

# Effects of Training and Testosterone on Muscle Fiber Types and Locomotor Performance in Male Six-Lined Racerunners (*Aspidozelis sexlineata*)

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## ABSTRACT

Testosterone (T) is thought to affect a variety of traits important for fitness, including coloration, the size of sexual ornaments, aggression, and locomotor performance. Here, we investigated the effects of experimentally elevated T and locomotor training on muscle physiology and running performance in a nonterritorial male lizard species (*Aspidozelis sexlineata*). Additionally, several morphological attributes were quantified to examine other characters that are likely affected by T and/or a training regimen. Neither training alone nor training with T supplementation resulted in increased locomotor performance. Instead, we found that T and training resulted in a decrease in each of three locomotor performance variables as well as in hematocrit, ventral coloration, and testis size. Strikingly, neither the size nor the fiber composition of the iliofibularis or gastrocnemius muscles was different among the two treatments or a group of untrained control animals. Hence, the relationships among T, training, and associated characters are not clear. Our results offer important insights for those hoping to conduct laboratory manipulations on nonmodel organisms and highlight the challenges of studying both training effects and the effects of steroid hormones on locomotor performance.

## Introduction

Because of its broad contextual use (Irschick and Garland 2001; Husak and Fox 2006) and direct fitness impacts (Husak et al. 2006; Irschick et al. 2008), locomotion has been suggested to be one of the most important traits on which selection acts to mold the phenotype of organisms (Dickinson et al. 2000). Although the morphological (Hildebrand 1985; Miles 1994; Bonine and Garland 1999; Bonine et al. 2005) and biomechanical (Biewener 1990; Alexander 2003) underpinnings of terrestrial locomotor performance have been extensively studied, it remains unclear how hormones such as testosterone (T) regulate locomotor performance in nonhuman animals (Husak and Irschick 2009). Circulating levels of T can be positively correlated with performance traits in lizards (Husak et al. 2007; Huyghe et al. 2009; but see Husak et al. 2006), but experimental elevation of T has produced equivocal results (Klukowski et al. 1998; Sinervo et al. 2000; Huyghe et al. 2010). The lack of consistent results among studies and performance measures in lizards is similar to results obtained in humans (reviewed in Husak and Irschick 2009). One potentially confounding factor in laboratory manipulation studies is that individuals involved in the study, including those receiving exogenous T, do not adequately “train” target muscles during the period of elevated T in order to detect a significant response (Huyghe et al. 2010).

Unlike the extensive work conducted on human athletes that shows training to be important for pronounced T effects on performance (e.g., Bhasin et al. 2001; George 2003; Hartgens and Kuipers 2004), surprisingly little research has tested for the effects of training and T on nonhuman animal locomotor performance, and even less has examined the effects of training on nonmammalian vertebrates (Husak and Irschick 2009). Only two studies to date have examined the effects of training on locomotor performance in lizards (Gleeson 1979; Garland et al. 1987), both examining territorial species. Garland et al. (1987) subjected lizards to a training regimen consisting of several 30-min treadmill endurance trials per week and found no change in endurance capacity. Garland et al. (1987) concluded that this regimen may have been excessive, because half of the lizards in the training group ( $n = 10$ ) experienced deterioration of hind limb joints and muscles. Gleeson (1979) ran lizards around a circular track 5 d/wk and gradually increased the total distance run (i.e., week 1 = 13 m, week 2 = 27 m, etc.). This training regimen led to reduced sprint speed. The author suggested that this was the result of decreased thigh muscle mass. Thus, to date, it remains unclear how training increases locomotor performance in lizards.

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Combined, these findings are somewhat surprising because several studies involving human subjects have examined how T supplementation coupled with training affects muscle properties (i.e., muscle fiber composition and muscle fiber diameter). However, these studies have used supraphysiological levels of T (Griggs et al. 1989; Sinha-Hikim et al. 2002) or T in combination with other hormones (Alén et al. 1984), and they have supplemented human diets with protein (George 2003). In humans, elevated T has been shown to increase muscle mass (Griggs et al. 1989), muscle fiber diameter (Alén et al. 1984; but see Griggs et al. 1989), muscle size (Bhasin et al. 1996), blood hematocrit (Griggs et al. 1989), and strength (Bhasin et al. 1996; Bhasin et al. 2001) in males. However, the cellular mechanisms by which these effects occur remain unclear or mixed, and the role of T in altering performance at physiological doses is also unclear (Husak and Irschick 2009).

Gowan et al. (2010) found covariation among plasma T levels, bite force, and running endurance in male racerunner lizards (*Aspidoscelis sexlineata*). However, this field study did not attempt to isolate the specific physiological mechanism by which T influences running or biting performance (i.e., increased muscle area, alteration of muscle fiber types, etc.). Hence, a pilot study was conducted where T levels were experimentally elevated within the physiological range for male *A. sexlineata* (O'Connor 2009). The aim of that study was to determine whether performance levels could be increased as a result of T supplementation at physiological levels. The results showed that although the T implants raised T levels, locomotor performance was unaffected. However, lizards were exercised only one time per week, and thus lack of activity, or training, was a likely explanation (see also Bhasin et al. 2001). Given these results, our study was conducted to examine the effects of training coupled with elevated T levels on the locomotor performance and muscle properties of male *A. sexlineata*. To the best of our knowledge, this represents the first study of the combined influences of training and T supplementation in a nonhuman vertebrate.

This study addresses three main questions. First, does T supplementation coupled with a training regimen produce greater increases in locomotor performance than training alone? Second, do elevated T levels or training affect endurance-associated traits such as oxygen carrying capacity (blood hematocrit) or muscle physiology (fiber type or fiber size)? Finally, does exogenous T affect primary or secondary sexual traits (i.e., testis size, ventral coloration) in a nonterritorial lizard species in the same way as in territorial males?

We predicted that lizards given exogenous T and subjected to a training regimen (expressed as T + training) would exhibit the greatest increase in endurance (Bhasin et al. 1996). This prediction was based on the knowledge that exogenous T affects several key morphological traits such as muscle mass (Griggs et al. 1989), fiber diameter (Alén et al. 1984; but see Griggs et al. 1989), and muscle size (Bhasin et al. 1996). Both training (in *Amphibolurus nuchalis*; Garland et al. 1987) and exogenous T (Griggs et al. 1989) have been shown to increase percent hematocrit; hence, we predicted that T supplementation cou-

pled with training should show higher hematocrit levels than training alone. To determine whether the elevated levels of T affected experimental animals, we also examined other traits known to be affected by exogenous T. Because T is known to enhance coloration in territorial lizards (*Sceloporus jarrovii* [Cox et al. 2008], *Sceloporus undulatus* [John-Alder et al. 1996]), we predicted that males implanted with T would have increased hue compared with males receiving an empty implant. Likewise, supraphysiological levels of exogenous T cause a reduction in testis size (Fusani 2008), and hence we predicted that T supplementation, even at physiological dosages, would similarly reduce testis size.

## Material and Methods

### Study Species

Whiptail lizards of the genus *Aspidoscelis* (previously *Cnemidophorus*; Teiidae) are quick-moving active foragers found throughout much of the United States and Mexico (Wright 1993). *Aspidoscelis sexlineata* is a nonterritorial bisexual species found throughout the southeastern United States (Ballinger et al. 1979; Conant and Collins 1998). Adults have an active season lasting from late April to early September (Etheridge et al. 1983; Johnson and Jacob 1984). Despite being nonterritorial, males display enhanced blue coloration in the breeding season when T levels are high, and they employ mate-guarding behavior (Gowan et al. 2010). Thus, they share key traits likely affected by T with territorial species. However, to date, the influence of T has largely been studied in territorial lizards from a single clade (Iguania). We use *A. sexlineata* (Teiidae) to examine patterns of variation in a species with a slightly different mating system and in another branch of the lizard phylogeny (Teiidae) to add perspective to the generality of these patterns across lizards.

### Field Site and Sampling

Adult male *A. sexlineata* (snout-vent length [SVL]  $\geq 54$  mm; Hoyt and Hoddenbach 1966) were captured in Ocala National Forest (29.173659°, -81.781019° Universal Transverse Mercator) in April 2009. The search effort was focused along jeep trails and the edges of sand pine scrub stands or long-leaf pine stands, where animals are abundant and found year to year. All study sites contained large areas of open sand, which is considered ideal habitat for *A. sexlineata*. Sampling was restricted to 0900–1400 hours.

Lizards were captured by noose within 5 min of being sighted, and blood samples were collected in heparinized capillary tubes from the postorbital sinus within  $\leq 3$  min of capture. On capture, the sex of each lizard was confirmed by everting the hemipenes. Blood samples were centrifuged immediately on site to isolate plasma and red blood cells. Hematocrit (packed red blood cell volume) was measured immediately after centrifugation by using dial calipers on a subset of lizards from each locomotor treatment group ( $n_{T+training} = 7$ ,  $n_{O+training} = 7$ ,  $n_{control} = 6$ ). For each lizard, mean percent hematocrit was

Table 1: Initial and final values ( $\bar{x} \pm 1$  SE) of snout-vent length (SVL), mass, and performance for the testosterone (T) implant (T + training) and empty-implant ( $\emptyset$  + training) treatment groups

	$\bar{x} \pm 1$ SE Pretreatment	$\bar{x} \pm 1$ SE Posttreatment
T + training:		
SVL (mm)	62.67 $\pm$ 1.94 (9)	62.67 $\pm$ 1.93 (9)
Mass (g)	6.99 $\pm$ .62 (9)	6.20 $\pm$ .43 (9)
Maximum burst distance (m)	43.44 $\pm$ 1.86 (9)	40.89 $\pm$ 2.24 (9)
Maximum treadmill endurance (s)	217.37 $\pm$ 21.89 (8)	261.33 $\pm$ 34.42 (9)
Maximum time (s)	88.78 $\pm$ 18.22 (9)	76.44 $\pm$ 19.91 (9)
$\emptyset$ + training:		
SVL (mm)	63.05 $\pm$ 1.83 (10)	62.6 $\pm$ 1.82 (10)
Mass (g)	7.02 $\pm$ .56 (10)	6.01 $\pm$ .61 (10)
Maximum burst distance (m)	39.7 $\pm$ 3.74 (10)	41.50 $\pm$ 4.73 (8)
Maximum treadmill endurance (s)	204.5 $\pm$ 36.11 (10)	212.0 $\pm$ 30.49 (8)
Maximum time (s)	103.7 $\pm$ 16.74 (10)	85.88 $\pm$ 10.5 (8)

Note. No differences were observed between the pre- and posttreatment values for either treatment group. Sample sizes are given in parentheses.

calculated from the two or three heparinized capillary tubes used to collect the blood sample. Average sample volume was 50–100  $\mu$ L (~50% red blood cells). All samples were stored on dry ice in the field and subsequently stored at  $-20^{\circ}\text{C}$  in the lab. Lizards were kept in cloth holding bags until they were returned to the animal facility at Georgia Southern University.

### Housing

Lizards were housed individually in glass aquariums (50.8 cm  $\times$  27.9 cm  $\times$  33.0 cm) with a sand substrate. Cardboard inserts between aquariums obscured sight lines and prevented agonistic interactions between lizards. Each aquarium contained a water dish, hide ( $\bar{x} = 33.9^{\circ}\text{C}$ ), and basking spot under a 100-W incandescent bulb ( $\bar{x} = 41.0^{\circ}\text{C}$ ). Lizards were fed commercially available crickets (dusted with Reptocal vitamins) and mealworms (*Tenebrio molitor*) every other day to satiation. This diet was selected because field crickets have high-protein (58%) and fatty-acid (10%; Wu et al. 2004) content, as do mealworms (~65% protein, 12% fatty acids; Ghaly and Alkokaik 2009). Lighting was controlled by automatic timers on an 11L : 13D light cycle, and UVB was provided daily. All lizards were maintained in the lab for 6 wk.

### Locomotor Performance

Performance trials were conducted 3 d after capture before T implantation and weekly thereafter for 42 d. The weekly training regimen is described below. Three locomotor performance traits were tested: maximum time run, burst distance, and treadmill endurance. Lizards were fasted for 12 h before performance trials because feeding negatively affects endurance (Huey et al. 1984). Lizards were warmed to their field-active body temperature ( $38^{\circ}$ – $41^{\circ}\text{C}$ ) for 30 min before each performance trial, and temperatures were verified with a cloacal ther-

mometer before and after each trial. All performance tests were conducted between 0900 and 1400 hours.

Maximum time run (s) was measured by chasing a lizard around a 5-m circular racetrack that had cardboard walls and an artificial turf substrate. Each lizard was chased around the track at or near its full speed until it began to slow. On slowing, tail taps were used to motivate the lizard to continue its forward motion. Throughout the trial, the total distance covered by the lizard was recorded every 30 s onto a voice recorder. The trial ended when the lizard failed to move forward after 10 consecutive taps on the tail and the lizard had lost its righting response (Cullum 1997, 1998; Gowan et al. 2010). If the lizard was able to right itself on being turned on its back, the trial continued until the lizard was exhausted. Burst distance (m) was measured using the same methods described for maximum time run except that the trial ended when the lizard's gait slowed to a walk. Treadmill endurance (s) was measured on a small-animal treadmill at a constant speed of 1.0 km/h (Garland et al. 1987). Lizards were encouraged to run by lightly tapping them on the tail. Minimal stimulation was used to motivate a lizard while on the treadmill because if frightened, the lizard would dart to the front of the treadmill, not maintain a constant speed, and fatigue quickly. The trial ended when the lizard was unable to maintain its position on the belt after 10 consecutive tail taps (Huey et al. 1984; Garland 1994). The longest trial of each performance test (before and after the training regimen) was retained for statistical analysis.

### Training Regimen

Each week, the training regimen consisted of three burst distance trials, two treadmill endurance trials, and one maximum time run trial. (The duration and distance of each trial type are reported in Table 1.) Lizards performed only one locomotor

trial type per day. We established this regimen on the basis of earlier training studies in lizards (Gleason 1979; Garland et al. 1987). However, because the regimens used in earlier work were quite different, we incorporated aspects of each. Gleason (1979) studied a sedentary territorial species (Phrynosomatidae: *Sceloporus occidentalis*) compared with *A. sexlineata*; hence, we increased the number of times per week that lizards were exercised compared with that study. However, Garland et al. (1987) concluded that treadmill running five times per week was likely overly strenuous for another sedentary lizard (Agamidae; *Amphibolurus nuchalis*). Thus, we ran lizards to exhaustion (max time) only once in 7 d; treadmill endurance was measured once every 4 d, and burst performance was conducted every other day. The order of trials was changed weekly. With this schedule, on a given day, individual lizards were not trained for more than one type of exercise. Each lizard was not run at all 1 d/wk. On the basis of this species' movement patterns, this regimen is reasonable. Males occur in densities of 15–24/ha and have home ranges of approximately 13,000 m<sup>2</sup> (Clark 1967). They are active for 3–4 h/d (L. D. McBrayer, personal observation) and are known to move at roughly 4 m/min while active (Cooper 2007). Also, they move 72% of their activity time (Cooper et al. 2001), so they move roughly 518 m/d.

#### Treatment Groups and Implant Construction

After initial performance trials when brought into the lab, lizards were randomly assigned to one of two treatment groups: T implant + training (T + training;  $n = 9$ ) and empty implant + training ( $\emptyset$  + training;  $n = 10$ ). A third group acted as a control ( $n = 10$ ); these lizards were kept isolated in cages as described above but were not given a T implant, and they were not trained. The function of the control group was to serve as a comparator for associated morphological traits (hematocrit, hind limb muscle histology, color, heart mass, testis volume) in a set of lizards that had not experienced the same handling stress as those undergoing the training regimen. Unfortunately, locomotor performance was measured on this control group at the start of the experiment and not again at the conclusion. Thus, our interpretations of variation in locomotor performance are limited to comparisons between T + training and  $\emptyset$  + training groups. Presumably, individuals of this highly active species kept isolated in cages for 6 wk would decline in all aspects of running performance, but we cannot know without the missing data on the control group.

For the T + training and  $\emptyset$  + training groups, subcutaneous implants were constructed using 4 mm of Silastic tubing (Dow Corning 508-006, 1.47 mm i.d.  $\times$  1.96 mm o.d.). One end of the implant was sealed with silicone sealant and cured for 2 h. Then, empty (sham) implants were filled with 3  $\mu$ L of dimethyl sulfoxide (DMSO), and the open end of the tube was closed with silicone sealant. Using a Hamilton syringe, we injected T implants with 3  $\mu$ L of a solution containing T (Sigma, T-1500) dissolved in DMSO at a concentration of 100  $\mu$ g T/ $\mu$ L of DMSO (following Cox et al. 2005). Then the open end of the implant was closed with silicone sealant. All sealed implants were then

placed under a fume hood for several days to cure and to allow the DMSO to diffuse out of the implants (Cox et al. 2005).

Before the implant surgery, lizards were anesthetized with ketamine injected intramuscularly. For each implant, a small slit was made in the right lateral fold of the lizard (slightly anterior to the hind limb). The implant (either empty or T) was inserted subcutaneously, and the incision site was sealed with 3M Vetbond tissue adhesive. Lizards were held in cloth bags for at least 1 h postsurgery to allow the adhesive to set. Lizards began the training regimen 5 d postsurgery. Blood was drawn from the postorbital sinus at 14 and 28 d postimplantation to measure hematocrit. Our focus in this study is not to compare circulating levels of T between groups but instead to contrast any morphological or physiological effects that raised T levels might have between groups. Therefore, we validated our implant methodology before the experiment. Circulating T levels of field-active lizards across the breeding-season average  $4.12 \pm 0.52$  ng/mL for this population (Gowan et al. 2010). While some variation in T levels existed, the variation between the implant group versus the empty-implant group in the lab was always consistent; 2 wk postimplant, T-implanted males always had T levels several times higher than those of empty-implanted males (at 2 wk postimplant, empty:  $\bar{x} = 0.28$ ; implant:  $\bar{x} = 2.75$  ng/mL) yet remained within the range of natural variation. Details of hormone assay methodology can be found in O'Connor (2009).

The duration of T exposure can also be important in determining the effects of T on performance levels. Exogenous T significantly increases locomotor performance in territorial lizards over shorter periods of time (30 d [John-Alder 1994], 14–23 d [Klukowski et al. 1998], 18 d [Sinervo et al. 2000]) than in our study (42 d). Hence, it is unlikely that we missed any peak effect of T. We initiated and completed our study during the early breeding season; thus, all animals should have had similar exposure times to natural T secretion. The peak of the breeding season occurs in late June in this population (Gowan et al. 2010), suggesting that a study duration exceeding 6 wk would have little effect on male condition during the breeding season but instead that any effects of T after the 42-d period here would be seen in the postbreeding season when performance declines (Gowan et al. 2010).

#### Measurement of Morphology

Pre- and posttreatment values for SVL and mass were recorded for all lizards. Also, a small set of fresh field lizards ( $n = 5$ ) were measured for testis volume and heart mass along with lizards in each experimental group to examine whether captivity reduced breeding-season condition. After the experiment, five lizards from the  $\emptyset$  + training and T + training groups were sent to Clemson University for histochemical analysis of the hind limb muscles (see below). The remaining lizards were euthanized with an overdose of sodium pentobarbital. Specimens were fixed in 10% buffered formalin and then preserved in 70% ethanol (EtOH). Testis length and width of each lizard were measured to the nearest 0.1 mm, using a dissecting mi-

croscope equipped with an ocular micrometer. Testis volume was determined by calculating the volume of an ellipsoid ( $V = 4/3\pi a^2b$ ; Mayhew 1963), and the average testis volume for each lizard was retained for statistical analysis. Heart mass was quantified using an electronic balance (to the nearest 0.0001 g).

#### *Tissue Preparation and Staining*

After 6 wk of training, a subset of lizards was sent to the Histology Core Facility at Clemson University for histochemical analysis of the hind limb muscles. Lizards were killed, and the right hind limb of each lizard was removed and frozen in liquid nitrogen. The upper (femur) and lower (tibia/fibula) hind limbs were then serially sectioned (10  $\mu\text{m}$  thickness) using a cryostat. Cross sections were mounted onto coverslips and stained for myosin adenosinetriphosphatase (mATPase) and succinic dehydrogenase/NADH diaphorase (SDH). After staining, the coverslips were mounted onto glass slides.

#### *Muscle Fiber Composition*

Glass slides containing cross sections of lizard hind limbs were photographed (using ACDSee, ver. 3.1, with QCapture plug-in) using a camera (Nikon QImaging Micropublisher) mounted above a compound microscope. A stage micrometer was photographed at each magnification for later use in determining muscle areas and fiber areas. The iliofibularis (IF) and gastrocnemius (G) of five lizards from each treatment group were later analyzed for fiber composition and fiber size. These muscles were selected because of the importance in the stance (G) and swing (IF) phases of locomotion (Higham et al. 2011). Each photograph was analyzed using ImageJ software (ver. 1.41o; Rasband 1997–2011; Abramoff et al. 2004). First, the outline of each muscle was traced and the area was calculated. Using the cell-counter plug-in, we characterized fibers as one of three fiber types—fast-twitch glycolytic (FG), fast-twitch oxidative glycolytic (FOG), or slow oxidative (SO)—on the basis of their staining characteristics (Bonine et al. 2001). FG fibers stain dark with mATPase and light with SDH (Bonine et al. 2001). Muscles with higher oxidative capacities stain more darkly with SDH, whereas muscles with low oxidative capacities are lighter in color (Gleeson and Harrison 1988). The diameter of approximately 20% of the fibers within each muscle was measured, and the proportion of each muscle occupied by the three fiber types (i.e., FG, FOG, and SO) was calculated.

#### *Color*

To examine changes in coloration due to T supplementation, we scanned the ventral surface of each lizard at the beginning and end of the experiment by using an HP Photosmart C4280 all-in-one scanner. Lizards were placed in cloth bags and briefly cooled (5 min at 5°C) to reduce activity. Lizards had similar body temperatures after cooling; cooling did not visually appear to alter coloration, and lizards recovered rapidly. Scanned im-

ages were saved onto a laptop computer, and color was analyzed using Adobe Photoshop (ver. 6.0; Cox et al. 2008). The elliptical marquee tool was used to select three areas across the width of the lizard's ventral surface. For each ellipse, the histogram tool was used to quantify the mean red, green, and blue values. Red, green, and blue values were entered in the color-picker tool in order to determine hue, saturation, and brightness. The mean value of hue, saturation, and brightness for each ellipse from each lizard was retained for statistical analyses.

#### *Analysis*

All statistical analyses were performed using JMP (ver. 7.0.1). Significance was accepted at  $P < 0.05$ , and all  $P$  values were two tailed. For locomotor performance variables, pretreatment and posttreatment comparisons were made via repeated-measures ANOVA (rmANOVA) between the T + training treatment group and the  $\emptyset$  + training treatment group. For morphological characters, rmANOVA was used to analyze differences in ventral coloration and blood hematocrit between the two treatment groups and the control group (i.e., no T + no training). At the conclusion of the experiment, groups were divided into subsets for hind limb muscle histology and estimating testis volume and heart mass. An additional set of fresh field-captured individuals was also included ( $n = 5$ ; captured on May 17, 2009, at the same study sites) for comparisons of testis volume and heart mass. Testis volume was not measured on the individuals used for muscle histology. Testis volume and body mass were  $\log_{10}$  transformed to yield normal distributions. A Kruskal-Wallis test was used to compare these groups when data could not be transformed to meet the assumptions of an ANOVA.

After all analyses, the data appeared strongly to suggest that a stress response had occurred over the course of the experiment. Thus, we analyzed corticosterone levels for a subset of lizards housed under identical conditions but without a training component (see O'Connor 2009). We used standard radioimmunoassay techniques (a direct assay, following Huyghe et al. 2009) to quantify corticosterone levels from plasma of a subset of males from an identical experiment but without a training regimen. Lizards in this post hoc data set were collected in the postbreeding season (i.e., low T) and returned to the lab where they were run and then implanted with either a sham (empty implant) or a T implant. After implantation, lizards were run only once per 14 d for 6 wk, and blood samples were drawn once every 14 d (sample sizes: sham = 12, T implant = 7). By analyzing corticosterone levels in these lizards, we reduce the added effect of the increased daily handling and chasing stress involved in training trials on 6 out of 7 d/wk for six consecutive weeks, as in the training experiment. When T levels are experimentally elevated, male lizards have lower corticosterone levels compared with those of controls (Klukowski et al. 1998), so our expectation was that males with a T implant would have lower corticosterone levels compared with those of males without a T implant. Inclusion of this group is beneficial

because it will provide stronger inferences into the possibility of a stress response under a training regimen.

**Results**

Treatment groups did not differ in initial SVL (Kruskal-Wallis,  $H = 0.03$ ,  $P = 0.983$ ; Table 1), mass (Kruskal-Wallis,  $H = 0.04$ ,  $P = 0.982$ ; Table 1), or blood hematocrit (ANOVA,  $F_{2,16} = 3.06$ ,  $P = 0.075$ ; Table 2). Posttreatment, males in the T + training and  $\emptyset$  + training groups did not differ in body mass (rmANOVA,  $F_{1,17} = 0.001$ ,  $P = 0.984$ ), yet both had significantly decreased in body mass ( $F_{1,17} = 34.281$ ,  $P < 0.0001$ ; time  $\times$  treatment,  $P = 0.838$ ; Table 1). Males that were kept in cages and not given a T implant or trained increased in body mass (ANOVA,  $F_{1,9} = 51.988$ ,  $P < 0.001$ ). Hence, captive conditions alone did not cause lizards to lose weight, but the training regimen and/or T supplementation may have.

*Locomotor Performance*

Body mass explained a significant amount of variation in pretreatment maximum burst distance ( $r^2 = 0.248$ ,  $P = 0.030$ ); therefore, residual values were calculated via regression (body mass  $\times$  burst distance) and used in all subsequent analyses. Pretreatment locomotor performance was not different between the treatment groups (ANOVA, maximum burst distance:  $F_{1,17} = 2.10$ ,  $P = 0.1433$ ; maximum time run:  $F_{1,17} = 0.82$ ,  $P = 0.378$ ; maximum treadmill endurance (s):  $F_{1,17} = 0.08$ ,  $P = 0.779$ ; Fig. 1). Posttreatment, the T + training group did not have greater locomotor performance than did the  $\emptyset$  + training group for any locomotor measure (Fig. 1; rmANOVA,

maximum burst distance:  $F_{1,14} = 0.217$ ,  $P = 0.648$ ; maximum time run:  $F_{1,15} = 0.875$ ,  $P = 0.366$ ; maximum treadmill endurance:  $F_{1,14} = 0.822$ ,  $P = 0.380$ ; for each time (within subject) and time  $\times$  treatment effect,  $P > 0.05$ ).

*Hematocrit, Heart Mass, Testis Size, and Coloration*

The analyses of hematocrit and other morphological variables allowed for the comparison of two treatment groups with a control group (no T, no training). Treatment groups differed in hematocrit (rmANOVA, T + training:  $F_{2,14} = 4.856$ ,  $P = 0.025$ ); however, each treatment group declined in hematocrit over time (within subject:  $F_{2,14} = 84.757$ ,  $P < 0.0001$ ; time  $\times$  treatment interaction:  $F_{2,14} = 1.214$ ,  $P = 0.236$ ). Over the training regimen, males in the T + training group had greater loss of hematocrit ( $45.6\% \pm 1.97\% \rightarrow 32.3\% \pm 0.941\%$ ) than did males in the  $\emptyset$  + training group ( $40.16\% \pm 0.901\% \rightarrow 30.7\% \pm 1.26\%$ ; Table 2). Control males also decreased in hematocrit ( $42.44\% \pm 1.84\% \rightarrow 34.29\% \pm 0.856\%$ ).

At the conclusion of the experiment, heart mass was not significantly different among the T + training,  $\emptyset$  + training, and control treatment groups (ANOVA,  $F_{2,14} = 3.80$ ,  $P = 0.06$ ). The  $\emptyset$  + training group had the smallest heart mass ( $\bar{x} = 23.4$  mg) compared with that of the other groups ( $\bar{x}$ : T + training = 34.4 mg; control = 39.0 mg). We also compared the heart mass of a sample of field-active males with that of the other groups and found an overall effect ( $F_{3,18} = 3.97$ ,  $P = 0.03$ ; Fig. 2A). Tukey-Kramer post hoc tests revealed that the field-active males had significantly greater heart mass than did the  $\emptyset$  + training group but not the other treatments

Table 2: Initial and final values ( $\bar{x} \pm 1$  SE) of hematocrit and ventral coloration (hue, saturation, and brightness) for lizards in each treatment group

	$\bar{x} \pm 1$ SE Pretreatment	$\bar{x} \pm 1$ SE Posttreatment
T + training:		
Hematocrit (%)	45.6 $\pm$ 2.0	32.3 $\pm$ .9*
Hue	266.1 $\pm$ 16.3	188.2 $\pm$ 4.7*
Saturation	22.9 $\pm$ 3.6	23.3 $\pm$ 2.6
Brightness	75.9 $\pm$ 2.9	77.8 $\pm$ 1.6
$\emptyset$ + training:		
Hematocrit (%)	40.2 $\pm$ .9	30.7 $\pm$ 1.26*
Hue	249.5 $\pm$ 28.8	178.9 $\pm$ 10.3*
Saturation	24.3 $\pm$ 5.0	21.1 $\pm$ 3.6
Brightness	81.8 $\pm$ 5.1	79.2 $\pm$ 5.7
Control:		
Hematocrit (%)	42.4 $\pm$ 1.8	34.3 $\pm$ .9*
Hue	249.3 $\pm$ 20.0	168.6 $\pm$ 12.8*
Saturation	17.5 $\pm$ 3.8	15.3 $\pm$ 2.1
Brightness	76.8 $\pm$ 2.1	77.6 $\pm$ 2.1

Note. Pre- and posttreatment values were compared using repeated-measures ANOVA. Hematocrit decreased significantly for all males. Hue decreased significantly also.

\* $P < 0.05$ .

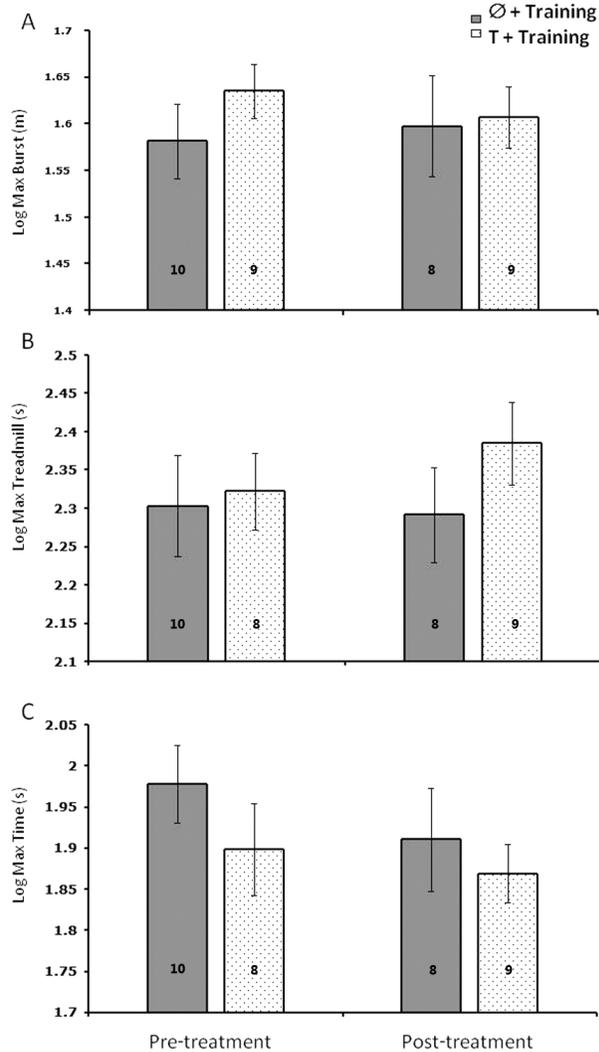


Figure 1. Pre- and posttreatment values of max burst (A), max treadmill endurance (B), and max time (C) for males in the Ø + training and T + training groups. Data are presented as mean ( $\pm 1$  SE). Sample sizes are given at the bottom of each bar.

( $P > 0.5$  for each). Hence, the large difference between the heart mass of field-active males ( $\bar{x} = 44.4$  mg) and that of the Ø + training group ( $\bar{x} = 23.4$  mg) generated the overall significant difference across all four groups.

Testis volume was significantly different among the groups (ANOVA,  $F_{2,10} = 8.06$ ,  $P = 0.008$ ) at the conclusion of the experiment. Males in the T + training and Ø + training groups had smaller testes than did males in the control group (Fig. 2B). Testis volume of each experimental group was also compared with that of field-active males ( $n = 5$ ) to determine whether captivity reduced breeding-season condition. Testis volume of field-active males was greater than that of T + training and Ø + training males (ANOVA,  $F_{3,14} = 12.69$ ,  $P < 0.001$ , Tukey-Kramer HSD; Fig. 2) but not the control males ( $P > 0.05$ ).

Pretreatment values of ventral coloration (i.e., hue, saturation, and brightness) were not different among treatment groups (Kruskal-Wallis, hue:  $H = 0.15$ ,  $P = 0.927$ ; saturation:  $H = 1.98$ ,  $P = 0.327$ ; brightness:  $H = 3.54$ ,  $P = 0.170$ ). Both saturation and brightness were not significantly different among the T + training, Ø + training, and control groups (rmANOVA, saturation:  $F_{2,24} = 1.477$ ,  $P = 0.248$ ; brightness:  $F_{2,24} = 1.255$ ,  $P = 0.303$ ; for both, the time [within subject] and time  $\times$  treatment interaction,  $P > 0.05$ ; Table 2). Hue was not different among groups, but it significantly declined over the course of the experiment (rmANOVA,  $F_{2,24} = 0.580$ ,  $P = 0.567$ ; time [within subject]:  $F_{2,24} = 35.58$ ,  $P < 0.001$ ; time  $\times$  treatment:  $P = 0.732$ ; Table 2).

#### Histochemical Analysis of Hind Limb Muscles

Treatment groups did not differ in total muscle area of the IF or G as a result of T supplementation or training (ANOVA,

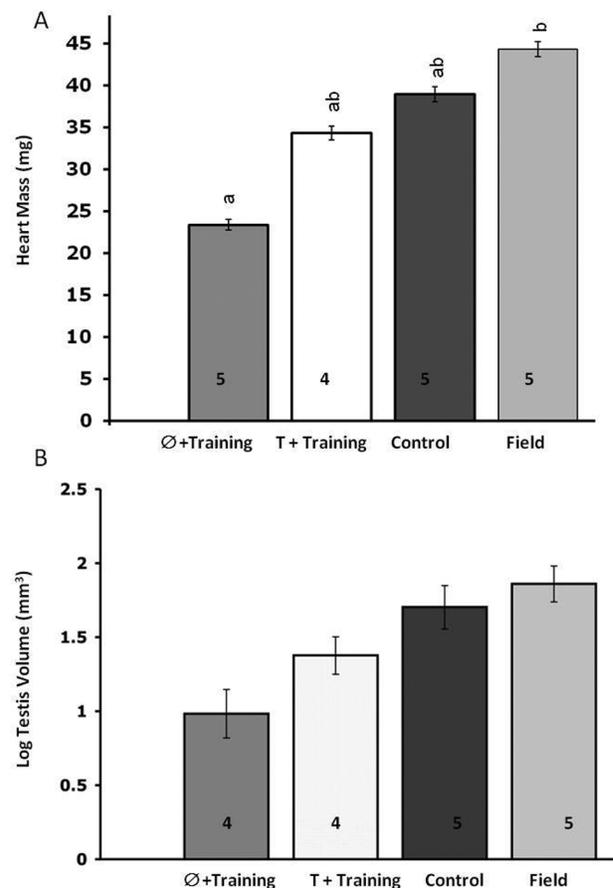


Figure 2. Heart mass (A) and testis volume (B) of lizards in each treatment along with a subset of adult male *Aspidoscelis sexlineata* ( $n = 5$  captured on May 17, 2009). Data are presented as mean ( $\pm 1$  SE). Heart mass did not differ among treatments. Testis volume was significantly different among groups. Columns not sharing the same letter are significantly different (Tukey-Kramer HSD). Sample sizes are given at the bottom of each bar.

IF:  $F_{2,7} = 0.20$ ,  $P = 0.821$ ; G:  $F_{2,4} = 3.63$ ,  $P = 0.126$ ). Muscle fiber composition of the IF was not different between the T supplementation and training groups (ANOVA, FG:  $F_{2,7} = 0.93$ ,  $P = 0.437$ ; FOG:  $F_{2,6} = 1.53$ ,  $P = 0.290$ ; SO:  $F_{2,6} = 0.85$ ,  $P = 0.473$ ; Fig. 3). Muscle fiber composition of the G was not different among treatment groups (ANOVA, FG:  $F_{2,9} = 1.34$ ,  $P > 0.05$ ; FOG:  $F_{2,9} = 1.34$ ,  $P > 0.05$ ; Fig. 3). Individual fiber areas of SO, FOG, and FG fibers in the IF and G did not differ among treatments as a result of training or T supplementation ( $P > 0.05$ ). Thus, somewhat surprisingly, no differences were observed in any muscle properties among lizards exercised six times per week for 6 wk with lizards that remained caged for 6 wk.

The results above for body mass, testis mass, and hue seemed to indicate a stress response. Thus, we collected an additional post hoc data set to examine this hypothesis. As expected, T-implanted males had significantly lower corticosterone levels than did sham (control) implanted males 2 wk after implantation (ANOVA,  $F_{1,13} = 4.77$ ,  $P = 0.04$ ; Fig. 4).

## Discussion

Although T has been suggested as a possible mediator of changes in whole-animal performance (e.g., sprint speed and bite force), the precise physiological mechanisms that may underlie those changes in performance remain obscure (Husak and Irschick 2009). We predicted that T supplementation and/

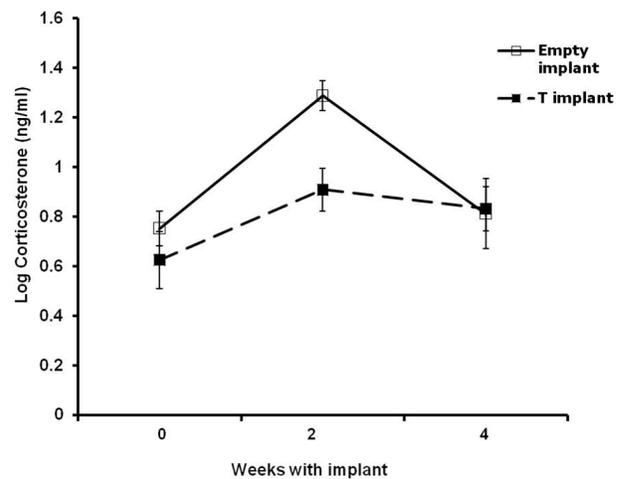


Figure 4. Corticosterone concentrations at 0, 2, and 4 wk postimplant for male *Aspidoscelis sexlineata* implanted with either an empty implant ( $n = 12$ ) or a T implant ( $n = 7$ ). T-implanted males had lower corticosterone concentrations than did empty-implanted males at 2 wk postimplant. These data indicate a stress response despite subjects not having undergone a training regimen.

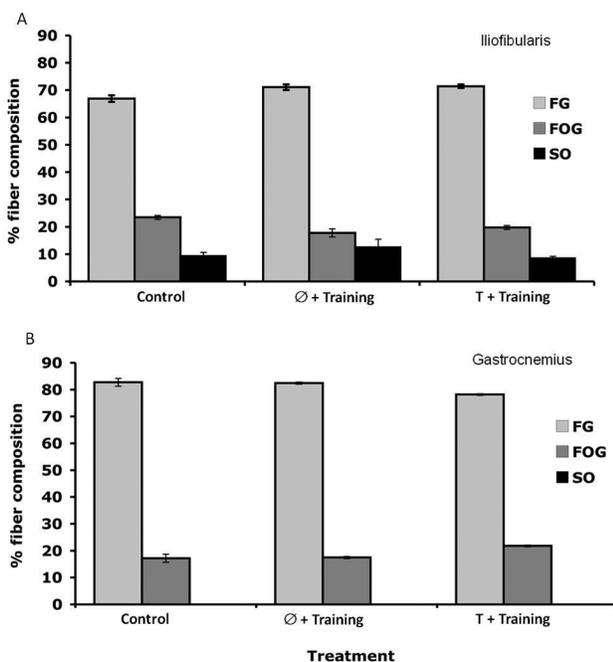


Figure 3. Muscle fiber composition of the iliofibularis (A) and gastrocnemius (B) for lizards in the control, Ø + training, and T + training groups. Muscle fibers were classified as fast-twitch glycolytic (FG), fast-twitch oxidative glycolytic (FOG), or slow oxidative (SO). Treatments were not significantly different for any fiber type in either muscle.

or training would enhance running performance via associated morphological and physiological changes in a species of lizard in which locomotion is likely important to fitness. In fact, the opposite occurred. Neither T nor a combination of T and training increased performance; similarly, morphological and physiological traits predicted to be increased by T supplementation declined (Table 2; Fig. 2). Taken together, these data add to evidence indicating that the influence of T and/or training on locomotor performance in nonhuman subjects is not straightforward. In many cases, and possibly here, laboratory training conditions may cause cascading interactions that function to obscure the relationship of T with locomotor performance.

In nonhuman subjects, training regimens lasting several weeks increase locomotor performance (Cummings 1979; Miller and Camilliere 1981; Pearson et al. 1990; Anttila et al. 2006), and training in combination with T produces greater increases in performance than does T or training alone (Bhasin et al. 1996). However, as in our study, two previous studies involving lizards (Gleeson 1979; Garland et al. 1987) failed to detect an increase in locomotor performance due to training. Our data show that lizards decreased in body condition (lost body mass, lowered hematocrit, loss of hue) but showed no sign of physical injury to indicate that the training regimen was excessive (e.g., Garland et al. 1987). Given that the control group gained body mass, it is likely that training stress led to the decrease in body condition of the training groups. However, the lizards were exercised well within their daily capacities; the distances run during training were relatively short (averages: burst trials = 38 m, treadmill = 55 m, maximum timed run = 40 m) compared with the distances they traverse in a typical day (~500 m; see Cooper et al. 2001; Cooper 2007). Both training and T supplementation should function to increase muscle size

(Bhasin et al. 2001), although some muscles have been shown to be more sensitive to T than others largely for unknown reasons (Sidor and Blackburn 1998; Neal and Wade 2007; Huyghe et al. 2009). We found that neither had an effect on the size of the G or IF or on their histochemistry. Given these physiological results, it is not surprising that locomotor performance was unaffected by either treatment. But why? Here we intentionally elevated T levels to the high end of physiological levels (O'Connor 2009). In contrast, most studies have used suprphysiologic doses of T whereby any effects of T may be greatly amplified (reviewed in George 2003; Hartgens and Kuipers 2004; Husak and Irschick 2009). Our results suggest that the effect of elevated T on particular physiological mechanisms is minor unless very high levels of T are present.

We anticipated significant differences between the two locomotor treatment groups in hematocrit, ventral coloration, and testis size (Alén 1985; Garland et al. 1987; Cox et al. 2008). Males receiving exogenous T + training maintained slightly greater hematocrit compared with the  $\emptyset$  + training group throughout the experiment, but hematocrit decreased over the course of the experiment for both groups and in the untrained controls (Table 2). Hematocrit varies seasonally in some species (Acuña 1974; John-Alder et al. 2009), but seasonal hematocrit profiles are unknown in *Aspidoscelis sexlineata*. Our study was executed entirely during the breeding season, so it is more likely that this decline was related to some other factor. Captive housing is one possible explanation. In support of this, each group also lost ventral coloration during the experiment (Table 2). However, housing conditions supplied UV lighting and access to their preferred activity temperatures ( $\sim 40^{\circ}\text{C}$ ). Supplementation of T is known to decrease testis volume through the inhibition of gonadotropin release from the pituitary (Narula et al. 2002; Fusani 2008). Males in both training groups (T + training,  $\emptyset$  + training) had significantly smaller testis volume than did field-active males and control males (Fig. 2). Additionally, control males had the largest testes at the end of the experiment and gained body mass, while the other groups lost body mass. Hence, it appears that decreased testis volume and our other results were influenced not only by exogenous T but also by increased stress levels due to the training regimen.

If a stress response is related to our unexpected results, then an increase in corticosterone is possible. Figure 4 shows that corticosterone levels are significantly elevated in control males 2 wk postimplantation of T supplementation. Supplemental T lowers corticosterone levels (Klukowski et al. 1998; Moore et al. 2000), and thus it is not surprising that the corticosterone levels are lower but still elevated in the T-implanted group shown in Figure 4. Therefore, these data strongly suggest that a stress response is highly likely in our training study because lizards were run six times per week as opposed to once per 14 d (as the lizards shown in Fig. 4 were). Elevated corticosterone levels not only would be present during our training study but also are likely to have influenced the effects of the T supplementation and/or training regimen. Indeed, Langkilde and Shine (2006) showed that chasing lizards to measure locomotor performance causes an increase in corticosterone levels for a

short time, and Gleeson et al. (1993) showed that corticosterone levels remain elevated in muscle for at least 2 h postexercise. An intensive training regimen would thus likely lead to extended elevation of corticosterone. Elevated corticosterone levels would also explain the reduction in body mass (via muscle breakdown, increased metabolic rate, etc.) we observed in each training group but not in the control group that did not undergo the training regimen. Future studies should quantify corticosterone in addition to T when interpreting results from laboratory studies of training and/or T supplementation because individual responses may vary (see Knapp and Moore 1997).

Finally, the role of a high-protein diet and training technique must be considered. George (2003) indicated that each was important to consistently see an effect of anabolic steroid use on human performance. *Aspidoscelis sexlineata* eats mostly planthoppers, grasshoppers, beetle larvae, and spiders (Paulissen 1987). Because we do not know the precise protein content of their natural diet, it is hard to judge whether the diet provided was high enough to see enhancement by training and T, but it is unlikely that the diet provided in the lab was insufficiently high in protein. Our laboratory diet likely had 58%–65% crude protein and thus seems high. We followed published accounts of training protocols for lizards and tried to vary the type of training among burst endurance, maximum endurance, and walking/running endurance on a treadmill (see Gleeson 1979; Garland et al. 1987; Cullum 1998; Klukowski et al. 1998; Garland 1999). The diverse training measures we employed may simultaneously but differentially affect certain muscles or muscle fibers and thereby preclude observation of increased performance in any of the three performance measures we quantified. In other words, not all of the performance traits or their underlying physiology would necessarily be affected in the same manner. Garland et al. (1987) argued that habituation to the training stimuli could result in lizards being “less motivated” to run as they progress through the training regimen, regardless of training effects. The fact that our histochemistry data revealed no differences among the training groups and control group make this scenario highly unlikely.

Although T has been shown to enhance muscle size and performance in some animal species, our data clearly show that elevating T to high physiological levels does not increase muscle size or alter fiber types in leg muscles of a lizard species. Furthermore, our data show that T and/or training are not sufficient to alter locomotor performance as expected. While increased endurance (as a result of elevated T; John-Alder 1994) allows territorial males to defend larger territories and increase display rates (i.e., head bobs and dewlap extensions; Perry et al. 2004), the role, if any, of T in mediating increases in locomotor performance deserves more attention. The lack of significant effects on a suite of locomotor and T-associated traits is striking. We echo the sentiment of Garland et al. (1987) in that results for nonhuman animals that differ widely from results obtained on human subjects may be due to interesting biological differences or stress associated with captivity and handling. Considering all of our results, we would suggest that

a stress response is likely in training studies of nonmodel organisms. Is there something truly different about the way human and nonhuman animals' performance responds to exogenous T, or are patterns masked by logistical constraints in experimental studies? Our consideration of elevated corticosterone during captivity and training provides a promising line of research for future investigators who seek to determine the interactive effects of T, stress, and training.

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