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Plasma DHEA levels in wild, territorial red squirrels: Seasonal variation and effect of ACTH

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ABSTRACT

In many species, territorial behavior is limited to the breeding season and is tightly coupled to circulating gonadal steroid levels. In contrast, both male and female red squirrels (*Tamiasciurus hudsonicus*) are highly aggressive in both the breeding and non-breeding seasons in defense of food stores on their individual territories throughout the boreal and northern forests of North America. Dehydroepiandrosterone (DHEA), an androgen precursor, is secreted from the adrenal cortex in some mammals, and DHEA has been linked to aggression in non-breeding songbirds. Here, we examined plasma DHEA levels in a natural population of red squirrels in the Yukon, Canada. Plasma DHEA levels in both males and females reached high concentrations (up to 16.952 ng/ml in males and 14.602 ng/ml in females), markedly exceeding plasma DHEA concentrations in laboratory rats and mice and similar to plasma DHEA concentrations in some primates. Circulating DHEA levels showed both seasonal and yearly variation. Seasonal variation in male plasma DHEA levels was negatively correlated with testes mass. Yearly variation in male DHEA levels was positively correlated with population density. In both males and females, circulating DHEA rapidly increased after ACTH treatment, implying an adrenal origin. This is the first examination of plasma DHEA concentrations in a wild rodent and the first field experiment on the regulation of plasma DHEA in any wild mammal. These data lay the foundation for future studies on the role of DHEA in non-breeding territoriality in this species and other mammals.

1. Introduction

Territoriality is a mechanism to secure exclusive access to resources related to reproduction during the breeding season. In mammals, the sex responsible for securing the territory may be the female, the male, or both (Clutton-Brock, 1989). The gonadal steroids directly related to reproduction (primarily testosterone in males; primarily estradiol and progesterone in females) underlie territorial behavior during the breeding season (Monaghan and Glickman, 1992; Bronson and Heideman, 1994; Wingfield et al., 2006; Demas et al., 2007). However, territoriality during the non-breeding season is also found in many taxa, including mammals, and is related to the protection of food resources essential for survival. For example, in the red squirrel (*Tamiasciurus hudsonicus*), both males and females are highly aggressive in defense of individual territories during the breeding season and the non-breeding season, the latter being critical to protect food stores essential for overwinter survival (Smith, 1968a,b; Kemp and Keith, 1970; Rusch and Reeder, 1978; Larsen and Boutin, 1994; McAdam and Boutin, 2003). These data raise the question of how aggression is regulated in non-reproductive contexts in this and other species.

One possible hormone regulating non-breeding season territoriality in mammals is the steroid dehydroepiandrosterone (DHEA), a sex steroid precursor produced by the zona reticularis of the adrenal cortex, the gonads, and/or the brain. Unlike testosterone and estradiol, DHEA does not have a known classical intracellular steroid receptor (Widstrom and Dillon, 2004; Labrie et al., 2005). However, DHEA can be readily converted to active sex steroids in tissues, such as the brain, that express the appropriate enzymes (Zwain and Yen, 1999). There are two lines of evidence that suggest that DHEA regulates territorial behavior in the non-breeding season. First, it is generally believed that only humans and higher primates form DHEA of adrenal origin and other mammals form DHEA of gonadal origin (Labrie et al., 2005), but recent evidence

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indicates that hamsters synthesize DHEA in the adrenals (Pieper and Lobocki, 2000; Demas et al., 2007). Under "winter-like" short-day photoperiods (typical of the non-breeding season), these hamsters exhibit high levels of territorial aggression, even though the gonads are regressed and circulating testosterone levels are basal (Jasnow et al., 2000, 2002). Moreover, under long-day photoperiods, melatonin treatment increases aggression by acting, at least in part, on the adrenal cortex (Demas et al., 2004).

Second, in some songbirds, non-breeding season male territoriality also occurs, for example in the Pacific Northwestern song sparrow (*Melospiza melodia morphna*) (Soma, 2006). The gonads in males are regressed during the non-breeding season, and the plasma concentrations of testosterone, 5α-dihydrotestosterone, androstenedione, 17β-estradiol, and estrone are basal (Wingfield and Hahn, 1994; Soma and Wingfield, 1999). Castration does not decrease aggressive behavior in the non-breeding season (Wingfield, 1994). Aromatase inhibitors decrease aggressive behavior at this time (Soma et al., 2000a,b), indicating that estrogens regulate non-breeding aggression. Estrogens might be produced locally in the brain via neural metabolism of DHEA synthesized in the adrenals, the regressed gonads, and/or the brain itself (Soma et al., 2004; Schlinger et al., 2008).

Here we investigate whether DHEA is present in the blood of both breeding and non-breeding red squirrels. First, we examine the effects of season, year, and sex. Second, we examine the effects of exogenous ACTH. This study represents the first examination of plasma DHEA concentrations in a wild rodent and the first field experiment on the regulation of plasma DHEA in any wild mammal.

2. Methods

2.1. Study site

The study site was located in an undisturbed northern boreal forest near Kluane Lake, Yukon Territory, Canada (60° 57′ N, 138° 12′ W) (Krebs et al., 2001). The climate is cold continental with a short growing season (mid-May–mid-August) and snow cover from October through early May. The mean monthly temperatures for the warmest (July) and coldest (January) are 12.8 °C and −21.9 °C, respectively, and total annual precipitation averages 284 ± 10 mm (mostly snow) (data from Burwash Landing Climatological Station 1967–1995). Day length in midwinter is short (5–6 h). The vegetation in this area is heterogeneous, being dominated by white spruce forests (*Picea glauca*), an understory of willow (*Salix* sp.) and birch (*Betula glandulosa*), willow shrub thickets, and lesser contributions of grass meadows and trembling aspen (*Populus tremuloides*) and balsam poplar (*Populus balsamifera*) stands.

2.2. Study species

The red squirrel is a diurnal, medium-sized (ca. 250 g) tree squirrel whose distribution in North America covers the entire boreal forest (5 million km^2), western cordilleran region, and northeastern mixed coniferous and hardwood forests (Banfield, 1974; Steele, 1998). Red squirrels are one of the most abundant small mammals in these forests (100–500/km^2—Obbard, 1987). Unlike many other sciurids (e.g. ground squirrels, marmots, and chipmunks), tree squirrels (including red squirrels) remain euthermic and active throughout the year (Obbard, 1987).

Red squirrels are granivores specializing in seeds from pine and spruce (Smith, 1968a,b; Gurnell, 1987; Obbard, 1987). They clip cones from mid-August to late September in the Yukon and store them in food hoards (middens) that form the center of individual-based territories (Smith, 1968a,b; Price et al., 1990; Steele et al., 2005). The seed is then extracted by husking cones at the midden and these stores of cones are utilized throughout winter and into the following spring.

The red squirrel breeding season in the Yukon commences in mid to late winter (late-January to mid-February; Lane et al., 2007), but reproductive chronology is highly dependent on food conditions. All males (including yearlings) are in reproductive condition from January to May and then the proportion of breeding males decline until early August, when all males are non-reproductive (testes regressed and abdominal; Boutin, unpublished data). In the Yukon, females typically produce a single litter that is conceived in March, born in April, and weaned by early July. If there is a mast year (a year of abundant, widespread, synchronous cone production; Lamontagne and Boutin, 2007) or if a female's first litter fails, then she may attempt a second litter (Boutin et al., 2006).

2.3. General field procedures

The University of Toronto Animal Care Committee approved all procedures in accordance with the guidelines of the Canadian Council on Animal Care. Wild red squirrels were captured in Tomahawk live-traps (14 × 14 × 40 cm or 16.5 × 16.5 × 48 cm; Tomahawk Live Trap Company, Tomahawk, Wisconsin, USA) baited with peanut butter between 07:40 and 9:30 h. Traps were set on or near each squirrel's midden and checked every 60 min; thus the exact time of trap entry is not known and the length of time in the trap is an estimate. Squirrels were captured either on three unmanipulated sites (each 40 ha) that have been monitored since 1987 (Boutin et al., 2006) or on random sites in nearby forest. Those captured on unmanipulated sites were released on their territories immediately after blood sampling and collecting body measures. Squirrels captured for the first time were given a numbered tag in each ear, weighed to the nearest 5 g with a Pesola spring-scale, sexed, and sexual condition determined (males: testes scrotal or abdominal [determined by palpation]; females: pregnant, lactating, or not in breeding condition). Those captured at random sites were sacrificed with a halothane overdose and paired testes mass (to 0.001 g) determined.

We captured males during (1) the pre-breeding season (February 2004), (2) the late breeding season May 1996 and 2004), (3) the post-breeding season (June 2004, most testes still scrotal but declining in size) and (4) the non-breeding season (August 1999, July and August 2003 and 2004). We captured females only during the non-breeding season (July and August 2003 and 2004). Traps containing squirrels were initially placed in a canvas bag and then transported to a nearby site for processing. Here, they were covered with a burlap bag to minimize stress and visual contact. The time between initial capture and the first blood sample was approximately 2 h. It is extremely difficult to bleed these animals immediately at the site of capture without anesthesia, particularly in harsh weather conditions.

Population density was determined by complete enumeration of all animals each year on the two most intensively studied unmanipulated sites (Sulphur and Kloo sites, McAdam et al., 2007). Individually marked territory owners were identified through a combination of live-trapping and behavioral observation of territorial behavior (McAdam et al., 2007). We censused the population in May (adults only) and August (adults and juveniles) every year. We used the mean estimate of density in each season from these two sites. The average density per ha on these two sites were similar from 1987 to 2005 (spring, $N = 19$ years: 2.25 ± 0.16 vs 2.31 ± 0.23 and late summer, $N = 18$: 2.58 ± 0.17 vs 2.63 ± 0.27, on Suphur and Kloo, respectively, Boutin unpublished data).

2.4. Blood sampling and hormonal challenge protocols

We collected blood samples from two groups of males: (1) a small sample was shot to compare with subjects captured by live-trapping, and (2) all other subjects were captured by livetraps and subsequently bled. Lethal samples were collected during 4–6 May 1999 ($N = 6$), 3–19 August 1998 ($N = 6$) and 16 August 1999 ($N = 1$) near the Alaska Highway by a gun shot (22 calibre) and bled within 2–3 min by heart puncture. DHEA levels from these animals were compared with those from animals captured at the same time by livetrap using procedures given above.

For livetrapped subjects, the animals were allowed to habituate to processing site for 1 h. Animals were anaesthetized prior to obtaining a blood sample using isoflurane USP delivered at 3.5% in air from a purpose-built portable anesthetic delivery unit. Anesthesia was induced within 15–30 s and blood samples were collected within 1 min of induction. Each squirrel was bled by a suborbital sinus puncture using a heparinized glass pipette and immediately returned to its holding trap. This blood sampling procedure did not affect survival (Boutin unpublished data).

To examine the effects of exogenous ACTH on plasma DHEA levels, we collected four blood samples over a 2-h period. Blood from the first sample (the BASE bleed, 400 μl) was used for measuring baseline DHEA levels. Immediately following the collection of the first sample animals received an intra-muscular injection into the thigh of 4 IU/kg of synthetic ACTH (Synacthen Depot, CIBA, Ontario, Canada). Subsequent blood samples (all 200 μl) were collected at 30, 60, 120 min post-ACTH injection (called the P30, P60, and P120 bleeds, respectively). This ACTH stimulation challenge was carried out on males in February, May, and June 2004 and on both males and females in July and August 2003 and 2004.

Blood samples were stored at 4 °C until being centrifuged (within 2 h). The separated plasma was then frozen at −20 °C, transported to Toronto, and stored at −80 °C until analysis. Steroids are very stable for long periods when stored below −40 °C (Schneider et al., 2007).

2.5. Measurement of plasma DHEA

DHEA was measured in duplicate using a double antibody radioimmunoassay (DSL-8900, Diagnostic Systems Laboratories, Webster, TX) and all assays were run from February to May 2007. The DHEA assay was modified to increase sensitivity (Granger et al., 1999), as done previously (Goodson et al., 2005; Newman et al., 2008). Briefly, supplied DHEA standards were diluted 10× in phosphate buffered saline with gelatin (PBSG), the primary antibody was diluted 4× in PBSG, and the tracer was diluted 4× in PBSG. Each sample (5 μl) was extracted with redistilled dichloromethane. Extracts were dried under nitrogen and reconstituted in assay buffer. Samples were incubated with the primary antibody for 30 min at 37 °C. Next, [^{125}I]-DHEA was added to samples, followed by an incubation for 180 min

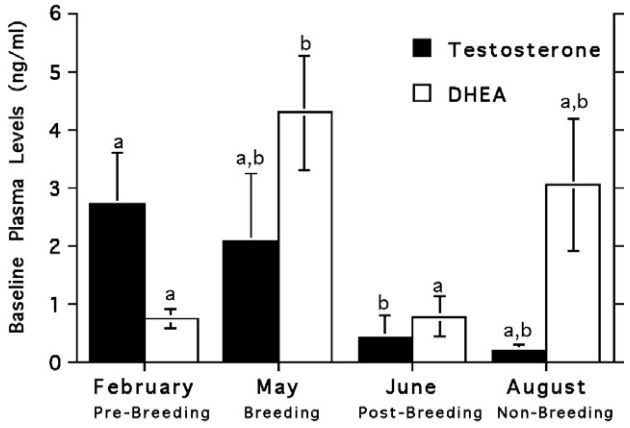

Fig. 1. Changes in baseline concentrations of plasma DHEA and testosterone (means ± SE) in wild male red squirrels sampled at four times over the year. Both steroids changed significantly over time ($p = 0.001$ and $p = 0.016$, respectively). Within-hormone comparisons were performed on $\log(x+1)$ transformed data with the Tukey–Kramer multiple range test, and bars with the same superscript letter are not significantly different. Samples sizes for DHEA in February, May, June, and August were 8, 10, 8, and 15, respectively, and for testosterone were 26, 10, 24, and 5, respectively.

at 37 °C. The secondary antibody/precipitating reagent mixture was then added. After a 20-min incubation at room temperature, the samples were centrifuged at 3000 rpm for 20 min. Samples were decanted, and the pellets were counted for 2 min on a gamma counter.

The DHEA antibody has a low cross-reactivity with DHEA-S (0.02%), cortisol (<0.001%), 16β-OH DHEA (0.041%), androstenedione (0.46%), testosterone (0.028%), and 17β-estradiol (<0.004%; E. Chin and K. Soma, unpublished data). Every assay included water blanks, standards, and quality controls. When multiple samples for the same animal were run (e.g. four sequential blood samples in response to the ACTH challenge), all samples were run within an assay to reduce between assay variation. The average recovery of the 100 pg quality control was 83.9% ± 0.013 (1 SE) ($N = 18$ runs; range 77–96%). The final concentration for each sample was adjusted for the within-assay recovery. The lowest point on the standard curve was 2 pg/tube. The intra-assay coefficients of variation were 4% and the interassay variation was 13.2% for the 25 pg quality control and 6.8% for the 100 pg quality control.

2.6. Measurement of plasma testosterone/dihydrotestosterone

Plasma testosterone plus dihydrotestosterone was measured in duplicate by radioimmunoassay. The protocol for the radioimmunoassay of testosterone was based on that of Abraham et al. (1971) with diethyl ether extraction (twice) of duplicate plasma samples (Boonstra and Boag, 1992). Prior to extraction each plasma sample (25 μl) was treated with 20 μl NH_4OH to saponify triglycerides. Every assay included water blanks, standards, and quality controls. The mean recovery of testosterone added to plasma was 99.7% ± 1.13 (range 95.9–101.4, $N = 5$) and no adjustment was made to the final concentration. The antibody (P43/11) was produced by Croze and Etches (1980) and has a low cross-reactivity to DHEA (0.75%, Boonstra unpublished data) and high cross-reactivity to 5α-dihydrotestosterone (62%). Assay sensitivity was 10 pg/25 μl plasma. Non-detectable samples were conservatively ascribed a value of 10 pg. The intra- and inter-assay coefficients of variation for testosterone were 5% and 6%, respectively.

2.7. Statistical analysis

All statistical tests were performed using StatView (Caldarola et al., 1998) and according to procedures in Zar (1999) and Sokal and Rohlf (1995). All data are presented as means ± 1 SE. Prior to analysis, we used the $\log(x+1)$ transformation to make the variances for the DHEA and testosterone data homogeneous. We used: (1) two-tailed *t*-tests to test for difference in testes mass with season; (2) one-way analysis of variance (ANOVA) to test for differences in DHEA and testosterone levels with season; (3) two-way ANOVA to test for differences in (i) DHEA levels with collection method (shot or livetrapped) and season; and (ii) DHEA levels with sex and year; (4) a repeated-measures ANOVA in males and in females to test for the effects seasonal differences following an ACTH injection; (5) regression analysis to examine the relationship between log DHEA levels and either testosterone levels or testes mass; and (6) Pearson product-moment correlation analysis to examine the relationship between DHEA levels and population density. Post hoc comparisons were performed using Tukey--Kramer multiple comparison test. The null hypothesis was rejected at $P < 0.05$.

3. Results

3.1. Impact of trapping stress on DHEA levels

We compared plasma DHEA concentrations in shot ($N = 13$) versus livetrapped male squirrels ($N = 15$). There was a significant treatment effect ($F_{1,24} = 17.75$, $P < 0.001$), no season effect ($F_{1,24} = 1.80$, $P = 0.19$), and no interaction effect ($F_{1,24} = 0.07$, $P = 0.80$). DHEA levels were significantly lower in shot (1.716 ± 0.428 ng/ml) than livetrapped animals (5.222 ± 1.030 ng/ml). Thus, livetrapping (capture in a trap and subsequent handling) increases plasma DHEA levels in these animals.

3.2. Overview of baseline DHEA levels in males and females

In livetrapped subjects, we analyzed plasma DHEA levels in 116 wild animals (72 males and 44 females) from four different years. In males, the overall mean baseline concentration was 1.783 ng/ml (±0.323, CV = 150%) with a range of 0.096–16.952 ng/ml. Of these, 31 (43%) had levels >1 ng/ml and 7 (10%) had values >4 ng/ml. In females, the overall mean baseline concentration was 1.532 ng/ml (±0.392, CV = 170%) with a range of 0.044–14.602 ng/ml. Of these, 16 (36%) had values >1 ng/ml and 3 (7%) had values >4 ng/ml.

3.3. Seasonal changes in baseline male DHEA and testosterone

We examined for the changes in DHEA levels with season (Fig. 1). For the August period, we only included males captured from 11 August onwards, when levels for both hormones were measured in males. Plasma DHEA concentrations varied significantly over the year ($F_{3,37} = 6.73$, $p = 0.001$), with those in February and June being low (<0.8 ng/ml), those in May high (4.2 ng/ml), and those in August intermediate (3.1 ng/ml). February and June samples differed significantly from May samples. However, the variation in DHEA levels, particularly in May and August, was high.

Plasma testosterone concentrations also varied significantly over the year ($F_{3,63} = 3.74$, $p = 0.016$, Fig. 1), with concentrations being high (>2.0 ng/ml) in the pre-breeding (February) and breeding (May) seasons and low at the end of the breeding (June) and non-breeding seasons (August: 0.20–0.42 ng/ml). Only February concentrations differed significantly from those in June. Again, the variation among males in testosterone concentrations, especially in May, was high. We could not test for variation among years (as we did for DHEA) because as we did not have sufficient sample volume. The changes in testosterone concentrations mirrored those in testes mass. From the males autopsied from 1996 to 2004 (collected only in May and August), paired testes mass declines significantly ($t = 8.14$, $p < 0.0001$) from May (0.87 ± 0.13 g, $N = 22$, range 0.195–2.112 g) to August (0.12 ± 0.01 g, N = 43, range 0.036–0.324 g).

Thus, these results suggest that concentrations of plasma DHEA and testosterone are inversely related. We examined this hypothesis in two ways. A number of males were sacrificed after the challenge protocol and we assessed the relationship between DHEA concentrations and (a) testes mass and (b) testosterone concentrations. Both latter values were not always available for each male and hence the samples sizes differ. Fig. 3 indicates that there is an inverse relationship between DHEA concentrations and testes mass (regression: testes mass = −0.804 (log DHEA concentration) + 0.998, $F_{1,25} = 5.88$, $p = 0.023$). However, there was no relationship between DHEA and testosterone concentrations ($F_{1,30} = 0.08$, $p = 0.78$).

3.4. Yearly variation in DHEA levels

Variation in DHEA concentrations among years was examined from baseline samples collected in August, the only month in

which males were bled in each of 4 years. DHEA concentrations varied significantly among years ($F_{3,29}$ = 8.11, p = 0.0004), with significantly higher concentrations in 1999 than in all other years (3-fold greater than in 1998 and ~14-fold greater than in 2003 and 2004; the latter 3 years did not significantly differ; Fig. 2). Marked differences among years in DHEA levels were positively correlated with mean population density, with the correlation being significant for the spring population density estimate that consisted only of overwintering adults ($F_{1,2}$ = 21.49, p = 0.019, r^2 = 0.96), but not for the late summer population density estimate that consisted of both adults and juveniles ($F_{1,2}$ = 5.60, p = 0.14, r^2 = 0.74). Thus yearly variation in DHEA levels in the non-breeding season is pronounced and positively correlated to population density.

3.5. Male–female DHEA comparisons

To assess whether males and females had similar plasma DHEA levels, we examined only baseline concentrations from animals from the summers of 2003 and 2004 when both sexes were sampled (Fig. 4). We found a significant sex effect ($F_{1,67}$ = 8.13, p = 0.006), no year effect ($F_{1,67}$ = 3.36, p = 0.071); and no interaction effect ($F_{1,67}$ = 0.39, p = 0.53). Females had concentrations that were over twice those of males (1.23 ± 0.25 vs 0.55 ± 0.10 ng/ml, respectively). The year effect is suggestive in that animals in 2004 had concentrations almost twice as high as those in 2003 (1.15 ± 0.26 vs 0.67 ± 0.10 ng/ml, respectively).

3.6. Impact ACTH stimulation challenge on DHEA concentrations

3.6.1. Males

Males subjected to the ACTH challenge were captured in 2003 and 2004 in three periods: the winter pre-breeding period (February 2004), the late spring post-breeding period (late May and June 2004), and the summer non-breeding period (late July and August

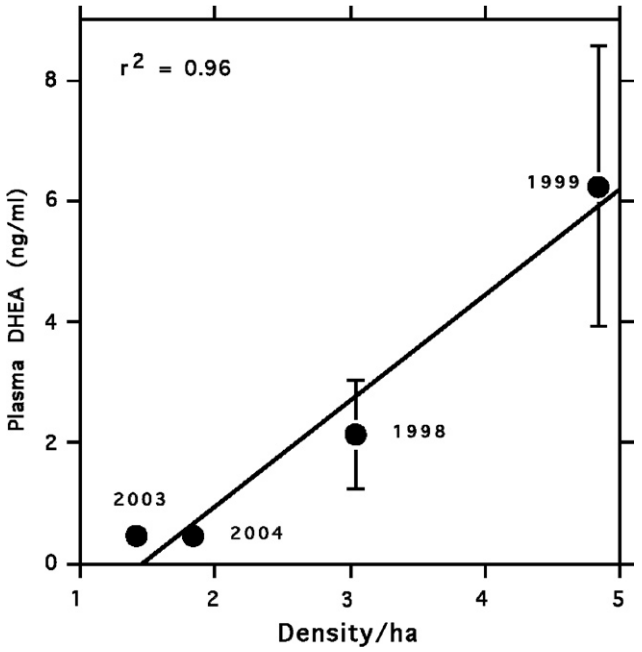

Fig. 2. The relationship between mean baseline concentration of DHEA (±SE) in non-breeding male red squirrels sampled in four different summers and mean population density in the springs of those years (p = 0.019). DHEA levels differed significantly among years (p = 0.0004; 1999 > 1998 = 2003 = 2004). The error bars for 2003 and 2004 were smaller than the size of the dot symbol and thus not obvious on the graph. Samples size for DHEA in 1998, 1999, 2003, and 2004 were 6, 6, 11, and 10, respectively.

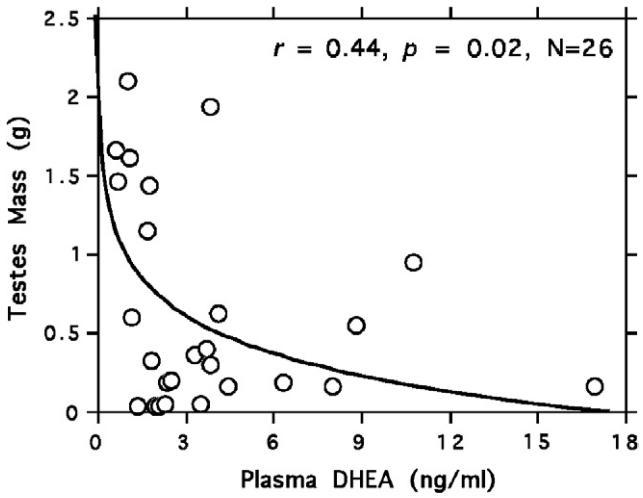

Fig. 3. The logarithmic relationship in male red squirrels between DHEA baseline concentrations and testes mass (p = 0.023).

2003 and 2004) (Fig. 5). Averaged over the entire experiment, there was a no significant difference among seasons ($F_{2,40}$ = 1.77, p = 0.18). The response to the ACTH injection over the experiment was highly significant ($F_{3,120}$ = 12.38, p < 0.0001), but there was no interaction effect between season and subject ($F_{6,120}$ = 0.40, p = 0.88). Averaged over all males (N = 43), ACTH caused an approximate doubling of plasma DHEA within 30 min of injection (P30) (Fig. 5). By P120, concentrations were declining but still above BASE concentrations, but lower than at P30 and P60 (Fig. 5).

3.6.2. Females

Non-breeding females were subjected to the ACTH stimulation challenge in the summers of 2003 and 2004. The data were split into those subjected to this challenge in mid-summer (28 July–5 August) and those challenged in late summer (6–23 August) (Fig. 6). Averaged over the entire experiment, there was no significant difference among seasons ($F_{1,40}$ = 0.007, p = 0.93). The responses to the ACTH injection over the time were highly significant ($F_{3,120}$ = 5.43, p < 0.002), but there was no interaction effect between season and subject ($F_{3,120}$ = 0.45, p = 0.72). Averaged over all females (N = 42), ACTH caused DHEA concentrations to increase about 54% within 30 min of injection (Fig. 6). By P120, concentrations were about 14% above BASE concentrations (Fig. 6).

4. Discussion

Our findings in red squirrels lead us to three major conclusions. First, plasma DHEA levels in both males and females can reach high concentrations (up to 16.952 ng/ml in males and 14.602 ng/ml in females), markedly exceeding concentrations found in laboratory rats and mice and similar to plasma DHEA concentrations in humans. This is the first report of such levels in a free-ranging wild mammal. Second, plasma DHEA concentrations show seasonal (Fig. 1) and yearly (Figs. 2 and 4) variation. Seasonal variation in males was inversely related to reproductive condition (Figs. 1 and 3). Yearly variation in males in late summer was positively related to population density (Fig. 2). Third, circulating levels of DHEA respond rapidly to ACTH treatment in both males and females (Figs. 5 and 6).

Our livetrapping protocol caused an increase in plasma DHEA levels, relative to those from shot red squirrels. Nonetheless, data from live-trapped subjects are useful for several reasons. First, many field studies focus on *relative* differences in hormone levels (e.g. Boonstra et al., 1998; Bradley 1990). What is critical is that pro-

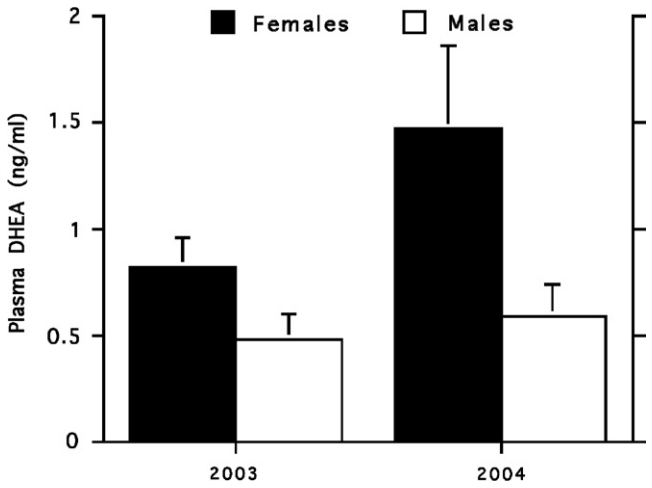

Fig. 4. Females have significantly higher DHEA concentrations in summer than males ($p = 0.009$) and concentrations in 2003 were lower than the same time in 2004 ($p = 0.053$), Samples sizes for males in 2003 and 2004 were 12 and 16, respectively, and for females, 16 and 27, respectively.

cedures are standardized and that there are no systematic biases across the groups being compared. Second, the marked seasonal, yearly, and sex differences cannot be explained by the livetrapping regime, which was standardized. Third, the livetrapping protocol did not block the capacity for exogenous ACTH to increase plasma DHEA levels even further than baseline levels.

Understanding the variation in DHEA levels within and among years is an important goal. First, the primary food ensuring winter survival—white spruce cones and their seeds—is highly variable. Cone production varies both in time (among years) and space (across the forest) (Lamontagne and Boutin, 2007). This can affect red squirrel population density through variation in rates of reproduction (Boutin et al., 2006) and survival (McAdam and Boutin, 2003). This density variation will influence territory size and hence the potential for territorial conflict as food supply waxes and wanes. Second, levels of DHEA and DHEA-S decline with age in primates (Sapolsky et al., 1993; Lane et al., 1997; Dharia and Parker, 2004). Red squirrels are long-lived (up to 8 years; McAdam et al., 2007) and for many of our subjects we do not know their age. Third, differences in capture history (new animals versus those captured repeatedly) and length of time in the trap prior may contribute to variation in DHEA levels.

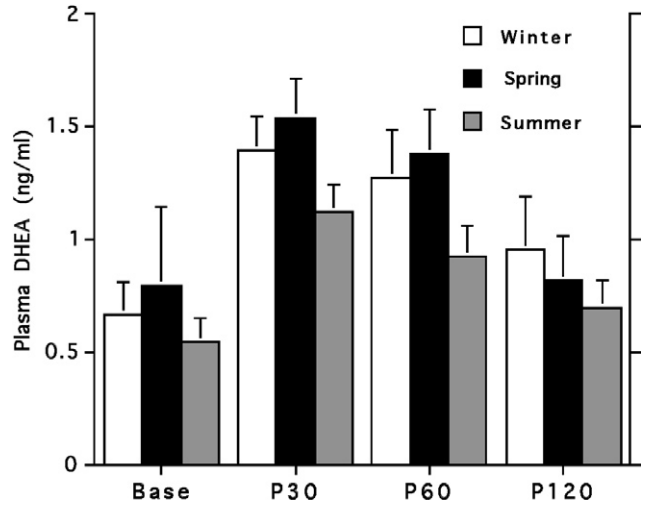

Fig. 5. ACTH stimulation challenge in male red squirrels. An ACTH injection after the BASE bleed causes a rapid increase in DHEA concentrations (repeated-measures ANOVA, $p < 0.0001$), but males did not differ across seasons ($p = 0.18$). P30, P60, and P120 represent times of bleeding 30, 60, and 120 min post-ACTH injection. Samples sizes for males in winter, spring, and summer were 7, 8, and 28, respectively.

The concentrations of plasma DHEA in red squirrels can be high and are comparable with those found in humans and other primates and are much higher than those found in most laboratory and domestic animals. Male golden hamsters (*Mesocricetus auratus*) also have relatively high levels of circulating DHEA and DHEA-S (Pieper and Lobocki, 2000). Importantly, these data and avian studies (Hau et al., 2004; Soma, 2006) indicate that adrenal synthesis of DHEA is not limited to primates. There are approximately 5400 extant mammalian species and 9000 extant bird species, and future studies should be extended to a broad range of species to understand species differences in DHEA synthesis and function.

Female red squirrels have higher DHEA concentrations in mid-to-late summer than males (Fig. 4). This may be related to differences in female and male behavior at that time. To increase juvenile survival, approximately one third of females (Berteaux and Boutin, 2000), but not males, bequeath part or all of their territory to one or more of their offspring (Price and Boutin, 1993; Boutin et al., 2000). Overwinter survival is absolutely dependent on owning a territory and a midden (Kemp and Keith, 1970; Larsen and Boutin, 1994) and thus this female behavior increases the survival probability of their offspring. However, bequeathing adult females must then secure another territory for their own overwinter survival. Thus, there may be increased intruder pressure by females than males, resulting in more conflict and higher DHEA levels.

The increase in plasma DHEA after ACTH injections suggests that the circulating DHEA is of adrenal origin. Exogenous ACTH caused plasma DHEA levels to increase by 103% (Fig. 5) in males and 54% in females (Fig. 6) within 30 min. In humans, where it is known that circulating DHEA originates from the adrenal cortex, plasma DHEA levels are increased by even by very low doses of ACTH (Arvat et al., 2000). In contrast, in rats, which have low or no adrenal production of DHEA, injections of ACTH have no effect on plasma DHEA levels (Bélanger et al., 1990). Furthermore, castration decreases plasma DHEA to non-detectable levels in rats, guinea pigs, dogs and rabbits (Schiebinger et al., 1981; Bélanger et al., 1989, 1990), but not in humans or hamsters (Bélanger et al., 1989; Pieper and Lobocki, 2000). Adrenalectomy decreases plasma DHEA in hamsters (Pieper and Lobocki, 2000). Future studies will examine the source of the DHEA in red squirrels.

Plasma testosterone levels are high in the breeding season (February and May) and low thereafter (Fig. 1), and testosterone levels are positively correlated with testes mass. The plasma testosterone levels mirror the results obtained by testes palpation. No other study has reported seasonal changes in red squirrel testosterone levels, although similar seasonal changes in testes size have been seen in other red squirrel studies (Rusch et al., 1982; Layne, 1954). Thus, male red squirrels are highly territorial in the non-breeding season, even though plasma testosterone levels are low and the testes are regressed.

Plasma testosterone and DHEA levels are elevated during the breeding season, and both might regulate territorial behavior at that time. Breeding males engage in scramble competition for females, and females mate with ~7 different males (Lane et al., 2008). Thus, ownership of a territory during the breeding season does not guarantee exclusive mating access to local females. Hence, territoriality even in the breeding season may be related to defense of food resources, as in winter.

Our results are consistent with the hypothesis that DHEA supports red squirrel territorial behavior during the non-breeding season. Importantly territorial behavior is essential to protect food resources that permit survival in a very harsh environment with long, cold winters. First, plasma DHEA levels were high in August,

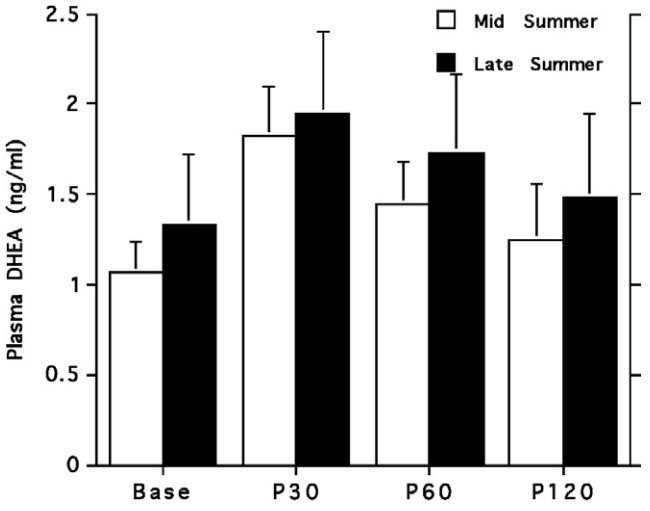

Fig. 6. ACTH stimulation challenge in female red squirrels. An ACTH injection after the BASE bleed causes a rapid increase in DHEA concentrations (repeated-measures ANOVA, $p < 0.002$), but females did not differ across seasons ($p = 0.93$). Samples sizes for females in mid-summer and late summer were 15 and 27, respectively.

over a month after reproduction was completed and when plasma testosterone levels were low. Second, DHEA levels in August were positively correlated with population density. Increased population density results in increased territorial interactions. We have observed a 5-fold difference in population density over the last 20 years, related to changes in cone production (Boutin unpublished data). The very high DHEA levels in May and August 1999 were associated with the highest population density that we have observed in our 20-year study, and the low DHEA levels in 2003 and 2004 were associated with the lowest population densities that we have observed. Thus, these data highlight the need for long-term field studies.

Non-breeding seasonal aggression and territoriality is common in many mammals, yet for virtually all, the hormonal basis for this behavior is unknown. Chipmunks and ground squirrels are among the best-studied group where we have some knowledge of both behavior and hormonal levels. At one end of spectrum are male yellow-pine chipmunks (*Tamias amoenus*), in which testosterone levels are high only during the mating period and then decline to low levels (mirroring testes size), irrespective of age (Place and Kenagy, 2000). Nevertheless, they are highly aggressive throughout the summer (Broadbrooks, 1970). At the other end of the spectrum are male arctic ground squirrels (*Spermophilus parryii*), in which testosterone levels are high both during the mating period and the pre-hibernation period in late summer (the latter occurs when the testes are regressed in both juvenile and adult males—Barnes, 1996; Boonstra et al., 2001). They are aggressive in the pre-hibernation period (Carl, 1971) and this is postulated to be related to a defense of an hibernacula near to females and to the protection their underground seed cache (Carl, 1971; Boonstra et al., 2001; Gillis et al., 2005). A similar late summer testosterone peak has been described for juvenile, but not adult, golden-mantled (*S. lateralis*, Barnes, 1996) and California ground squirrels (*S. beecheyi*, Holekamp and Talamantes, 1991). In *S. beecheyi*, Holekamp and Talamantes (1991) suggested that the late summer rise functions in the onset of puberty. Thus, in these sciurids in the non-breeding season, there is no consistent pattern between testosterone levels and spacing behavior. The difficulty will be to tease out correlation from causation (as has been done in the dusky woodrat, Neotoma fuscipes, in which seasonal aggression is not dependent on the presence of testes—Caldwell et al., 1984), and DHEA may play a role in some or all of these species.

In red squirrels, circulating DHEA, rather than testosterone, may regulate territorial behavior in the non-breeding season because of the negative costs that would occur with high systemic testosterone levels in winter (increased energetic costs, increased muscle growth, immune suppression, etc.) (Wingfield et al., 2001; Reed et al., 2006). Winter is a time of severe food limitation and extreme temperatures, and territorial defense of food stores permits winter survival with a small body mass. Most small mammals and birds enhance thermogenic activity and immune activity to promote survival in winter (Nelson et al., 2002). Circulating DHEA, but not testosterone, may permit those activities because DHEA acts as a pro-hormone. The effects of DHEA on specific regions of the brain and body are contingent on metabolic enzymes in target tissues (Soma, 2006).

To further explore the role of DHEA in regulating red squirrel territorial behavior, we suggest three lines of future research. First, field experiments should examine the behavioral effects of DHEA manipulations. Second, gonadectomy and adrenalectomy experiments should address the source of circulating DHEA in red squirrels. Production of DHEA in the brain as a neurosteroid should also be explored (Baulieu and Robel, 1996). Third, experimental simulated territorial intrusions and experimental food manipulations should examine the relationships among intruder pressure, food supply, and DHEA levels.

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