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Seasonal changes in plasma testosterone and glucocorticosteroids in free-living male yellow-pine chipmunks and the response to capture and handling

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Abstract We measured plasma levels of testosterone, corticosterone, and cortisol in free-living male yellow-pine chipmunks to demonstrate the patterns of seasonal variation and to assess the effects of capture and handling on hormone levels. We achieved the latter by modifying our standard trapping technique (blood samples collected within 1–3 h of capture) to obtain blood samples that allowed measurement of hormone levels within 3 min of capture (basal) and again 30 min later. By alternating the modified and standard trapping techniques over 7 months of the active season we demonstrated that seasonal patterns of variation in steroid hormone levels can be accurately described with the simpler, standard trapping technique. Basal and 30-min post-capture testosterone levels were high during mating and dropped to a persistently low level thereafter. Conversely, both cortisol and corticosterone were at their seasonal low during mating and climbed to peak levels in June following reproduction. Plasma glucocorticosteroid levels increased during the 30 min after capture and handling at all times of the active season, and these elevated levels were similar to the levels obtained by standard trapping. Testosterone levels during the mating period also increased in response to capture and handling. The contrasting patterns of seasonal variation in glucocorticosteroid and testosterone levels and the changes induced by capture and handling suggest that when testosterone concentration is high, adrenocortical activity is suppressed.

Key words Testosterone · Glucocorticosteroids · Seasonality · Male chipmunks

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Abbreviations *CBG* corticosteroid-binding globulin · *GC* glucocorticosteroid · *T* testosterone

Introduction

Temperate-zone environments are seasonal and the environmental variability associated with these environments challenges animals to make behavioral and physiological adjustments over a broad temporal scale, ranging from seconds and minutes to weeks and months. Hormones regulate a diverse suite of physiological and behavioral activities associated with mating, foraging, migration and hibernation. Changes in hormone levels often reflect the physiological adjustments that animals make as the environment changes (Wingfield and Kenagy 1991). Thus, endocrinological studies, particularly when based in the field, can provide insights into the ways animals anticipate and adjust to changing conditions (Wingfield et al. 1992; Astheimer et al. 1995).

We measured the gonadal steroid testosterone (T) and the glucocorticosteroids (GCs) corticosterone and cortisol in free-living male yellow-pine chipmunks, *Tamias amoenus*, to determine if changes in levels of T and the GCs are associated with changes in reproductive condition. In previous laboratory and field studies of larger scuirids (ground squirrels and marmots) in which the seasonal variation of plasma steroids was described, the investigators did not account for the potential effect of capture and handling on hormone levels (Barnes 1986; Armitage 1991; Holekamp and Talamantes 1991; Boswell et al. 1994). The standard trapping technique used by the field investigators resulted in the collection of blood samples an hour or more after capture. Animals may respond to laboratory or trap confinement as a stressful event, which would activate GC synthesis and secretion from the adrenal cortex; this response may in turn have inhibitory influences on reproductive physiology and endocrinology (Greenburg and Wingfield 1987; Sapolsky 1987).

In this study we have assessed the interrelations of the GCs and T in male chipmunks as they respond to a potential acute stress (capture and handling) while making the transition from reproductive to nonreproductive condition during their active season. We used a modified trapping technique to measure plasma levels of GCs and T within 3 min of capture (basal) and to quantify the effects of capture and handling on those levels. We also compared hormone levels from this modified trapping technique to those of standard trapping (1–3 h in trap before blood sampling) to assess the ability of these methods to describe the patterns of seasonal hormone variation accurately.

Materials and methods

Animals

We studied individually marked male chipmunks by live-trapping, examination, and release from autumn 1995 through autumn 1996 at a previously described study site (Kenagy et al. 1989; Kenagy and Place 2000). The site is montane forest (elevation 670 m) in Chelan County, Washington, with an area of 3.8 ha covered by a 14×14 grid of 196 Sherman live traps on 15-m centers.

Sampling

Following initial observations and trapping in fall 1995, we conducted a general trapping study to assess seasonal activity, reproductive condition, and plasma levels of T and GCs in spring through early autumn of 1996, consisting of 37 trapping days over 7 months from late March through September. We began collecting blood samples from juvenile males in July, after they had been observed above ground for about a month. For our standard seasonal trapping on the grid, traps were generally baited in the early morning with a bird seed mix and collected about 2 h later; we took all traps to a central area for processing. The chipmunks were weighed with a Pesola spring balance, examined for reproductive condition and other external information. We used a 4-point grading system (0–3) as a measure of testicular size; we took enlarged, scrotal testes (grades 2–3) as an indication of mating condition, while regressed, non-scrotal testes were assigned grades 0 or 1. Animals were lightly anesthetized (10- to 20-s exposure) with ethyl ether inhalant before we took a blood sample from the infraorbital sinus, which was accomplished within 3 min of removing the animal from its trap. Despite rapid handling of individuals under routine conditions, we recognize that animals were typically held in the traps for 1–3 h before blood sampling, and therefore it is unlikely that these hormone concentrations were basal.

To measure basal hormone levels and examine the adrenocortical response to capture and handling, we used a modified trapping protocol on 16 days separate from routine trapping of the grid. We quickly obtained blood samples within 3 min of capture from animals that we directly observed entering strategically set traps. An animal was returned to and held within its trap for an additional 30 min, after which we obtained a second blood sample. A tight cluster of up to 40 specially placed traps for each of one to three observers was continuously monitored, beginning early in the morning and continuing no later than noon. As soon as a trap door closed, we noted the time, ran to obtain the trap, and began processing the animal in the usual manner. To assess the effects of reproduction on the adrenocortical response, we conducted these tests both during and after the reproductive season.

We captured 37 individual adults and 46 young-of-year males. We report data on plasma hormone titers and body mass by combining data obtained over short calendar intervals (1–2 weeks) and reporting them for a single median monthly date. Animals were often caught multiple times during the active season, but rarely was an individual caught and bled during each of our seven sampling intervals. Specifically for seasonal hormone titers of animals captured using standard trapping, most samples came from males bled three or fewer times (20 of 28 animals), while only two animals were bled during each of the 7 months. Eight of 15 juvenile males were sampled once or twice over a 4-month period, seven individuals were bled on three occasions. Similarly when we obtained samples to measure basal hormone levels and study the effect of capture and handling on those levels, we bled most adults and juveniles during only one or two of the four sampling intervals.

Blood samples of about 400 µl were taken for routine seasonal sampling on the grid, and samples of 200 µl for the two samples at 30-min intervals for assessment of the response to capture. Blood was stored in a cooler for several hours and centrifuged. Plasma was stored in 200-µl plastic microcentrifuge tubes at −20 °C until analysis.

Hormone radioimmunoassays

Plasma samples were thawed and assayed for testosterone and corticosterone as described by Wingfield and Farner (1975) and Ball and Wingfeld (1987); samples with sufficient residual volume were frozen and later assayed for cortisol (see below). Briefly, plasma samples of 50–100 µl were extracted in dichloromethane, dried under N_2 and reconstituted in 10% ethyl acetate:90% isooctane. Steroids were separated via Celite (Sigma) chromatography; recovery of testosterone and corticosterone was measured for each sample. After separation samples were dried under N_2, reconstituted in phosphate-buffered saline, and allowed to equilibrate overnight. Samples were incubated with a primary antibody to corticosterone or T (Endocrine Sciences, Tarzana, Calif.) and tritiated-labeled hormones (NEN Research Products, Boston, Mass.); dextran-coated charcoal was used to separate bound and free-labeled hormone. Intra- and inter-assay variations were 8.9% and 17.4%, respectively, for corticosterone and 11.9% and 11.1%, respectively, for T.

Cortisol was measured using a solid-phase ^{125}I radioimmunoassay (RIA) kit (DiaSorin, previously Incstar, Stillwater, Minn.). Plasma samples were diluted fivefold with the serum blank provided and dispensed in aliquots of 10 µl into duplicate assay tubes. Cross-reaction of the cortisol antibody with corticosterone is $< 0.4\%$, as reported by the manufacturer. A dilution curve of chipmunk plasma was parallel to the standard curve of the kit. Cortisol was not detected in charcoal-stripped chipmunk plasma. Intra- and inter-assay variations were 5.6% and 6.1%, respectively.

Statistics

Results were analyzed with Statview 5.0 (SAS, Cary, N.C.) using analysis of variance (ANOVA). Because most of the seasonal trapping samples (seven different periods, by month) were from animals that were bled once or twice, we used an ANOVA design without repeated measures for hormones. Likewise, we represented body mass over the course of 7 months by entering the first value obtained for an individual in a given month, also applied to an ANOVA without repeated measures. This approach preserves strong sample sizes across seasons and is a practical compromise to the randomness of captures within a large natural population and the low incidence of consistently repeated individuals. A repeated measures ANOVA was used for the 0- to 30-min comparisons of hormones within the same individual, in which blood sampling time was the repeated measure and season was examined as a factor. Paired *t*-tests were used when results from the 0- to 30-min comparisons were analyzed within a single sampling interval. The Fisher's PLSD was used as a post-hoc test.

Results

Adult body mass showed significant seasonal variation ($F_{6,94} = 7.10$, $P < 0.0001$) over the active season (Fig. 1), with the lowest values in early spring following emergence from hibernation. Body mass increased thereafter, reaching a peak in late June. Juvenile males were first captured in early June when their mass was about half that of adult body mass. Juveniles gained weight rapidly, but their body mass remained significantly different ($P < 0.05$) from that of adults until early September (Fig. 1).

All males had fully enlarged, scrotal testes (grade 3) during March and April. Testicular size gradually declined in May, and testes had fully regressed (grade 0) in all males by early June. We never found enlargement of testes in young-of-year males. Adult male levels of T showed a distinct seasonal pattern of significant change ($F_{6,62} = 26.81$, $P < 0.001$), with the highest T levels during mating in March and uniformly low T levels in post-reproductive adults and juveniles (Fig. 2).

Plasma levels of corticosterone and cortisol from chipmunks captured and bled using the standard trapping method also showed significant seasonal variation ($F_{6,62} = 12.55$, $P < 0.0001$, and $F_{6,57} = 4.73$, $P = 0.0006$, respectively), with maximum levels in June (Fig. 3). Plasma levels of cortisol were 25 to 50 fold higher than those of corticosterone (Fig. 3). Levels of corticosterone increased sharply from April to May (Fisher PLSD, $P < 0.0001$), following mating, and showed nearly a tripling from April to June. Levels of cortisol rose more gradually to their peak in June showing a more modest but significant increase of about

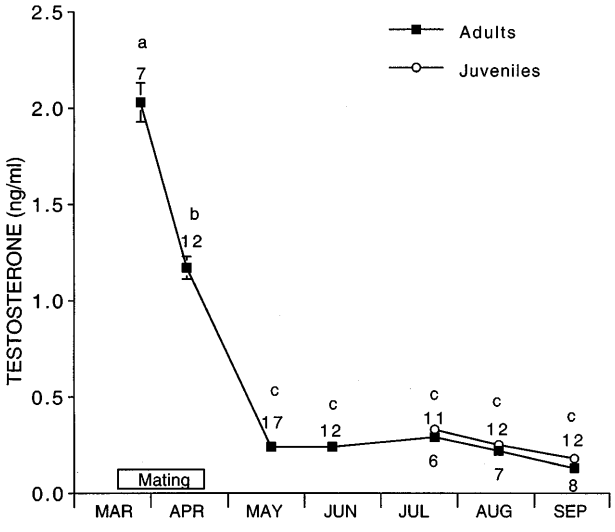

Fig. 2 Seasonal patterns of plasma testosterone (T) in male chipmunks according to age-class. Data are for chipmunks captured in a natural population by standard trapping (see Materials and methods). Data points for adults are significantly different when the letters above differ (Fisher PLSD < 0.05). Symbols and reproductive season as in Fig. 1

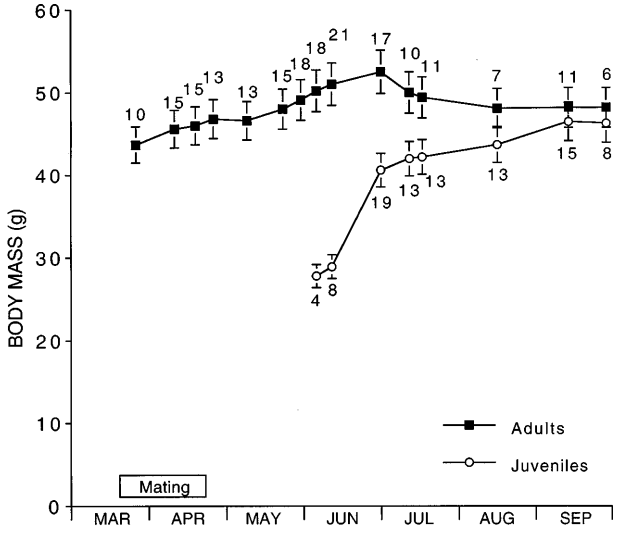

Fig. 1 Seasonal patterns of body mass in male chipmunks according to age-class. Values are mean ± SEM, and sample sizes are indicated. Mating for the population as a whole is indicated by *horizontal bars*, as obtained by observation of individuals and extrapolations based on the individual duration of gestation (~4 weeks) and lactation (~7–8 weeks) (Kenagy and Barnes 1988)

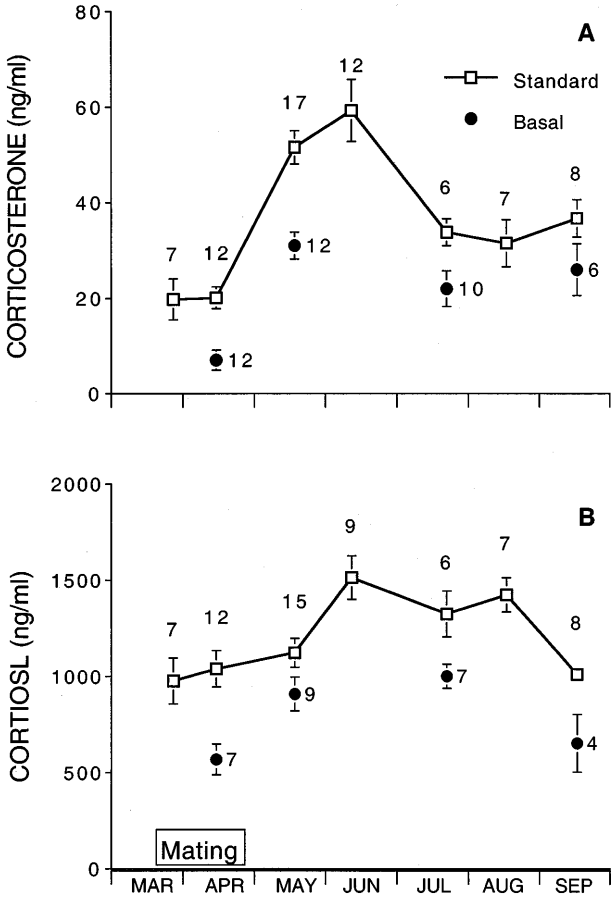

Fig. 3 Seasonal patterns of the plasma (**A**) corticosterone and (**B**) cortisol in adult male chipmunks. Data are for chipmunks captured in a natural population by standard trapping (*open squares*) and by a modified trapping protocol (*filled circles*) in which basal hormone level was determined by sampling an animal within 3 min of its observed capture (see Materials and methods)

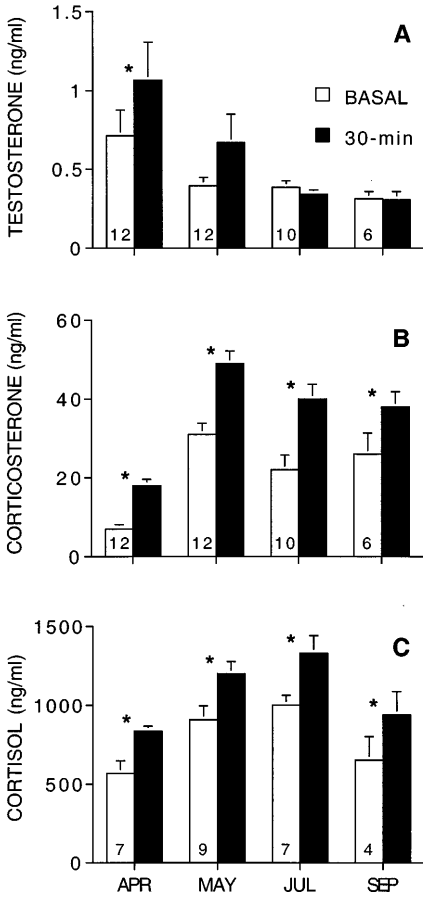

Fig. 4 Basal plasma concentrations (*open bars*) of (**A**) testosterone, (**B**) corticosterone, and (**C**) cortisol and of hormone levels after being held in the trap for 30 min (*solid bars*) following capture and handling in adult male chipmunks over 4 months of the active season. Values are mean + SEM, and sample sizes are indicated. An *asterisk* above pair of columns indicates significant difference between basal and 30-min levels. Basal values of corticosterone and cortisol are repeated in Fig. 3 for comparison to standard values

50% (Fisher PLSD, $P = 0.002$). The decline after the June peak was also relatively greater for corticosterone than for cortisol. We found no significant correlation between body mass and GC levels in adult males either during or after mating ($P = 0.13$ and 0.42 for corticosterone, respectively, and $P = 0.10$ and 0.09 for cortisol, respectively).

The basal levels of corticosterone and cortisol from animals captured using our modified trapping technique also showed significant seasonal variation ($F_{3,36} = 13.03$, $P < 0.0001$ and $F_{3,22} = 5.35$, $P = 0.0063$, respectively; Fig. 3). Basal levels of corticosterone and cortisol were significantly lower than the levels in animals trapped by the standard method ($F_{1,75} = 32.42$, $P < 0.0001$ and $F_{1,59} = 23.00$, $P < 0.0001$, respectively), but the general seasonal patterns were similar in form (Fig. 3).

During the mating period T levels 30 min after capture increased significantly above basal levels (paired *t*-test, $P = 0.018$), while in the months following mating no significant change in T levels occurred over 30 min post-capture (Fig. 4a). However, despite this apparent effect of capture and handling on T levels during mating neither basal nor 30-min post-capture levels were significantly different from levels from the standard trapping method ($F_{1,75} = 0.004$, $P = 0.94$). Regardless of trapping method the post-mating decline in T levels was readily apparent (Figs. 2, 4a).

Capture and handling induced a significant increase in corticosterone and cortisol levels above basal 30 min after capture and handling at all times of the active season ($F_{1,36} = 105.20$, $P < 0.0001$ and $F_{1,23} = 93.05$, $P < 0.0001$, respectively; Fig. 4b, c). The lowest basal and 30-min GC levels were measured during April, while males were still reproductively active. In general the individual basal and 30-min GC concentrations showed a significant positive correlation ($r = 0.820$, $P < 0.0001$ corticosterone; $r = 0.879$, $P < 0.0001$ cortisol).

Young-of-year males also significantly increased their corticosterone and cortisol levels in response to capture and handling ($F_{1,10} = 24.09$, $P = 0.0006$, and $F_{1,9} = 28.17$, $P = 0.0005$, respectively), and as with the adults basal GC levels were significantly lower than levels obtained by standard trapping ($F_{1,31} = 40.54$,

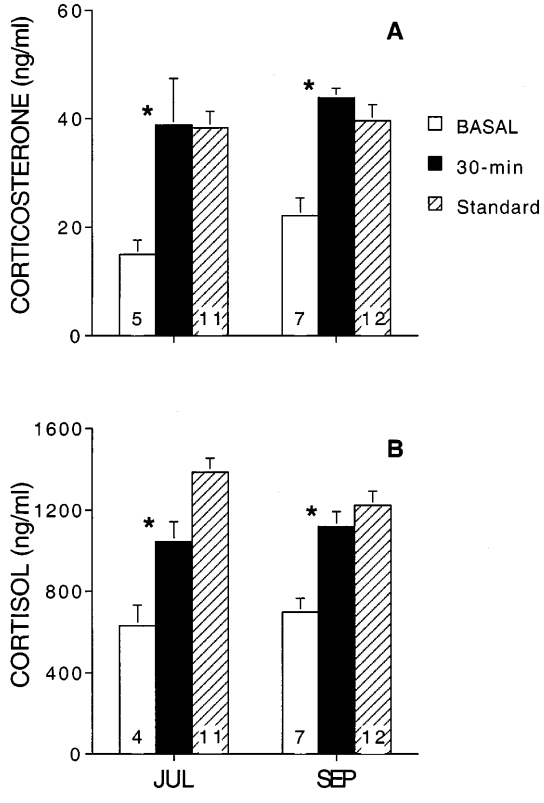

Fig. 5 Basal plasma concentrations (*open bars*) of (**A**) corticosterone and (**B**) cortisol and of hormone levels after being held in the trap for 30 min (*solid bars*) following capture and handling in juvenile male chipmunks at 2 months and 4 months after emergence from their natal burrow. Data from juveniles captured in a natural population by standard trapping in the same month are also shown (*hatched bars*). Values are mean + SEM, and sample sizes are indicated. An *asterisk* above pair of columns indicates significant difference between basal and 30-min levels

$P < 0.0001$, and $F_{1,30} = 69.64$, $P < 0.0001$, respectively; Fig. 5). Despite the significant change in body mass of juveniles from July to September ($P = 0.0045$), GC levels changed very little and did not correlate with body mass on an individual basis ($P = 0.9011$ for corticosterone, and $P = 0.1768$ for cortisol).

Discussion

Capture and handling consistently increased plasma GC levels above basal and induced a rise in T levels during mating. Yet despite the "non-basal" hormone levels obtained by standard trapping with delayed blood sampling our results demonstrate that these easier-to-obtain samples can provide reliable data on general patterns of seasonal change in free-living animals. Regardless of the trapping and blood sampling method used, plasma T concentrations were high during mating and low thereafter, while GC levels were low initially, high after breeding, and then low again before hibernation.

The seasonal increase and decrease in T levels in the yellow-pine chipmunk, *T. amoenus*, coincide with the changes in reproductive state associated with testicular growth and regression (Kenagy and Barnes 1988). T levels dropped promptly after the mating season and remained uniformly low thereafter in both adults and juveniles. The persistently low T levels of post-reproductive and juvenile chipmunks contrast with patterns in ground squirrels (*Spermophilus* spp.). In the arctic ground squirrel, *S. parryii*, adult males experience a second peak in T well after the mating season has ended; this second T peak has been associated with the time of acquisition of a hibernation burrow (Barnes 1996). Juvenile arctic ground squirrels also demonstrate a prehibernation rise in T (Barnes 1996). A similar prehibernation (prepubertal) T peak has been described for juvenile golden-mantled, *S. lateralis* (Barnes 1996), and California ground squirrels, *S. beecheyi* (Holekamp and Talamantes 1991). Unlike arctic ground squirrels, no late season peak was noted in adult golden-mantled and California ground squirrels. The late summer rise in T of juvenile hibernating sciurids has been suggested to function in the onset of puberty (Holekamp and Talamantes 1991). Male chipmunks from our study population typically achieve sexual maturity in their first spring, at ca. 10 months of age, without showing a late summer "T-pulse". Chipmunks and ground squirrels belong to the family Sciuridae, and though their life histories share much in common, many differences exist. Chipmunks, compared to ground squirrels, depend much more on food caches rather than body fat stores for overwinter energy supply (Stebbins and Orich 1977) and have a longer active season (Broadbrooks 1958). The absence of a late-season, prepubertal T-pulse in chipmunks may be related to these differences in overwintering strategy.

Capture and handling induced a significant increase in T levels during the mating period despite the coincident increase in GC levels. Sapolsky (1986) also noted a transient increase in T levels in male olive baboons, *Papio anubis*, during the 1st hour after capture. Baboons experienced a steady decline in T concentrations thereafter. In chipmunks, however, levels of T associated with the two trapping techniques were similar, suggesting that chipmunks left undisturbed within a trap for several hours did not experience marked changes in plasma T concentration. The transient increase of T in baboons was mediated by an attenuated response to the suppressive effects of cortisol (Sapolsky 1985) and a heightened sensitivity to catecholamines (Sapolsky 1986). No similar studies exist for chipmunks, but GC levels did not seem to influence T levels nor affect the T response to capture and handling, as we detected no significant correlations between T and GC levels from individual animals during the mating period.

Based on total (bound + free) GC concentrations, cortisol's role as the principal plasma GC in yellow-pine chipmunks is now well established, both in females (Kenagy and Place 2000) and males (present study). Even though the absolute levels of cortisol and corticosterone differ by 25- to 50-fold, both GCs varied seasonally in synchrony. In the golden-mantled ground squirrel (*S. saturatus*) cortisol levels were only twice as high as corticosterone (Boswell et al. 1994). It is also interesting that in chipmunks both GCs consistently increased in response to capture and handling across all time periods tested (Figs. 4B, 4C), suggesting some role for corticosterone, as well as cortisol, in the response to stressful events. Determining the relative importance of these two GCs within a given species will require studies that quantify free hormone and corticosteroid-binding globulin (CBG) levels, GC-receptor and CBG-binding affinities, and metabolic clearance rates. These studies should also provide insights into how and why chipmunks operate with such remarkably high levels of cortisol, since these levels would be considered pathological in some other animals (Keightley and Fuller 1996).

Some of the seasonal variation in GC levels may be due to the seasonal changes in T. High T levels during mating may suppress GC levels, as described in the rat (Kitay 1963). In mammals it is generally thought that T suppresses total GC levels by reducing the synthesis of CBG by the liver; as a result free GC concentrations remain relatively stable (Gala and Westphal 1965). The semelparous marsupial *Antechinus stuartii* is an exception; T induces a decrease in CBG, while corticosterone secretion is increased. This results in strikingly high free GC levels that are thought to play a causal role in the programmed death of post-reproductive male *Antechinus* (McDonald et al. 1981). Male yellow-pine chipmunks, in contrast, are iteroparous; they mate annually and may live as long as 3–4 years (Broadbrooks 1958). We have not measured CBG or free GC levels in this species; despite their rather high total cortisol levels, we do not expect free GC levels of chipmunks to be unusually high.

Body mass of *T. amoenus* varied over the active season, and the peak coincided with peak GC levels in late June. Despite the generally recognized association of body mass changes with regulation of energy expenditure and intake (Mrosovsky 1990), we found little evidence in chipmunks of a correlation between body mass and GC levels. This point is further demonstrated by comparing GC levels in adults to those of juveniles. Despite the significant differences in mass between adults and juveniles in July, we found their GC levels were remarkably similar, regardless of which trapping and blood-sampling technique we used.

In this study we were able to measure basal plasma steroid levels and demonstrate that capture and handling not only affects plasma levels of cortisol and corticosterone but also increase T levels of yellow-pine chipmunks during mating. Field-based endocrinology studies of small mammals have traditionally used trapping and bleeding techniques like the one we used (Barnes 1986; Armitage 1991; Holekamp and Talamantes 1991; Boswell et al. 1994), and basal hormone levels were probably not obtained. Until recently the effects of capture and handling had not been quantified (Romero et al. 1997; Kenagy and Place 2000). Research on large, free-living mammals (baboons, carnivores, ungulates) has addressed the effects of potential capture-induced stress on plasma steroid levels (Sapolsky 1985; Brown et al. 1991, 1993; Hastings et al. 1992; Van Jaarsveld and Skinner 1992; De Villiers et al. 1995), but basal hormone levels were rarely measured because the need to immobilize these animals resulted in delayed blood sampling. Large size, nocturnality, low capturability, and other logistical problems may prevent investigators from collecting blood samples within three minutes of capture from some mammals. For studies of these kinds of animals hormone data obtained via standard trapping methods will continue to provide useful information about seasonal patterns of variation and insights into the ways that animals make behavioral and physiological adjustments to changing environmental conditions.

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