RESEARCH ARTICLE

Effects of Testosterone on Morphology, Performance and Muscle Mass in a Lizard

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ABSTRACT

Because sexual selection pressures are high in sexually dimorphic organisms, morphological, physiological and performance traits are often studied in a sexual selection context. The proximate mechanisms underlying evolutionary change in these traits, however, remain largely unstudied. Here, we examined the role of steroids in shaping morphology and physiological performance in males of a sexually dimorphic lizard (*Gallotia galloti*). We compared morphology and physiological performance of males with experimentally elevated testosterone levels to sham-operated males. Before surgery, inter-individual variation in plasma testosterone levels correlated positively with bite force capacity. Administration of exogenous testosterone resulted in an increase of the mass of both jaw closing and locomotory muscles compared with sham-operated individuals, but the responsiveness varied considerably among muscle groups. In contrast to our expectations, the dramatic testosterone-induced changes in muscle masses did not result in concordant changes in bite force performance or sprint speed. *J. Exp. Zool. 313A:9–16, 2010.* © 2009 Wiley-Liss, Inc.

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Sexual selection has often been demonstrated to target animal performance through intrasexual competition. The degree to which an animal can conduct an ecologically relevant task when pushed to its maximal limits (Arnold, '83; Irschick and Garland, 2001), is an important target of selection in polygynous, territorial species where males compete, sometimes fiercely, to defend a territory or to maintain a certain dominance status. When selection acts on performance in the context of intrasexual competition, traits such as morphology and physiology can be shaped accordingly to provide the best possible "design" for the best possible "performance," given constraints imposed by other selective pressures. Morphological, physiological and performance traits are therefore often studied in a sexual context as possible predictors of the outcome of male–male competition or female mate choice events (Irschick et al., 2007). The proximate mechanisms underlying this selection for bigger, faster, more attractive males, however, remain largely unstudied. A potential mechanism that may mediate differences in performance among males is the endocrine system, specifically the effect of circulating testosterone levels on an individual's morphology, physiology, performance and ultimately competitive abilities.

Although the effect of testosterone on aggressive behavior has been documented across a variety of vertebrate taxa (reviews in Oliveira, 2004 and Hau, 2007), it remains somewhat ambiguous, which phenotypic traits change with increased circulating testosterone levels and how those result in increased dominance.

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In addition, testosterone is known to affect morphological and physiological traits that may contribute to variation in performance (e.g. number of muscle units: Tobin and Joubert, '91; enzymatic activity in muscles and neurons: Luine et al., '80; contractile properties: Girgenrath and Marsh, 2003) and reproductive system (e.g. penis size: Guillette et al., '99). However, evidence for testosterone affecting relevant animal performance traits is scant and equivocal. In lizards, experimental elevation of testosterone levels has been demonstrated to improve running endurance capacity in lizards (Klukowski et al., '98; Sinervo et al., 2000), but Husak et al. (2006) found no relationship between sprint speed and natural variation in testosterone levels in the lizard *Crotaphytus collaris*. In *Anolis carolinensis*, bite force performance is an important trait in predicting the outcome of contests (Lailvaux et al., 2004). Concordantly, plasma testosterone levels are correlated with bite force capacity in this species, but largely owing to their mutual correlation with body size (Husak et al., 2007). Lacking from these studies are details of how, mechanistically, testosterone might influence performance. That is, what component of the underlying morphology is altered by testosterone to result in performance differences?

We used both a correlational and an experimental approach to investigate the role of testosterone as a predictor of performance traits (sprint speed and bite force capacity) and morphological traits (muscle mass) functionally related to those performance capacities in adult males. The Tenerife lizard, *Gallotia galloti*, is an excellent species for these types of study, because males are highly aggressive, and bite force capacity determines fighting ability and predicts the outcome of contests (Molina-Borja et al., '98; Huyghe et al., 2005). We tested several hypotheses concerning the role of testosterone in shaping morphological and performance traits, often suggested to be targets of sexual selection. First, we predicted a positive relationship between baseline circulating testosterone levels and size-corrected morphological traits (head size, jaw muscle mass, hind limb muscle mass) and between testosterone and each performance capacity. Secondly, we predicted that experimentally elevated testosterone levels would increase muscle mass and performance.

MATERIAL AND METHODS

Study Species

G. galloti is a medium-sized (mean male snout–vent length, SVL: 107 mm; Molina-Borja and Rodríguez-Dominguez, 2004) diurnal lacertid lizard endemic to the Canary Islands. This species is sexually dimorphic in body size and coloration. Adult males are larger than females, have relatively larger heads and possess bluish patches that stand out against the dark ground color of the head and flank region (Font and Molina-Borja, 2004). In September 2005, 21 adult males were caught using tomato-baited pitfalls at Punta Prieta (Tenerife, Canary Islands) and transported to the University of Antwerp, Belgium. Lizards were kept individually in glass cages ($40 \times 40 \times 40$ cm), provided with a sandy substrate and hiding places (rocks and pieces of bark). A light bulb (100 W) provided light (14 hr dark vs. 10 hr dark) and heat, allowing lizards to maintain their body temperatures at preferred levels. Water was available ad libitum, and lizards were fed twice a week with pieces of banana and tomato and once a week with vitamin-dusted crickets. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Antwerp.

Plasma Testosterone Levels

To document basal plasma testosterone levels (in captivity) and to check whether implants effectively released testosterone, 50–70 μL of blood was collected from all individuals before implantation (March 2006) and after the second set of performance measurements (August 2006). As far as we know, no information is available on the natural seasonal hormone cycle in this species. The postorbital sinus was punctured with a needle (0.4×20 mm) and blood collected in heparinized microhematocrit capillary tubes. Blood was immediately centrifuged for 15 min at 7000 rpm to separate the plasma fraction (mean volume $\pm$ SEM = 26.91 ± 0.94 μL). Subsequently, the samples were stored at -80°C until assays were conducted.

Concentrations of total circulating plasma androgens (5α-dihydrotestosterone [DHT] and testosterone) were measured by direct standard radioimmunoassay techniques (Wingfield and Farner, '75; Moore et al., 2000). Because DHT concentrations typically parallel that of testosterone, but at much lower concentrations, we refer to our final values as concentrations of testosterone as in previous studies (Cox and John-Alder, 2005). All samples were run in one assay. For individual extraction efficiency determination, we equilibrated each sample overnight with 2000 cpm of tritiated steroid. Each sample was extracted twice with 4 mL of diethyl ether with the ether phase removed and dried in a warm water bath, under a stream of nitrogen gas, and resuspended in 600 μL phosphate buffered saline, and maintained overnight at 4°C. Individual extraction efficiency for each steroid was determined from 100 μL of the sample, whereas 200 μL of the sample was allocated to each of two duplicates for the assay. Serial dilutions for the standard curve were performed in triplicate (range of curve: 500–1 pg). All samples were then incubated overnight with 100 μL of antiserum (WLI-T-3003S, Fitzgerald Industries, Concord, MA) and 100 μL of tritiated steroid (10,000 cpm). Unbound steroid was separated using dextran-coated charcoal and the bound steroid decanted into scintillation vials. Samples were counted on a liquid scintillation counter and final concentrations corrected for individual extraction efficiency (mean recovery was 80%). The intra-assay coefficient of variation, using six standards, was 5%.

Testosterone Implants

The 21 males were randomly assigned to one of two treatments: sham operated (control group: C-males, $N = 11$) and testosterone implanted (T-males, $N = 10$). Ten males were implanted subcutaneously with a 15 mm flexible silastic tube (Degania silicone, Regensburg, Germany, inner diameter 1.47 mm, outer diameter 1.96 mm), filled with crystalline testosterone (Sigma-Aldrich, Schnelldorf, Germany) and sealed with silastic medical adhesive (Dow Corning, Wiesbaden, Germany). This type of implants slowly releases testosterone, resulting in an increased testosterone levels over several months (De Ridder et al., 2002). Lizards were cooled to 5°C before surgery. Implants were inserted dorsolaterally through a 3 mm incision in the skin, which was sealed with tissue adhesive (Braun Histoacryl, Aesculap, Tuttlingen, Germany). The other 11 males were sham operated. For these males we performed identical incisions, insertion and removal of a tube with no testosterone, and wound sealing. The whole procedure took less than 5 minutes and all individuals appeared healthy after surgery.

Morphometrics and Performance

External morphometrics and performance capacities were assessed both before (March 2006) and several months after (August 2006) implantation. The following measurements were taken, using digital calipers (Mitutuyo, Telford, UK, precision: 0.01 mm): SVL, head length, head height, head width, and hind limb length. Before every performance trial, lizards were placed in individual cloth bags and placed for at least 1 hr in an incubator, set at 34°C (Huyghe et al., 2005).

Maximal sprint speed capacity was determined by chasing the lizards down a 2 m race-track, following standard procedures for quantifying sprint performance (Vanhooydonck et al., 2001). Eight pairs of photocells placed at 25 cm intervals signaled passing lizards to a PC that calculated speed over the consecutive 25 cm intervals. The fastest speed over any 25 cm out of three trials was used as an estimate of maximal sprint speed.

Maximal bite force capacity was estimated by the highest of five recording trials of a lizard biting on two metal plates connected to an isometric force transducer and a charge amplifier (see Herrel et al., '99 for more details on the experimental setup).

Muscle Masses

After completion of morphometric and performance measurements (August 2006), lizards were sacrificed by an overdose of ketamine. Specimens were preserved in 10% aqueous formaldehyde solution for 24 hr, rinsed, and transferred to a 70% aqueous ethanol solution. All jaw closers (i.e. adductor externus, adductor internus and adductor posterior groups sensu Lakjer, '26), a jaw opener (musculus depressor mandibulae), the principal femur retractor (musculus caudofemoralis longus), and the principal knee extensor (musculus ambiens) were removed on one side in each individual. Additionally, one of the two penises (hemipenes) was removed. Muscles and penis were blotted dry and weighed using a Mettler MT5 microbalance (precision: 0.01 mg).

Statistical Analyses

All data were logarithmically transformed to fulfill normality assumptions. Correlation analyses were used to investigate the relationships between testosterone and performance variables, testosterone and morphological variables, and the two performance variables. The effect of testosterone on changes in the external morphological and performance traits was estimated using repeated measures analyses of variance, with treatment (T vs. C) as the among-subjects factor. Analyses of covariance were done to test possible differences between treatment groups after implantation. SVL was used as a covariate. We first tested for treatment–covariate interactions, but in no cases were these significant. The assumption of homogeneity of slopes was met. Therefore, we report results from models without the interaction factor. Size-corrected bite force capacity was estimated by retaining the residuals from a regression of bite force capacity (dependent variable) on SVL (independent variable). Body condition was estimated by retaining the residuals from a regression of mass (dependent variable) on SVL (independent variable).

RESULTS

Baseline Testosterone Levels

C- and T-males did not differ in SVL, size-corrected bite force performance or sprint speed capacity before experimental manipulations (all $F < 2.70$, all $P \geq 0.12$). Correlation analyses including all individuals revealed a positive relationship between initial testosterone levels and size-corrected bite force capacity (Fig. 1a, $r = 0.51$, $P = 0.036$), and a nonsignificant, negative trend between testosterone and sprint speed capacity (Fig. 1b, $r = -0.43$, $P = 0.074$, power analysis: $1-\beta = 0.44$). No relationship was found between testosterone and SVL ($P = 0.35$) or any of the size-corrected external morphological traits (all $P > 0.20$). Additionally, there was a nonsignificant, negative trend between the two performance traits (bite force and sprint speed) (Fig. 1c, $r = -0.45$, $P = 0.063$, power analysis: $1-\beta = 0.51$).

Testosterone Treatment

One implant failed to release testosterone, so this individual was excluded from post implant analyses. T-males had significantly higher (repeated measures Anova, $F_{1,16} = 100.37$, $P < 0.001$) postimplant testosterone levels (mean ± SE: 62.42 ± 7.81 ng/mL) than C-males (mean ± SE: 0.73 ± 0.14 ng/mL). Testosterone levels of T-males were high, but within the natural physiological range of Lacertid lizards (e.g. concentrations up to 85 ng/mL occur in *Podarcis sicula sicula*: Manzo et al., '94). To our knowledge, no data are available on natural testosterone levels in *G. galloti* lizards and levels of control males are likely lower than natural

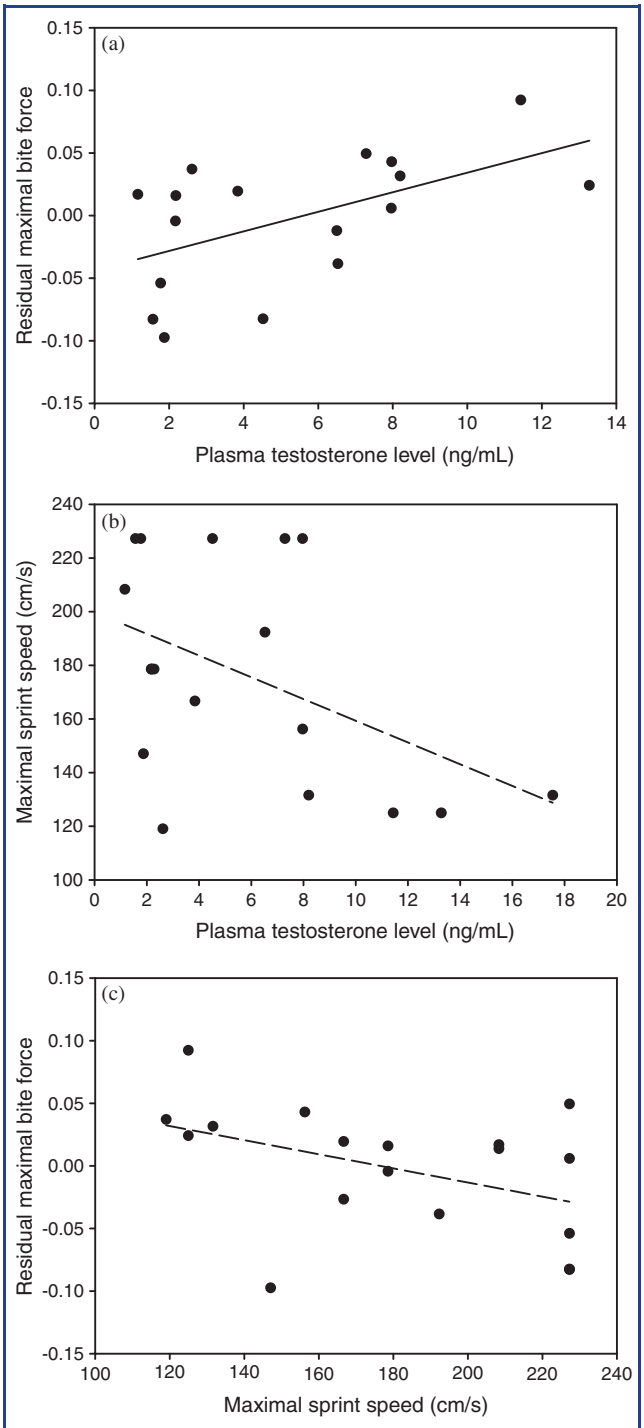

Figure 1. Pre-implant correlations between testosterone and residual bite force capacity ($r = 0.51$, $P = 0.036$) (a), between testosterone and sprint speed capacity ($r = -0.43$, $P = 0.074$) (b) and between residual bite force and sprint speed capacity ($r = -0.45$, $P = 0.063$) (c). Non significant relations are indicated by a dashed line, significant by a solid line.

levels owing to prolonged captivity with no social cues to maintain natural levels. There was no treatment effect on SVL ($F_{1,18} = 0.96$, $P = 0.34$), body condition ($F_{1,18} = 1.55$, $P = 0.23$), hind limb length or head dimensions (all $F_{1,16} < 2.51$ and all $P > 0.13$). Treatment did not affect either maximal sprint speed ($F_{1,16} = 0.52$, $P = 0.48$) or maximal bite force capacity ($F_{1,16} = 0.028$, $P = 0.87$). However, T-males had a greater relative penis size (analysis of covariance $F_{1,16} = 37.71$, $P < 0.001$) and relative adductor muscle mass ($F_{1,17} = 12.39$, $P = 0.003$, see Table 1 for means and statistics of individual muscles). Although no differences were found in the relative muscle mass of the jaw opener (m. depressor mandibulae), T-males did have heavier leg muscles than C-males (both $F_{1,17} > 5.02$ and both $P < 0.039$).

DISCUSSION

Baseline Testosterone Levels and Performance

Baseline testosterone levels are positively correlated with bite force, and show a nonsignificant tendency to be negatively related to sprint speed capacity. Sprint speed tended to be negatively correlated with bite force capacity. In male *G. galloti* lizards, testosterone seems to be a mediator between these two performance measures, with high levels favoring high bite force, and lower levels favoring high sprint speed. As testosterone was independent of body size and all individuals were tested on the same day, there is no ontogenetic or seasonal effect that may affect this apparent tradeoff. Given that bite force is an important predictor of the outcome of male interactions in *G. galloti* (Huyghe et al., 2005), that there is high sexual dimorphism in head size (Molina-Borja et al., '97, '98) and bite force (Herrel et al., '99), and that there is a positive correlation between bite force and testosterone, we assume that intrasexual selection is acting strongly on this suite of traits. Sprint performances, and also other locomotor performance measures such as acceleration and endurance capacity, do not contribute to a positive outcome for a male involved in an aggressive interaction with a rival (Huyghe et al., 2005). On the contrary, of the two fighting males with equal head size, the one with the best locomotor capacities is more likely to lose the fight (Huyghe et al., 2005). It should be noted, however, that despite showing a strong trend, the negative correlations between testosterone and sprint speed and bite force and sprint speed were not significant. Furthermore, in northern fence lizards, locomotor performance increases when testosterone concentrations are experimentally elevated (Klukowski et al., '98). A larger sample size might bolster these findings. Nevertheless, it remains compelling that testosterone levels positively affect animal performance traits proved to be important for males (bite force) and that our data at least suggest the opposite for performance traits not important during dominance disputes (sprint speed). Possibly, testosterone is not a direct mediator of this opposing effect, but acts through its effect on other unidentified traits. As there is a clear positive

Table 1. Descriptive statistics (means and standard errors), *F*- and *P*-values of univariate analyses of co-variance on the masses of the leg and jaw adductor muscles for C- and T-males ($N = 11$ and $N = 9$, respectively).

Muscle (group)	Mean ± SE C-males (mg)	Mean ± SE T-males (mg)	$F_{1, 17}$	P
m. caudofemoralis longus	112.70 ± 13.54	153.08 ± 12.65	5.02	0.039
m. ambiens	75.86 ± 6.68	120.24 ± 10.44	20.93	<0.001
m. depressor mandibulae	7.92 ± 1.09	9.05 ± 0.91	2.03	0.17
m. adductor mandibulae externus superficialis	69.89 ± 7.28	95.40 ± 11.42	7.11	0.016
m. adductor mandibulae externus medialis	29.13 ± 4.42	45.18 ± 10.32	5.76	0.028
m. adductor mandibulae externus profundus	11.72 ± 1.93	13.19 ± 1.65	1.82	0.195
m. pterygoideus lateralis	124.48 ± 16.91	182.51 ± 26.03	8.89	0.008
m. pterygoideus medialis	23.43 ± 3.58	46.73 ± 7.44	11.44	0.004
m. pseudotemporalis superficialis	21.58 ± 2.89	33.12 ± 4.12	9.49	0.007
m. pseudotemporalis profundus	39.91 ± 4.17	50.77 ± 6.16	9.09	0.008
m. adductor mandibulae posterior	8.87 ± 1.49	17.06 ± 2.23	14.48	0.001

SVL was used as a covariate.

relationship between head size and bite performance, there might be an indirect negative relationship between head size and sprint performance (as in López and Martín, 2002), owing to e.g. a lowered physiological condition.

Effects on Muscle Mass

In accordance with the human medical literature (e.g. Bhasin et al., '96, review in Hartgens and Kuipers, 2004) and experimental studies on fish (e.g. Brantley et al., '93), frogs (e.g. Regnier and Herrera, '93, Girgenrath and Marsh, 2003), birds (Fennell and Scanes, '92) and small mammals (Tobin and Joubert, '91, Schulte-Hostedde et al., 2003), testosterone administration resulted in a general increase in muscle mass in male *G. galloti*. The effect of the testosterone implant was striking, almost doubling the mass of some of the muscles considered. Mass increases of similar magnitude have been reported for grasping muscles (e.g. Sidor and Blackburn, '98) and trunk muscles (Girgenrath and Marsh, 2003) of male frogs.

However, the effect of testosterone on muscle mass was not the same for all muscles considered (Table 1). The mechanistic basis for the variation in bite force capacity can be assessed by studying the morphology of the jaw apparatus, e.g. by the examination of the mass distribution of the muscles that may contribute to bite force. Although the total adductor mass likely determines how hard a lizard can bite, mechanical modeling has shown that not all muscles contribute evenly to the moments generated at the jaw joint and thus to the generated bite force (Herrel et al., '99). Interestingly, experimentally elevated testosterone levels caused an increase of the total jaw adductor (closer) muscle mass, but not of the mass of the jaw opener (m. depressor mandibulae). The elevated testosterone levels also did not increase the mass of all jaw closing muscles evenly. For example, the posterior adductor (MAMP) differed most strongly between C- and T-males, followed by the pterygoideus group (MPtmed, MPtsup), the pseudotemporalis group (MPsTP, MPsTS), and the external adductors (MAMES, and MAMEM). Interestingly, no difference was found in the mass of the deep external adductor (MAMEP). As this is the deepest of the external adductors, space constraints may prevent an increase in mass of this muscle. Thus, the biggest differences in muscle mass between treatment groups were found in the so-called internal jaw adductor muscles (pterygoideus and pseudotemporalis groups, Lakjer, '26), which share innervation by the mandibular ramus of the trigeminal nerve (Schwenk, 2000). Both muscle groups are large, with the pseudotemporalis contributing significantly to generating bite force, but the pterygoideus, although being the largest muscle in the head, having little or no contribution to the moments delivered at the jaw joint, and thus bite force (Herrel et al., '99). The latter is sexually dimorphic and hypertrophied in males of many lizard species (Schwenk, 2000). This is also the case in *G. galloti*, and has been suggested tobe associated with a display function (Herrel et al., '99). The muscles are ideally positioned to make the head look bigger, a signal emphasized by the blue and ultraviolet spots on the males' cheeks (Font and Molina-Borja, 2004; see also Lappin et al., 2006 for a similar argument in *Crotaphytus* lizards).

Unexpectedly, the greatest effect of increased testosterone levels was on the posterior adductor, a muscle typically small and contributing little to overall bite force generation. The position of this muscle suggests that it acts mainly to stabilize the quadrate upon forceful biting as it crosses the quadratomandibular joint. As male–male combat in these animals involves biting the opponent at wide gapes to try to inflict wounds upon the opponent, an increase in the size of these muscles would functionally stabilize the jaw joint, thus preventing jaw dislocation and serious damage.

To summarize, the mass of all but one jaw closer muscle (MAMEP) increased differentially after administering exogenous testosterone, suggesting nonspecificity of testosterone's influence on muscle mass. Indeed, increased testosterone levels also caused an increase in both hind limb muscle masses investigated (femur retractor and knee extensor) but not the jaw opener.

Effects on Animal Performance

Unexpectedly, the increased limb and jaw muscle mass did not result in better (or worse) performance as C- and T-males did not differ in maximal sprint speed or maximal bite force capacity. With respect to the hind limb muscles, this could potentially be explained by having quantified the wrong aspects of locomotor performance or by not including the appropriate muscles in our analysis. Indeed, in a comparative study of locomotor performance in *Anolis* lizards, Vanhooydonck et al. (2006) found that of nine functional muscle groups of the hind limb, knee extensor mass explained significant variation in acceleration capacity, but not in sprint speed. Femur retractor mass was a predictor of neither locomotor performance trait. However, we do expect an increase of bite performance with increasing jaw muscle mass, as we found a positive correlation between the two traits in the unmanipulated males.

There are at least two potential explanations for not finding differences between C- and T-males in biting capacity: although muscle mass is a major determinant of muscle force generating capacity and consequently performance, previous studies have shown that both (1) fiber type, and (2) training may also be important. In a review of human skeletal muscle studies, Harridge (2007) provides evidence that muscles are remarkably sensitive to the mechanical loads placed upon them, and once the normal mechanical signals that are provided by every day activities are absent, a muscle will atrophy and become weaker. Moreover, Bhasin et al. ('96) showed that exogenous testosterone in men, combined with strength training, resulted in a greater effect on body mass, quadriceps cross-sectional area and the capacity for lifting a weight, than in men that were administered testosterone and did not exercise, or in men that exercised but had no extra testosterone. These studies demonstrate the importance of exercise for improving muscle performance. Moreover, in humans, much of the early adaptation to strength training does not result from an increase in muscle size but from improved activation of the muscle (Harridge, 2007). As our lizards were kept in captivity, away from the challenges they are confronted with in their natural environment, such as fighting, and crushing tough food items (plants), they potentially lacked the appropriate mechanical load stimuli needed to strengthen their jaw muscles and consequently increase their biting capacities, despite the fact that testosterone induced a significant increase in muscle size. However, the few studies available on training and performance in lizards found that training did not have a significant effect on running performance (Gleeson, '79) or endurance capacity (Garland et al., '78). Thigh muscle mass on the other hand, did increase significantly after training, resulting in a decrease in sprint speed ability (Garland et al., '78). Finally, steroids such as testosterone may cause an increase of water retention (Eisenberg and Gordan, '50) thus potentially biasing our estimates of force production using muscle mass.

Additionally, it should be noted that in green anole lizards (*A. carolinensis*) testosterone manipulation increased the percentage of fast oxidative fibers in the branchiohyoideus muscle that controls dewlap (throat fan) extensions during courtship displays. Yet, little to no effect was found on copulatory muscle fiber type (Holmes et al., 2007). These results suggest that testosterone can also mediate changes in fiber type among muscle groups and body regions, and thus muscle performance. This needs to be investigated further for the animals included in our study, as well as in other vertebrate systems.

Effects on Penis Size

Finally, the addition of exogenous testosterone caused an increase in penis size. As in other vertebrates, the development of male genitalia in reptiles is dependent on elevated testosterone concentrations during growth (Raynaud and Pieau, '85). In juvenile alligators, body size and plasma testosterone levels explained the existing variation in phallus size (Guillette et al., '99). Our results suggest that (seasonal) changes in testosterone levels may also result in considerable growth of the penis in adult male lizards. We know of no studies that have examined the functional significance of penis size in lizards, so we can only speculate on whether and how the change in penis size helps a male lizard's fertilization success. Bigger penises could facilitate intromission in spite of resistance of the female (as seen in some insects, Bertin and Fairbairn, 2005) and/or may function in postcopulatory processes of sperm competition (e.g. copulatory plug removal, see Moreira et al., 2007) or cryptic female choice (e.g. Olsson et al., '96).

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