

Effects of Experimentally Elevated Testosterone on Plasma Glucocorticoids, Body Mass, and Recapture Rates in Yellow-Pine Chipmunks, *Tamias amoenus*

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ABSTRACT In male yellow-pine chipmunks plasma levels of glucocorticoids (GCs) are low while plasma testosterone (T) levels peak during the mating season, suggesting that T suppresses GC levels. To test this hypothesis, free-living, post-reproductive males were implanted during summer with either a T-filled (T-males) or an empty silastic implant (controls or C-males). Body mass and plasma levels of corticosterone, cortisol, and T were measured immediately before and 1 month after implantation. Exogenous testosterone increased T to high physiological levels typical of reproductively active males. By 1 month after implantation, T-males decreased their mean body mass and plasma GC levels, while C-males maintained their mean body mass and GC levels. Even though T-males lost mass, recapture success 1 month after implantation for T-males (71%) was equal to that of C-males (71%). However, the overwinter recapture rate of C-males (83%) was significantly greater than that of T-males (20%). The results support the hypothesis that high plasma T of males during mating has a suppressive effect on plasma GC levels. Additionally, experimentally elevated T significantly reduced the rate of recapture during the following spring, and this may reflect a reduction in local overwinter survival. The suppression of adrenocortical activity by T may contribute to the reductions in prehibernation body mass and post-emergence recapture success. *J. Exp. Zool.* 287:378–383, 2000. © 2000 Wiley-Liss, Inc.

Chipmunks, ground squirrels, and marmots demonstrate pronounced seasonal changes in plasma levels of glucocorticoids (GCs), and these hormone concentrations are generally lowest just after emergence from hibernation (Armitage, '91; Boswell et al., '94; Kenagy and Place, 2000). Conversely, levels of testosterone (T) are relatively high at this time, as mating occurs shortly after emergence (Holekamp and Talamantes, '92; Barnes, '96; Place and Kenagy, 2000). In male yellow-pine chipmunks, *Tamias amoenus*, T levels decline and GC levels tend to peak after the mating period (Place and Kenagy, 2000). In vertebrates generally, elevated GCs consistently suppress plasma T levels and also inhibit behaviors associated with reproduction (Sapolsky, '85; Tokarz '87; Denardo and Licht, '93; Denardo and Sinervo, '94). The reciprocal effect of T on the level of GCs seems to be more complex and varies depending on vertebrate class and species (Silverin, '86; Wingfield and Silverin, '86; Ketterson et al., '91; Denardo and Sinervo, '94; Klukowski et al., '97).

In mammals T is thought to suppress total (free+bound) GC levels (Kitay, '63; Gala and Westphal, '65; Handa et al., '94), but evidence is

generally limited to laboratory animals and to humans. This may be true of chipmunks as well, but an alternative hypothesis warrants consideration. Relatively low GC levels of chipmunks in the field during spring could be a consequence of a hibernation-induced regression of the adrenal cortex. This is supported by Sheppard's ('68) observation of relatively small adrenal glands in chipmunks after emergence from hibernation, i.e., during mating. Thus the inverse relationship of T and GC levels in *T. amoenus* may be a simple correlation alone, such that levels of T may not influence GC levels at all.

In this study the effect of T on GC levels was experimentally assessed by implanting post-reproductive, male chipmunks with T during summer. This design was meant to distinguish between the two principal hypotheses that explain low plasma

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GC levels during mating. If exogenous T induces a decrease in plasma GC levels, then low GC levels noted during mating (Place and Kenagy, 2000) are likely due to suppressive effects of T. Conversely, if T-implants do not decrease plasma GC levels, then the effect of hibernation on adrenocortical activity would warrant further investigation. Additionally, potential costs of exogenous T in chipmunks were assessed by measuring changes in body mass and rate of recapture 1 month after implantation, as experimental elevation of plasma T in nonmammalian vertebrates has been associated with life history costs (Hegner and Wingfield, '87; Marler and Moore, '88). Finally, because T-implants have been shown to inhibit hibernation in ground squirrels in the laboratory (French, '86; Lee et al., '90), overwinter recapture rates were also measured by trapping animals through the following spring.

MATERIALS AND METHODS

Animals

Individually marked, male yellow-pine chipmunks were studied by live-trapping from July 1997 to April 1998 at two ecologically similar, nearby (3.2 km) areas of montane forest (elevation 670 m) in Chelan County, Washington. The principal site (3.8 ha) was covered by a 14 × 14 grid of 196 Sherman live traps on 15-m centers and has been described previously (Kenagy and Place, '99). A second site (2.8 ha), which was covered by 200 traps on approximately 10-m centers, was added to increase sample sizes. Traps were generally baited with birdseed in early morning or mid-afternoon and collected about 2 hr later. All animals were taken to a central area for processing. Chipmunks were weighed with a Pesola spring balance and examined for reproductive condition (size and location of testes).

Implantation

Thirty-eight male chipmunks with fully regressed testes were captured in the field, implanted, and released August 19–27, 1997. Following collection of an initial blood sample from the infraorbital sinus, animals were randomly assigned to receive either an empty ($n = 18$) or T-filled implant ($n = 20$). After Metofane anesthesia, a 400- μ l blood sample was collected and an implant was then inserted subcutaneously between the scapulae. The skin was sutured (0000 Vicryl) and animals were returned to their trap site within 1 h after recovery. Implants were made of medical grade silastic

tubing (10 mm total length; 1.98 ID/3.18 OD) and sealed with medical-grade silicone sealant (Dow Corning); implants were left empty or filled with 6 mm of crystalline T (Sigma). Implant diameter and length were estimated using values described by Bartness ('96) for Siberian hamsters.

Animals were left undisturbed for 1 month, when trapping was resumed during 7 days in September 1997. Twenty-six (68%) of the 38 animals implanted during August were recaptured, weighed, and bled as before prior to release. Rate of recapture, which does not distinguish between losses due to mortality and dispersal, was calculated as a percent based on the number of animals recaptured divided by the total number of animals that were captured at the beginning of the study. The maximum number of chipmunks available for recapture was decreased to reflect loss of animals due to loss of implant or trap-related deaths. Implants were lost from three T-males and one C-male, which were thus excluded from analysis. Two T-males with intact implants were lost due to trap-related deaths.

The majority of blood samples were obtained between late morning and mid afternoon in both groups and during both months. Mean time-of-day in which blood samples were obtained was not significantly different within or between treatment groups (data not shown). Blood was stored in a cooler for about an hour after collection and then centrifuged. Plasma was separated and stored at -20°C until analysis.

Hormone radioimmunoassays

Plasma samples were thawed and assayed for testosterone, 5 α -dihydrotestosterone (DHT), and corticosterone as described by Wingfield and Farner ('75) and Ball and Wingfield ('87). Samples with sufficient residual volume were frozen and later assayed for cortisol (see below). Briefly, plasma samples of 50–100 μ l were extracted in dichloromethane, dried under nitrogen, and reconstituted in 10% ethyl acetate/90% isooctane. Steroids were separated via Celite (Sigma) chromatography; recovery of T, DHT, and corticosterone was measured for each sample. After separation, samples were dried under nitrogen, reconstituted in phosphate-buffered saline, and allowed to equilibrate overnight. Samples were incubated with a primary antibody to corticosterone or testosterone (Endocrine Sciences, Tarzana, CA) and tritiated-labeled hormones (NEN Research Products, Boston, MA); dextran-coated charcoal was used to separate bound and free-labeled hormone. All samples were run in a single assay; the intra-assay coefficients

of variation were 11.9% for T, 9.6% for DHT, and 10.5% for corticosterone.

Cortisol was measured using a solid-phase ^{125}I radioimmunoassay (RIA) kit from DiaSorin (previously Incstar, Stillwater, MN). Plasma samples were diluted 5-fold with the serum blank provided and aliquots of 10- μl were added to 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. The intra-assay coefficient of variation was 5.6%.

The study was completed according to the Laboratory Animal Care and Use Protocol of the University of Washington, under a Washington State collecting permit, and it conformed to NIH guidelines.

Statistics

Body mass and plasma hormone data were analyzed with a commercial statistical program (Statview 5.0, SAS, Cary, NC) using a repeated measures analysis of variance (rANOVA). The F and P values for the interaction effect of treatment (T- or blank-implant) and time (before or after implantation) are reported, and bar graphs for interaction effects for plasma T, GCs, and body mass are displayed in Fig. 1. Fisher's exact test was used to compare recapture data.

RESULTS

T-implants increased plasma levels of T by about 15-fold, while blank implants had no effect ($F_{1,19} = 166.1$, $P < 0.0001$; Fig. 1A). Plasma T levels in T-males reached concentrations typical of the mating season (Place and Kenagy, 2000). However, neither T- nor blank-implanted animals demonstrated a significant change in plasma DHT levels ($F_{1,19} = 2.6$, $P = 0.12$). Thus, any effect of T on plasma GC levels, body mass, or local survival is not likely to require the conversion of T to DHT.

Mean cortisol level dropped by 23% one month after implantation in T-males, while mean cortisol level was unchanged in C-males ($F_{1,18} = 12.1$, $P = 0.027$; Fig. 1B). Similarly, mean corticosterone level decreased by 44% in T-males and showed no change in C-males ($F_{1,19} = 11.8$, $P = 0.003$; Fig. 1C).

T-males decreased body mass during the month following implantation, and body mass of C-males

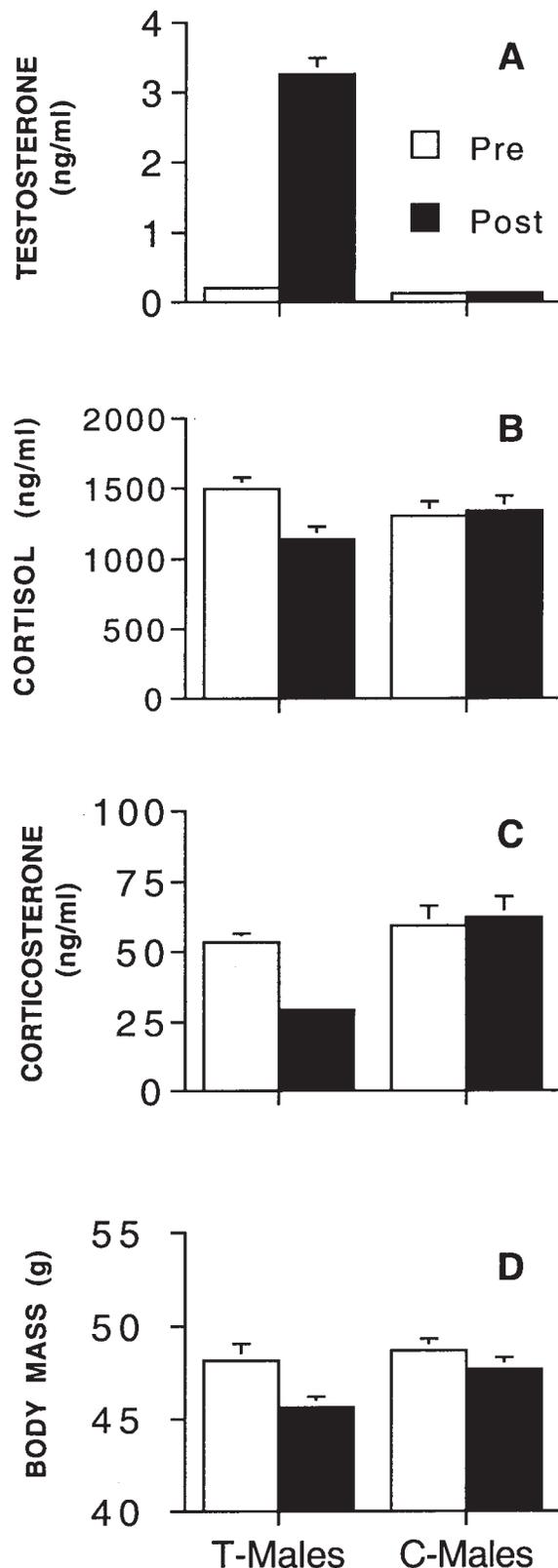


Fig. 1. Plasma concentrations of (A) testosterone, (B) cortisol, and (C) corticosterone, and (D) body mass for chipmunks

immediately before (open bars) and 1 month after (solid bars) implantation. Values are mean \pm SEM; $N = 11$ for T-males and $N = 10$ for C-males (except for cortisol data, $N = 9$).

did not change ($F_{1,19} = 4.9$, $P = 0.040$; Fig. 1D). Despite the decrease in body mass, the recapture rate of T-males (71%) during the first month post-implantation was identical to that of C-males (71%). However, the recapture rate was significantly reduced in T-males compared to C-males ($P = 0.008$; Table 1). Interestingly, all males caught in April had fully enlarged, scrotal testes, save for the two T-males. These two T-males showed no signs of testicular growth at any time during spring 1998; when their T-implants were removed, they still contained crystalline T. Owing to the paucity of T-males captured in April, body mass and hormone data are not reported.

DISCUSSION

Exogenous T significantly reduced plasma corticosterone and cortisol levels in post-reproductive male chipmunks. This is consistent with the hypothesis that naturally high T levels during mating suppress plasma GC levels. As such, chipmunks demonstrate the same inverse relationship between T and GCs described in laboratory rats (Handa et al., '94). The suppressive effect of T on plasma GC levels in chipmunks may result from the ability of T to reduce corticosteroid binding globulin (CBG) synthesis in the liver, as it does in rats (Matarazde et al., '92). Decreased CBG levels increase the ratio of free to CBG-bound GCs, but the metabolic clearance rate of free GCs is more rapid and results in an overall reduction in total GC concentration. Semelparous marsupials represent an exception to this general observation (Bradley et al., '80; Bradley, '87); the small marsupial *Antechinus stuartii* has high T levels during mating that suppress CBG synthesis, as expected, but its increased production of corticosterone outpaces any potential increase in GC clearance rate. The resulting high free GC concentrations of *Antechinus* have been suggested as a link to the post-reproductive programmed death of these animals (Bradley et al., '80). However, chipmunks are iteroparous and mate annually during their 3- to 4-year life

span (Broadbrooks, '58). Thus, when total (bound + free) GC levels rise in chipmunks following mating, most of the plasma GC is probably bound to CBG, which should increase in response to lower plasma T levels.

The GC levels described in this paper reflect the effects of capture and handling stress, because blood sampling occurred more than an hour after collection of animals from traps. Therefore, T-induced suppression of GC levels could have resulted from a dampened adrenocortical response to the stress of capture and handling. However, Place and Kenagy (2000) demonstrated that both basal and post-capture levels of corticosterone and cortisol were relatively low during mating, when T was high. Additionally, Place and Kenagy (2000) showed that on an individual basis a chipmunk's basal GC level correlated well with its post-capture hormone level. Thus, T probably suppresses basal GC levels as well, but the ability to increase GC levels in response to trap-related stressor remains intact in chipmunks, even when T levels are high.

The reduced recapture success in April 1998 may reflect a decrease in local overwinter survival of T-males. Increased late summer dispersal is unlikely to explain the poor recapture success of T-males, because recapture success one month after implantation was identical in the T- and C-male groups. The reduced rate of recapture may have resulted from a T-induced disruption of hibernation, as described in ground squirrels (French, '86; Lee et al., '90). In chipmunks, the effect of T on hibernation has not been investigated, but the reduced overwinter recapture success of T-males noted in this study is consistent with an inhibitory effect of T. Alternatively, reduced post-emergence recapture success may not be directly related to hibernation per se. For example, reduced efficiency of foraging and caching, increased metabolic rate, increased mate searching or other seasonally inappropriate activity, and increased thermoregulatory demands due to disruption of molt may have adversely affected local overwinter survival. All or some of these effects could be induced directly by T or indirectly via a T-induced reduction in plasma GC levels. T-males did demonstrate a significant loss of body mass 1 month after implantation, suggesting that T-males would have entered hibernation (if they entered torpor at all) in relatively poor condition. Loss of mass could have resulted from a T-induced reduction in the ratio of body fat to muscle ratio (Wade and Gray, '79). Alternatively, the decrease in mass may have been behaviorally induced. Glucocorticoids act di-

TABLE 1. Rate of recapture (%) of male chipmunks 1 month after implantation (September 1997) and 7 months later, after winter (April 1998)¹

Date	T-males	C-males	Fisher's exact test
Sep 1997	70.6% (12/17)	70.6% (12/17)	$P = 1.0$
Apr 1998	20.0% (2/10)	83.3% (10/12)	$P = 0.008$

¹Number of animals recaptured out of total in parentheses.

rectly on the hypothalamus to promote feeding behavior (Green et al., '92), and T may have reduced foraging and caching indirectly by decreasing GC levels. Also, T-implants may have directly promoted behaviors such as mate seeking and male-male aggression that are seasonally inappropriate and reduce foraging efficiency.

In summary, this study provides evidence consistent with the hypothesis that the relatively low concentrations of cortisol and corticosterone observed in reproductively active male chipmunks are due, at least in part, to high plasma T levels. The plasma GC levels measured in post-implanted T-males approached, but did not reach the low levels previously measured in male chipmunks during the breeding season (corticosterone 19.8 ng/ml and cortisol 977 ng/ml, Place and Kenagy, 2000). Though year-to-year variation in plasma GC levels may be a possible explanation, further studies to test the effects of hibernation on adrenocortical activity are warranted. Such an experiment might compare springtime GC levels between intact and gonadectomized males. Should castrated males have GC levels that are low and comparable to intact animals, than the effects of hibernation on GC levels would be supported. Additionally, the hypothalamic-pituitary-adrenal axis may respond differently to T in the summer compared to the spring. The overwinter reduction in adrenal mass reported in this species (Sheppard, '68) may render the adrenal cortex particularly sensitive to the suppressive effects of high plasma T levels, but higher centers may be involved as well. Demonstrating the disruption of hibernation by T in chipmunks would require experimental studies, either in the laboratory or perhaps in the field by using radio telemetry and body temperature sensing equipment (DeCoursey and Krulas, '98). Regardless of whether exogenous T acts directly or indirectly via reductions in plasma GC levels, its administration during the late active season of the yellow-pine chipmunk appears to be costly, as evident by reductions in prehibernation body mass and post-emergence recapture success.

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