 1987) and captivity of the brush-tailed phascogale (*Phascogale tapoatafa)* prevents significant increase in GCs during the breeding season as well as preventing death, which normal follows immediately after (Schmidt et al., 2006). Yet, sex hormones also have a direct relationship to elevated GCs: castration of *A. stuartii* reduces free GCs by allowing an increase in corticosteroid-binding globulin (CBG) levels, whereas testosterone injections decrease CBG levels (Bradley et al., 1980). Though testosterone and CBG levels are not correlated across individual arctic ground squirrels (Boonstra et al., 2001a; Delehanty and Boonstra, 2011), breeding males have lower CBG levels than those of non-breeding males, and the latter also have lower testosterone levels (Boonstra et al., 2001b; Buck and Barnes, 2003). Thus, in breeding male arctic ground squirrels, high testosterone may cause elevated free cortisol levels by driving down CBG production.

If elevated cortisol during the breeding season is adaptive, rather than pathological, in male arctic ground squirrels, the increase in cortisol should be present as a seasonal change in reproductive males even when they are removed from the actual stress of male-male aggression. To test this hypothesis, we captured and separated newly emerged breeding males prior to fighting. If their cortisol levels do not differ from wild males who are exposed to the stress of the breeding season, this would provide evidence that the stress response during the breeding season is anticipatory, rather than a consequence of competition. Furthermore, if environmental stressors do not trigger this chronic stress response, a potential candidate for regulating this change is the sex hormones, which are in higher concentrations in breeding animals and appear to permit the increase in GCs during breeding in Sockeye salmon and *Antechinus*. Male arctic ground squirrels are known to have increased androgen levels during the peak of the breeding season and also following encounters with other males during spring breeding (Buck and Barnes, 2003). To examine this relationship, an additional group of captive males was castrated, and plasma cortisol and fecal cortisol metabolite (FCM) levels were compared with those of wild males and captive-intact males.

2. Methods

2.1. Trapping and handling

Male arctic ground squirrels (n = 20) were captured at emergence at the Yukon Wildlife Preserve, located approximately 30 km west of Whitehorse, Yukon Territory. They were trapped with Tomahawk Live Traps (Tomahawk, WI, USA) baited with peanut butter. Trapping occurred on April 7th and 8th, 2015, at the onset of the breeding season. Males emerge from hibernation before females (McLean and Towns, 1981), so we were confident that males had just recently emerged, as only 8 of 28 of trapped animals were female. In contrast, the sex ratio once females have emerged is 1:1 (Boonstra et al., 2001b). At capture, squirrels were tagged with monel ear tags (National Band and Tag, Newport, KY, USA) and weighed. Squirrels were briefly anesthetized using a nose cone with cotton soaked in isoflurane (Benson Medical Industries Inc., Markham, ON, Canada) and a 0.3 mL blood sample was taken from the suborbital sinus. Fecal samples were collected from traps. These represent baseline GC levels of individuals in their wild state, as feces reflects plasma cortisol levels 7 h prior to the stress of capture (Bosson et al., 2009). Within 48 h of trapping, animals were transported by vehicle to Kluane Lake Research Station, Yukon, where they were kept in captivity for four weeks. Squirrels were kept indoors in polypropylene cages (47 cm × 26 cm × 20 cm) with mesh lids, in natural light provided by a bank of windows and under ambient temperature. Cages were spaced about a foot apart and were opaque, so squirrels had no visual contact with one another. A wooden board was placed over half of the cage to provide a fully covered area, and cedar bedding and cotton nesting material was provided. Water and food was available ad libitum. Squirrels were fed apple slices, lettuce, and a mixture of Step Right Premium "Classic Sweet" feed and "Complete Pellet" feed (Hi-Pro Feeds, Okotoks, AB, Canada). This feed was composed of grains and molasses with a minimum of 12% crude protein, 2.5% crude fat, and a maximum of 9% crude fiber for the former feed and 14% crude fiber for the latter. All animal care procedures were approved by the University of Toronto Animal Use Committee (protocol 20010861).

2.2. Surgery and sample collection

On April 10th, animals were randomly assigned to the two captive treatments. Half were castrated (n = 10) and a 0.3 mL blood sample was collected from the suborbital sinus. They were anesthetized using 3.5% isoflurane delivered from a portable anesthetic delivery unit and injected with a ketoprofen analgesic (Anafen, Merial, Baie-d'Urfé, QC, Canada). Testes were removed and vas deferens and scrotum were sutured shut. Captive animals to be left intact (n = 10) were also anesthetized in the same manner and a blood sample was taken. One animal from the intact group died under anesthesia. All other animals recovered rapidly from surgery without further complications. Following surgery, blood and fecal samples were collected on a weekly basis (April 17th, April 25th, May 1st, May 8th). On the days of sampling, starting

at approximately 8:00, animals were removed from cages using a pillow case one at a time in a random order, taken to the laboratory, and briefly anesthetized to collect a blood sample from the suborbital sinus. While it took <3 min to remove the squirrel from its cage and anesthetize it in the laboratory, plasma samples still likely reflect handling stress as the squirrels were aware of the presence of the researcher in the holding room (alert, some alarm calling) removing and returning other animals. Immediately after blood samples were taken, glucose concentration (mg/dL) was measured with a FreeStyle glucose meter (Abbott Laboratories, Alameda, CA) and hematocrit levels, representing percent packed red blood cell volume relative to total blood volume, were measured in duplicate using microhematocrit tubes and a microhematocrit (IEC Micro-Hematocrit Centrifuge at 13,460g). To obtain leukocyte profiles (white blood cells, WBC) blood-smear slides were created in duplicate and stained using Diff-Quick (Dade International, FL, USA). The slides were then examined under a microscope, and 100 WBC were counted and identified as neutrophils, eosinophils, monocytes, or lymphocytes.

On the morning of blood sample collection, fecal samples were also collected from cages. The night before sampling, cages were cleaned and bedding was changed to ensure no old feces was collected and that samples were not contaminated with urine. Between weekly sampling, animals were not handled or disturbed.

During the period of captive monitoring, wild male squirrels (n = 11) in the vicinity of Kluane Lake Research Station were live trapped and fecal and blood samples were collected in the same manner as described above. These wild males were clearly in the midst of the breeding season: testes were scrotal, severe wounding was noted, and the population was predominantly female.

2.3. Steroid analyses

At the time of collection, blood samples were centrifuged to separate plasma, which was then stored at −80 °C until analysis at University of Toronto. Total plasma cortisol was measured using a commercially available ^{125}I radioimmunoassay kit (MP Biomedicals, USA). The minimum detection limit of the kit is 1.7 ng/mL and the mean recovery of ^{125}I cortisol added to plasma is 102.4% (range = 91–117%). Parallelism has previously been determined for arctic ground squirrel plasma with this kit (Edwards and Boonstra, 2016). Cross-reactivities of the antibody to relevant steroids are low (11-desoxycortisol – 12.5%; corticosterone – 5.5%; 17α-hydroxyprogesterone – 1%; all other steroids <1%). All samples were measured in duplicate within a single run. To determine plasma CBG concentrations we used the maximum corticosteroid-binding capacity (MCBC) as determined by using dextran-coated charcoal to separate bound hormone (detailed in Delehanty and Boonstra, 2009; Delehanty et al., 2015). Free cortisol levels were calculated using the equation developed by Barsano and Baumann (1989). For each sample, this requires the CBG concentration, total cortisol concentration, and a species-specific dissociation constant (Kd) of CBG. For arctic ground squirrels, a Kd of 4.0 nM was used, as determined using the charcoal method (Delehanty et al., 2015).

Fecal samples were freeze-dried using a lyophilizer (LabConco, MO, USA) and homogenized by crushing with a mortar and pestle. We weighed out 0.05 g of feces per sample and suspended them each in 0.5 mL of 80% methanol. Samples were shaken for 30 min on a multi-vortexer (IKA VXR Basic Vibrax, 1450 r.p.m.) and centrifuged (15 min at 2500g) and an aliquot of the supernatant was stored at −20 °C until analysis. Extracts were diluted (1:100) in assay buffer, and assessed with an 11-oxoetiocholanolone EIA (Möstl et al., 2002; validated for arctic ground squirrels in Sheriff et al., 2012). The inter-assay and intra-assay CVs were determined by repeated measures of pooled samples, and were 14.2% (n = 5 plates) and 4.7% respectively.

Testosterone metabolites were also measured in fecal extracts. Extracts were diluted (1:400) in assay buffer and measured with an epiandrosterone EIA (measuring androgen metabolites with a 17-oxo group) on a single plate. The intra-assay CV was 8.6%. The EIA was

first described by Palme and Möstl (1994) but validated for arctic ground squirrels by Bosson et al. (ms in prep). As part of that validation, arctic ground squirrels (not used in this study) were injected with [^{3}H]-testosterone, and radioactive fecal metabolites were characterized by HPLC separation. Those HPLC immunograms revealed that several radiolabeled testosterone metabolites were present in the feces, and some of them (including one of the dominant metabolites) were recognized by the epiandrosterone EIA.

2.4. Statistical analysis

Data were tested for normality with the Shapiro-Wilk test and for homogeneity of variance with Levene's test. Plasma hormone data met these assumptions, but fecal hormone data did not, and thus were log transformed. Plasma measures of total cortisol, CBG, free cortisol, and fecal cortisol metabolites (FCM) were compared one-week post-surgery in the three groups by ANOVA, and eta-squared was used to measure effect size. Four wild males and two captive-castrate males did not have sufficient amounts of plasma to measure MCBC, so for CBG and free cortisol comparisons, sample size was reduced. One captive-castrate male was an outlier (more than two standard deviations above the mean) for total cortisol levels and removed from analysis. This allowed total cortisol data to fit the assumption of normality for ANOVA. Inclusion of the outlier did not alter the significance of the results.

Longitudinal treatment differences between captive-castrate and captive-intact males were compared by a repeated measures ANOVA. To examine if captivity itself was a stressor, FCMs in samples from intact males collected at time-points across captivity were compared to baseline FCMs levels at capture using Dunnett's test. Fecal epiandrosterone metabolite differences and week-one post-surgery weight loss differences were compared between captive-castrate and captive-intact males by t-test and Cohen's d was calculated as a measure of effect size. For WBC profile analysis, neutrophil to lymphocyte ratio (N:L ratio) was calculated for each individual. Evidence indicates that under conditions of chronic stress, the number of lymphocytes decrease relative to neutrophils in the blood (reviewed in Davis et al., 2008). Analysis was done using JMP (version 12, SAS Institute Inc., Cary, NC, USA) and figures created with Prism (version 4.0, Graphpad Software, La Jolla California, USA). Data are reported as means ± SE.

3. Results

3.1. Condition of animals at capture

The 20 males captured directly after emergence (prior to the breeding season) appeared in good condition with no visible wounding. The average weight was 664.8 ± 23.0 g. Blood glucose concentration averaged 105.3 ± 3.6 mg/dL, and hematocrits averaged 51.8 ± 1.7%. These males would become the two captive treatments. Wild males sampled in the midst of the breeding season were all reproductive (testes descended into the scrotum) and had instances of severe wounding (7 cm^2 or more of visible wounding; open or scabbed tears on the skin), averaged 564.5 ± 23.1 g, and had higher blood glucose concentrations of 128.5 ± 6.3 mg/dL, and lower hematocrits of 43.0 ± 1.8% relative to the recently emerged males.

3.2. Effect of captivity

Captivity did not appear to be a confounding factor for stress levels. The experiment rests on the assumption that captivity is not inherently more "stressful" for arctic ground squirrels than wild conditions. To test this assumption, FCMs of the intact males over captivity were compared to the fecal samples collected from traps at capture. Concentrations of FCMs reflect baseline wild levels without handling stress (fecal cortisol levels reflect the circulating levels 7 h prior in the closely related Columbian ground squirrel; Bosson et al., 2009). If captivity is not stressful,

FCMs should return to or fall lower than this wild baseline. Animals appeared to habituate to captivity. Intact males showed a significant increase in FCMs 48-h post-capture (P < 0.0005) but returned to baseline levels by one-week post-sham surgery (Fig. 1).

3.3. Effect of castration

Castration significantly reduced fecal androgen metabolite (FAM) levels relative to intact males one-week post-surgery (P < 0.005, d = 1.32). However, by week four, FAMs no longer significantly differed between treatments (P = 0.19, d = 0.71) (Fig. 2). 70% of the castrate males increased in FAM levels between weeks one and four, and two males increased such that they were above the original (week one) range of castrate males. Furthermore, two males in the intact group experienced FAM declines by week four of the study, though the FAM average for intact males as a whole did not decline between week one and week four. For this reason, we compared intact versus castrate male cortisol levels and downstream measures at one-week post-surgery.

3.4. Hormonal measures

From one-week post-surgery to the end of the study (four-weeks post-surgery), intact and castrate males showed no treatment differences in FCMs ($F_{[1,15]}$ = 0.13, P = 0.18). Since male androgen production rebounded after castration, further comparisons will focus on one-week post-surgery, when treatments still showed a clear difference (Fig. 2). In samples collected one week after surgery, wild, captive-intact, and captive-castrate males did not significantly differ in plasma levels of total cortisol ($F_{[2,26]}$ = 0.38, P = 0.69, η^2 = 0.03), CBG ($F_{[2,21]}$ = 0.30, P = 0.75, η^2 = 0.03), or free cortisol ($F_{[2,21]}$ = 0.82, P = 0.46, η^2 = 0.07) (Fig. 3). Of the total cortisol in plasma, 67% ± 4.6 was free. These plasma samples reflect handling stress. However, FCMs, which reflect stress levels prior to handling, also showed no differences amongst treatments ($F_{[2,24]}$ = 0.07, P = 0.93, η^2 = 0.006). (Fig. 4). Thus, both plasma and fecal GC measures are concordant.

3.5. Downstream indicators of stress

Though captive males were fed ad libitum, on average, both groups lost weight over the first week post-surgery, with intact males losing 34.4 ± 17.2 g and castrate males 56.7 ± 15.9 g. Weight loss did not differ between captive-intact and captive-castrate males (P = 0.36, d = 0.45). There was high variation in both treatments, and though the majority of males lost weight, not all did, with the maximum weight gain of 30 g. Captive males did not tend to regain weight: by week four, most males were still ~100 g below their initial weight at capture, with an

Fig. 2. Concentrations of fecal androgen metabolites (FAM) (log ng hormone/g feces) at post-surgery weeks one and four. Asterisks denote a significant difference between treatments (P < 0.005).

average weight of 568.2 ± 17.7 g compared with the capture average which was 664.8 ± 23.0 g. Across captivity (from week one to week four), weight declined, but not significantly (P = 0.10, d = 0.53). Wild males were not recaptured frequently enough to collect longitudinal weight loss data.

There were no differences amongst treatments in blood glucose concentration ($F_{[2,27]}$ = 0.05, P = 0.96, η^2 = 0.003) or hematocrits ($F_{[2,27]}$ = 0.79, P = 0.46, η^2 = 0.06). In measures of immune function, N:L ratio did not differ between the three treatments ($F_{[2,24]}$ = 2.73, P = 0.09, η^2 = 0.19). Average N:L ratios for castrate, captive-intact, and wild males were 1.4 ± 0.34, 1.6 ± 0.36, and 0.68 ± 0.38, respectively. Overall, neutrophils and lymphocytes made up approximately equal proportions of WBC, with an average of 33% of WBC being neutrophils, 39% lymphocytes, and 23% monocytes.

4. Discussion

We found that the high GC levels in breeding male arctic ground squirrels were not induced by stressful competition in the wild environment, but rather were reflective of a seasonal pattern that persisted even when contest competition was removed. It has previously been demonstrated that wild, breeding male arctic ground squirrels have free cortisol levels that are higher than post-breeding males, females, and juveniles (Boonstra et al., 2001a, 2001b). We assume that the wild males in our study are comparable to these previously documented
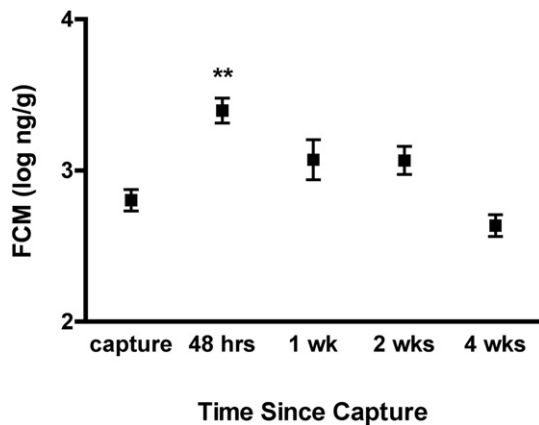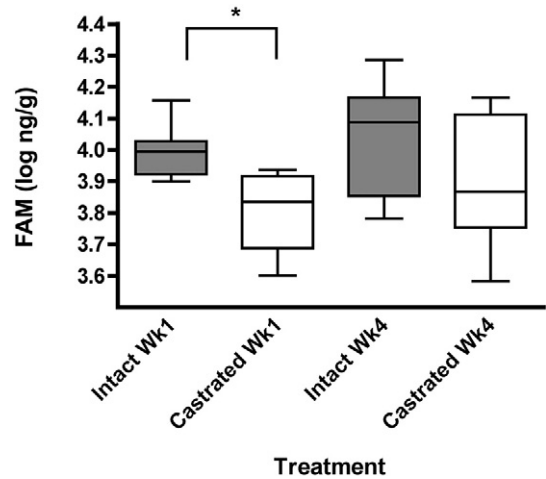

Fig. 1. Concentrations of fecal cortisol metabolites (FCM) (log ng hormone/g feces) from capture to post-surgery week four in intact captive males. Asterisks denote a significant difference from baseline FCM levels (P < 0.0005).
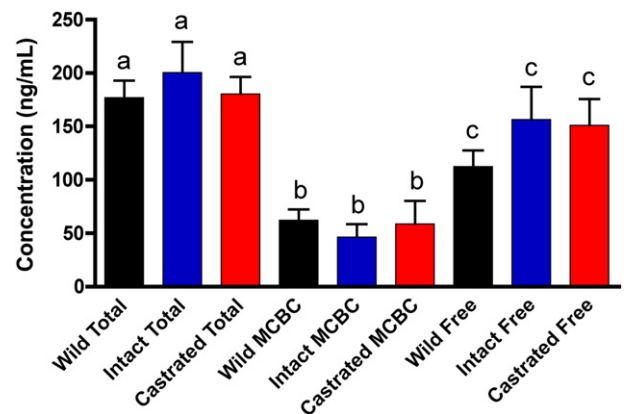

Fig. 3. Plasma total cortisol, MCBC, and free cortisol (ng hormone/mL plasma) at post-surgery week one. Same letters signify no significant difference within a plasma measure.
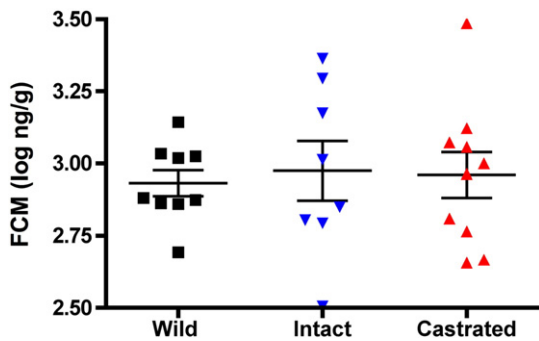
Fig. 4. Concentrations of fecal cortisol metabolites (FCM) (log ng hormone/g feces) at post-surgery week one compared amongst treatments. There were no significant differences amongst groups.

wild males that were also captured in the southern Yukon with the same live-trapping methods. If we accept the assumption that our wild males reflect the "normal" hormonal state of wild males during the breeding season in general, then the captive and castrated males in our study, which do not differ hormonally from wild males, reflect GC levels that are equally elevated over non-breeders, females, and juveniles. Further supporting this idea is the fact that squirrels in all three treatments had downstream measures that were remarkably similar to those of wild male arctic ground squirrels previously monitored during the breeding season (Delehanty and Boonstra, 2011; Delehanty, 2012), bolstering the idea that the physiology of all males in this study reflected the typical "stressed" condition of wild males during breeding. Thus, all males in this study have elevated GCs relative to other sex and age groups, and this persists when they are removed from male-male agonistic interactions or when testosterone is reduced.

We do not believe the high GCs in captive males were a result of captivity being an equal stressor to that of wild competition. First, it is clear that GC secretion had not hit a ceiling, as captivity resulted in a significant increase 48-hours post-capture and then declined. Second, comparable species have also been shown to habituate to captivity within a few days. In captive Columbian ground squirrels (*Urocitellus columbianus*; a species which does not exhibit seasonal breeding stress) kept in the lab under similar conditions, there was a significant increase in fecal GCs on day 2 after capture, but by days 7 and 8 GCs had returned to baseline, non-stressed levels (Bosson, 2007). These Columbian ground squirrels lost weight from capture to day 2, but after day 2 consistently gained weight throughout the remainder of captivity (14 days), also reflecting low stress levels and habituation (Bosson, 2007; Bosson et al., 2009). Furthermore, isolated captivity of the dasyurid marsupials *A. stuartii* and *P. tapoatafa* were able to attenuate the increase in GCs associated with seasonal breeding competition (Scott, 1987; Schmidt et al., 2006). If competition was also a primary cause of breeding stress in arctic ground squirrels, captivity should have similarly reduced GC levels.

High GC levels were also not related to food restriction and necessity of catabolizing body reserves. Our captive animals were fed ad libitum, whereas wild animals must continue to forage while simultaneously engaging in breeding activities. If high GC levels were related to a lack of food, we would expect captive animals to have lower GCs than wild ones, but this was not the case.

Therefore, high GCs do not appear to be a result of intrasexual competition or of food limitation, perhaps because these changes are programmed as a seasonal change for the breeding period. Though high free cortisol appears to be related to internal changes during the breeding season, it is not maintained by elevated testosterone levels either (Fig. 3, Fig. 4). Decreased testosterone in castrated arctic ground squirrels has no apparent effect on the stress axis, in contrast to the situation found in *Antechinus* where castration increases CBG production, consequentially decreasing free cortisol (Bradley et al., 1980). Castrate arctic

ground squirrel males had no increase in CBG production, and there was no decline in free cortisol nor FCMs (Fig. 3). Some castrate males began to rebound in FAM, indicating that there was an eventual compensatory production of androgens by the adrenals, a known androgen production site in this species (Boonstra et al., 2011).

It is possible that testosterone could have a permissive effect on HPA-axis changes in breeding males, which would not have been detected by our study. In the sockeye salmon gonadectomy study (Robertson, 1961) gonads were removed before individuals reached sexual maturation. We could not perform such a study in arctic ground squirrel males as they become euthermic three weeks prior to emerging from their hibernaculum in order to develop their quiescent gonads (Barnes and Ritter, 1993). It is also possible that some other seasonal change separately cues breeding males to elevate both testosterone and cortisol levels. For males, the onset of euthermy appears to be triggered by an endogenous circannual clock, as opposed to weather conditions (Sheriff et al., 2013). The appropriate hormone changes in males may be also triggered by this endogenous clock and return to euthermy.

The elevated free cortisol levels during breeding may have adaptive value for male arctic ground squirrels. Glucocorticoids mobilize energy, and can increase vigilance, activity, and aggression or attention to social threat (Breuner et al., 1998; Mikics et al., 2004; Roelofs et al., 2007). More energetic, alert, and reactive ground squirrel males may be more successful at defending a territory and the females who live on it, reaping the reproductive payoff, though they may die after one or two years. Males that have lower GCs may live longer, but also may not have the opportunity to reproduce at all. This would lead to the trade-off of long-term survival for short-term breeding success, through the beneficial changes induced by high GCs in a stressful environment. It has been suggested that ancestral mammals with a peak in GCs during breeding periods could be precursors to the evolution of semelparity (Romero, 2002), so it is possible that male arctic ground squirrels, because of their narrow breeding window and high-stakes reproductive skew, reflect one of these evolutionary half-way points between iteroparity and true semelparity.

Although we have demonstrated the persistence of high GCs after removal of competition stress, this study does not directly demonstrate the adaptive value of chronic stress in arctic ground squirrels. Future work directly linking variation in arctic ground squirrel male GC levels with reproductive success would more conclusively address this question. For example, if higher male GC levels were found to be associated with higher rates of paternity, or alternatively, if artificially reduced GC levels were associated with reduced rates of paternity, this would demonstrate a clear causal role between male GCs and reproductive success.

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