Manipulating Testosterone to Assess Links between Behavior, Morphology, and Performance in the Brown Anole *Anolis sagrei*

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ABSTRACT

Survival and reproductive success are determined by the complex interplay between behavior, physiology, morphology, and performance. When optimal trait combinations along these various phenotypic axes differ between sexes or across seasons, regulatory mechanisms such as sex steroids can often facilitate sex-specific and/or seasonal trait expression. In this study, we used surgical castration and replacement of exogenous testosterone in adult male brown anoles (*Anolis sagrei*) to simultaneously examine the effects of testosterone on a suite of morphological (dewlap area, body size), physiological (immune function), behavioral (dewlap, head bob, and push-up displays), and performance (stamina, sprint speed, bite force) traits. We show that testosterone increases (or castration reduces) growth rate, dewlap area, and bite force. Treatment effects on bite force may simply reflect underlying treatment differences in growth combined with allometry of bite force. Other traits, such as stamina, sprint speed, and rate of behavioral displays, were largely independent of circulating testosterone levels. Although we did not observe significant treatment effects on immune function, we found negative correlations between growth and immune function consistent with the hypothesis that testosterone mediates trade-offs between these competing aspects of energy allocation. Overall, our results demonstrate that testosterone can exert pleiotropic effects on a variety of morphological, physiological, behavioral, and performance traits that are known to influence survival and reproductive success.

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Introduction

Survival and reproductive success are determined by the complex interplay between behavior, physiology, and morphology, which can be viewed as facets of the phenotype that interact to influence whole-organism performance (Arnold 1983; Garland and Losos 1994; Brodie and Ridenhour 2003). Selection acting through variation in survival and reproductive success is therefore expected to generate optimal combinations among these various aspects of the phenotype. However, given their fundamentally divergent reproductive roles, males and females often differ in their optimal combinations of behavior, physiology, morphology, and performance (Badyaev and Martin 2000; Fairbairn 2007; Calsbeek and Bonneaud 2008). Moreover, phenotypes that maximize reproductive success often incur survival costs, so the optimal expression of these correlated traits can differ considerably between reproductive and nonreproductive stages. In situations where an entire suite of traits yields different fitness effects depending on the sex and/or season in which it is expressed, selection should favor the evolution of an underlying regulatory mechanism (or mechanisms) that facilitates the sex-specific and/or seasonal expression of these traits (Badyaev 2002).

Sex steroids (i.e., androgens, estrogens, and progestins) are excellent candidates for such regulation because they are produced and secreted by the gonads in sex-specific patterns that vary seasonally and ontogenetically. For example, in lizards, testosterone mediates sexual differences in growth and body size (Cox and John-Alder 2005; Cox et al. 2005*a*), coloration (Rand 1992; Hews and Quinn 2003; Cox et al. 2005*b*), and behavior (Moore and Lindzey 1992; Smith and John-Alder 1999; Lovern et al. 2001). Experiments on this group have also begun to investigate the effects of testosterone on ecologically relevant aspects of physiological and whole-animal performance such as metabolic rate (Marler et al. 1995; Oppliger et al. 2004), immune function (Belliure et al. 2004; Oppliger et al. 2004), and locomotor performance (John-Alder et al. 1996; Klukowski et al. 1998; Sinervo et al. 2000; Uller and Olsson 2003). Collectively, these studies indicate that testosterone can have pleiotropic effects on diverse components of the phenotype. Hormonal pleiotropy is widely viewed as an important evolutionary mechanism for phenotypic integration (Finch and Rose 1995; Flatt et al. 2005; Hau 2007; Zera et al. 2007), but few studies have simultaneously considered multiple aspects of behavior, morphology, physiology, and performance in a single experiment.

In this study, we manipulated testosterone levels in adult male brown anoles (*Anolis sagrei*) and simultaneously examined treatment effects on a suite of morphological (dewlap area, body

size), physiological (immune function), behavioral (dewlap, head bob, push-up displays), and performance (stamina, sprint speed, bite force) traits. We focused on these traits for several reasons. First, many of these traits are sexually dimorphic in *A. sagrei.* Males are substantially larger than females, possess larger and more conspicuous dewlaps, and conduct more frequent and intense agonistic behavioral displays. Second, body size, locomotor performance, bite force, and agonistic behavioral displays are all known to influence the outcome of social interactions that determine territory acquisition in *A. sagrei* and related anoles (Tokarz 1985; Jenssen et al. 1995; Lailvaux et al. 2004; Perry et al. 2004). Hence, many of these traits are probably subject to sexual selection (but see Tokarz 2002; Tokarz et al. 2003, 2005). Finally, traits such as body size, immune function, stamina, and sprint speed are also subject to natural selection arising from differential survival (Calsbeek and Irschick 2007; Calsbeek 2008; Calsbeek and Smith 2008; Calsbeek et al. 2008). Moreover, combinations of these traits that maximize fitness in males often differ from those that are favored by selection in females (Calsbeek and Bonneaud 2008).

Testosterone is a likely candidate for sex-specific and seasonal regulation of these diverse phenotypic traits in *A. sagrei* because circulating plasma testosterone levels are known to differ dramatically as a function of sex and season (Tokarz et al. 1998). Moreover, previous studies have demonstrated that testosterone influences the rate of dewlap extension (Tokarz et al. 2002) and physiological endurance (John-Alder 1994) in this species. Given these results and the fundamental sexual differences in expression of and selection on the traits that we investigated, we predicted that testosterone would stimulate (and castration would reduce) dewlap size, sprint speed, stamina, bite force, and the frequency of male display behaviors. However, suppression of the immune system is one potential cost of elevated testosterone levels (Folstad and Karter 1992), and previous studies have shown that testosterone suppresses immune function and/or increases parasitism in lizards (Belliure et al. 2004; Oppliger et al. 2004; Cox and John-Alder 2007). Thus, we further predicted that testosterone would suppress immune function and that immune function would correlate negatively with other sources of energy allocation stimulated by testosterone (i.e., growth rate). We tested these predictions by surgically castrating captive adult male brown anoles and restoring their plasma testosterone levels with exogenous hormone implants.

Material and Methods

Animals

All procedures involving animals were reviewed and approved by the Dartmouth College Institutional Animal Care and Use Committee (protocol 07-02-03). We obtained adult *Anolis sagrei* males from Carolina Biological Supply (Burlington, NC). Animals were housed together (three males per cage) in 10-gal glass terraria with pine-mulch bedding and a potted plant for perching. We watered cages and plants daily and provided crickets ad lib. (Fluker Farms, Port Allen, LA; dusted weekly with Fluker's Repta-Vitamin dietary supplement). Each cage was placed under a 40-W incandescent bulb in a reflective hood and two Repti Glo 5.0 full-spectrum fluorescent bulbs (5% UVB, Hagen, Montreal) for heat and ultraviolet radiation. Daytime temperatures within cages spanned a gradient from 26° to 35°C, bracketing the mean body temperature ($T_b = 29.2$°C) of active *A. sagrei* in the wild (Lee 1980). We initiated our experiments in February and collected data through June, a period that corresponds to natural seasonal peaks in circulating testosterone that occur in February and persist through August (Tokarz et al. 1998).

We measured each animal's snout-vent length (SVL) to the nearest 1 mm using a ruler and its body mass to the nearest 0.01 g using an electronic balance. Males initially ranged in size from 52 to 67 mm (mean ± 1 SE = 57.9 ± 0.4 mm), all of which fall within the size range for sexually mature adult males in wild populations. We collected pretreatment measures of behavior, morphology, and performance over a 30-d period and then assigned each male to one of three size-matched treatment groups: castrated males receiving a placebo implant (CAST; $n = 14$), castrated males receiving a testosterone implant (TEST; $n = 13$), and intact control males receiving a "sham" castration surgery and a placebo implant (CON; $n = 14$). Following surgery, three males (one per treatment) were housed together in each cage, and posttreatment measures were collected over a period of 108 d.

Testosterone Implants

We constructed tonic-release testosterone implants from 5-mm lengths of Silastic tubing (Dow Corning, Midland, MI; 1.47 mm i.d., 1.96 mm o.d.). After sealing one end of each tubule with silicone adhesive gel (Dow Corning), we used a Hamilton syringe to inject 3 μL of a solution of testosterone (T-1500, Sigma-Aldrich, St. Louis, MO) dissolved in dimethyl sulfoxide (DMSO; 100 μg T/μL DMSO) into the open end of each implant. We then sealed each tubule with silicone adhesive and waited several days for the DMSO to evaporate and diffuse through the tubing, leaving 300 μg of crystalline testosterone within the lumen (ca. 1.5 mm length) of each implant. We constructed placebo implants in an identical fashion but injected them with pure DMSO, which left an empty tubule after evaporation and diffusion. Previous experiments on other lizard species of similar size verify that these implants reliably elevate plasma testosterone of castrated males to physiologically relevant levels typical of breeding males (Cox and John-Alder 2005; Cox et al. 2005*a*, 2006).

Surgical Procedures

Animals were fasted for 1 d before surgery. We first applied local anesthesia at the site of incision with an intraperitoneal injection of lidocaine (2 μL of 2% lidocaine HCl; Phoenix Pharmaceutical, St. Joseph, MO). We then immobilized the animals by placing them in a freezer at −20°C for ca. 5 min and performing surgeries atop a slightly thawed reusable chem-

ical ice pack. We exposed the testes with a single ventral incision and bilaterally castrated (orchiectomized) CAST and TEST males by ligating each spermatic cord with surgical silk, ablating each testis, and cauterizing each ligated spermatic cord after removal of the testes. For CON males, we performed "sham" surgeries in which we made identical incisions to expose and manipulate the testes while leaving them intact. We then inserted either a testosterone implant (TEST) or a placebo implant (CAST and CON) into the coelomic cavity and closed the incision with Nexaband surgical glue (Veterinary Products Laboratories, Phoenix, AZ). Animals were allowed to recover in sterile plastic containers overnight before being returned to their cages.

Testosterone Assay

At 56 and 108 d posttreatment, we used heparinized microhematocrit capillary tubes (22-362-566, Fisher Scientific, Pittsburgh, PA) to collect blood samples from each animal via the postorbital sinus. We then centrifuged these samples and stored the separated plasma at −20°C until subsequent assays. Radioimmunoassays were performed following methods reported elsewhere (Smith and John-Alder 1999; Cox and John-Alder 2005; Cox et al. 2005*a*). We conducted two separate assays for samples collected at 56 and 108 d posttreatment. Plasma samples were extracted twice in diethyl ether, with mean 70.4% and 81.6% extraction efficiency for each of the two separate assays. Extracted samples were dried under a stream of ultrafiltered air and reconstituted in phosphate-buffered saline (PBS) with gelatin. Reconstituted samples were assayed with ^{3}H-testosterone as a radiolabel (PerkinElmer Life Sciences, Boston, MA) and testosterone antiserum (1 : 18,000 initial dilution) developed in rabbits by A. L. Johnson (University of Notre Dame, IN). Limits of detection were 2.72 and 2.90 pg testosterone per assay tube. For those replicates below the detection limit ($n = 12$, six animals per assay, all in the CAST treatment group), plasma testosterone levels were estimated using the detection limit as the potency value. Typical intra-assay variation is 6.7% (Smith and John-Alder 1999), while the interassay variation for these assays compared with previous assays was 6.9% and 11.1%.

Size and Growth

We measured SVL and body mass of each animal at 36, 64, and 98 d posttreatment to assess the allometry of performance traits and, when necessary, to serve as covariates in subsequent analyses. We also used these data to calculate linear growth rates by dividing change in size by elapsed time. Growth data are reported and analyzed in detail elsewhere (Cox et al. 2009) and are included in this article primarily to test the prediction that growth and immune function are traded off as competing targets of testosterone-mediated energy allocation.

Stamina

We measured stamina (i.e., endurance) by running lizards to exhaustion (loss of righting reflex) on a motorized treadmill rotating at 0.3 km/h. In a previous study of a similar *Anolis* species, this velocity optimized the inherent trade-off between obtaining sufficient variance among individuals (maximized at low speeds) and reducing the duration of individual trials (Perry et al. 2004). At this velocity, our trials typically lasted 3–9 min (median 5 min, 40 s), a period that was sufficiently long to induce substantial aerobic metabolism yet short enough to facilitate measurement of a large number of animals (Perry et al. 2004). Although several previous studies of anoles used an inclined treadmill to measure stamina (Perry et al. 2004; Calsbeek and Irschick 2007; Calsbeek and Smith 2008), we used a horizontal surface to facilitate direct comparison with a previous study that also manipulated testosterone in *A. sagrei* (John-Alder 1994).

Lizards were placed atop the moving treadmill and encouraged to run by gentle tapping of the tail and hind limbs. Lateral movements were constrained by confining lizards within a small open-bottom wire cage suspended just above the surface of the treadmill. A heat lamp was suspended above the cage and connected to a temperature sensor via a rheostat set to maintain ambient temperature at 32°C within the cage. Duration from initiation of the trial to loss of righting reflex was recorded as the measure of stamina. We conducted one trial before treatment and another at 72–76 d posttreatment to assess treatment effects. Animals were allowed to recover from stamina trials for at least 24 h before any other behavior or performance assay.

Sprint Speed

We measured maximal sprint speed by running lizards up a dowel (2 m length, 5 cm diameter) that was covered in cork for traction. Because anoles often hop on horizontal surfaces, we inclined the dowel at an angel of 45° from horizontal (Vanhooydonck et al. 2006). Lizards were placed at the base of the dowel and chased along its entire length and into a shelter at the top of the dowel. Pairs of infrared photocells and receptors were stationed at 25-cm intervals along the dowel to record the passage of lizards. These sensors were connected to a computer equipped with the program RaceTrack (ver. 2.0; Comet Controlling and Measuring Technologies, Wilrijk, Belgium), which logged data and calculated velocity over each 25-cm interval. We conducted three successive trials on each lizard and used the single greatest velocity achieved over any 25-cm interval as the measure of maximal sprint speed for that animal. We measured each lizard once before treatment and again at 44 d posttreatment. Our use of the term "maximal" follows common usage in the literature and refers to the fact that we used the single greatest velocity achieved by each animal. We have no means of assessing whether each animal was motivated to perform at its inherent maximal level.

Bite Force

We measured bite force using metal bite plates connected to a force transducer (type 9023, Kistler, Wintherthur, Switzerland). In turn, the force transducer was connected to a charge amplifier (5058a, Kistler) that recorded bite force (N). Handheld lizards were induced to gape and bite bilaterally on the plates. On each measurement date, we acquired five bites per lizard in two bouts (two or three bites per bout) with at least 30 min rest between bouts. This method has been shown to elicit repeatable and maximal bite force from lizards (Herrel et al. 2001; Irschick et al. 2006). On any given measurement day, we used the single greatest bite force achieved over five trials as the measure of maximal bite force for that animal. We measured bite force once before treatment and again at 66 d posttreatment. Our use of the term "maximal" refers to the fact that we used the single greatest bite force achieved by each animal and does not imply that each individual was motivated to perform at its inherent maximal level.

Dewlap Area

We measured dewlap area by placing each animal on its left side and using forceps to fully extend the dewlap by gently pulling forward on the second ceratobranchial cartilages (paired structures at the outer margin of the dewlap) near their articulation with the basihyal. Digital photographs of the extended dewlap were then taken with a Nikon L12 digital camera mounted on a tripod, and the area of the dewlap was calculated using ImageJ software (1.40g; W. Rasband, National Institutes of Health). This method yields repeatable measurements of *Anolis* dewlaps (Vanhooydonck et al. 2005). We measured dewlap area once before treatment and again at 66 d posttreatment.

Immune Function

We assayed immune function by injecting males with the novel mitogen phytohemagglutinin (PHA-P; L8754, Sigma-Aldrich). We measured the thickness of each animal's right hind foot to the nearest 0.01 mm at a standardized location (between the first and the fifth digits) using dial calipers and then injected 0.2 mg phytohemagglutinin (PHA) dissolved in 0.01 mL PBS into the foot. We measured the foot again at 24 h postinjection and calculated swelling response to PHA as the difference between pre- and postinjection foot thickness. We used the mean of three replicate measurements of foot thickness for each estimate. Estimates of foot swelling were highly repeatable (repeatability = 0.92). We measured swelling response to PHA at 64 d posttreatment. Unlike most other variables, we did not measure immune function before treatment.

PHA directly stimulates mitosis of T lymphocytes and is therefore commonly used to assess acquired cell-mediated immune function. However, PHA influences a variety of vertebrate cell types, and the swelling response that we measured probably reflects a combination of cellular responses, including both innate and acquired components of the immune system (Kennedy and Nager 2006; Martin et al. 2006). For these reasons, the interpretation of our results with respect to organismal immunocompetence is complex. Nonetheless, the PHA assay is informative because it provides an in vivo measure that can be quickly obtained with minimal stress to the animal. Hence, this assay is widely used as a surrogate for immunocompetence in many vertebrates, including lizards (Svensson et al. 2001; Belliure et al. 2004; Oppliger et al. 2004; Calsbeek et al. 2008).

Behavior

We measured agonistic behaviors by placing each animal in a cage that was opaque on the sides and equipped with a full mirror on the back wall to simulate a size-matched opponent and minimize variation due to the opponent's motivation to display (Brandt 2003). A heat lamp suspended above the cage maintained ambient temperature between 28° and 32°C, near the mean active body temperature for wild *A. sagrei*. Lizards were placed in the cage facing the mirror, which was initially obscured from view by an opaque container placed over the lizard. After a 2-min acclimation period, the container was removed so that the lizard could view its own reflection. An observer behind a blind then recorded the occurrence of three behaviors over a 10-min trial: (1) dewlap extension—extension and retraction of the dewlap; (2) push-up—elevation and lowering of the anterior portion of the body using the forelimbs; (3) head bob—rapid elevation and lowering of the head. These behaviors are frequently used in social communication among male anoles, particularly in the context of territorial defense and agonistic interactions (Jenssen et al. 1995).

Statistical Procedures

We compared differences in pre- and posttreatment values among groups using ANOVA with treatment as the main effect. When response variables were correlated with size, we compared groups using ANCOVA with SVL or body mass as a covariate. We verified homogeneity of slopes among groups (i.e., nonsignificant treatment-by-SVL interactions) before conducting ANCOVA. When pretreatment values were available, we assessed treatment effects using repeated-measures ANOVA with treatment as a between-subjects effect and time and the time-by-treatment interaction as within-subjects effects. Behavioral responses were nonnormally distributed, so we used nonparametric Kruskal-Wallis analyses to compare groups. In addition to ANOVA and ANCOVA tests across treatment groups, we examined correlations between individual plasma testosterone levels and response variables. These correlative analyses always gave conclusions identical to those obtained by comparing treatment groups. All statistical analyses were conducted using JMP, version 6.0.2 (SAS Institute, Cary, NC).

Results

Plasma Testosterone

Surgical castration and hormone replacement had pronounced effects on circulating testosterone levels measured at 56 d posttreatment ($F_{1,37} = 14.86$, $P < 0.0001$) and at 108 d posttreatment ($F_{1,37} = 16.02$, $P < 0.0001$). Castration reduced plasma testosterone of CAST to basal levels that were significantly lower than those of either TEST or CON (Fig. 1). Exogenous hormone implants restored plasma testosterone of castrated TEST males to levels that were statistically indistinguishable from those of intact CON (Fig. 1).

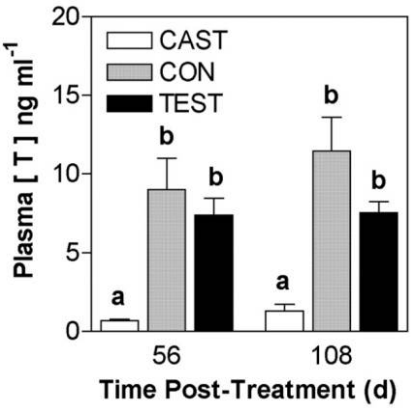

Figure 1. Mean (+1 SE) plasma testosterone levels in each treatment group at 56 and 108 d posttreatment. Lowercase letters denote statistical separation based on ANOVA with Tukey's post hoc test.

Stamina

Pretreatment stamina increased weakly with SVL ($F_{1,37} = 3.83$, $P = 0.06$). Stamina did not differ among groups before treatment ($F_{2,37} = 1.28$, $P = 0.29$). Posttreatment stamina was positively correlated with SVL ($F_{1,37} = 4.88$, $P = 0.03$; Fig. 2*A*), but groups did not differ in posttreatment stamina ($F_{2,37} = 1.90$, $P = 0.16$), even when including SVL as a covariate ($F_{2,36} = 2.22$, $P = 0.12$). Similarly, we did not find a time-by-treatment interaction when using repeated-measures ANCOVA with SVL as a covariate ($F_{2,35} = 0.78$, $P = 0.47$). Finally, we found only a very weak trend toward stamina increasing with circulating plasma testosterone levels across treatment groups ($F_{1,38} = 3.18$, $P = 0.08$, $r^2 = 0.08$; Fig. 2*B*). Thus, our data do not support the prediction that testosterone increases stamina in *Anolis sagrei.*

Sprint Speed

Sprint speed did not differ among groups before treatment ($F_{2,37} = 0.05$, $P = 0.95$) or at 44 d posttreatment ($F_{2,37} = 0.99$, $P = 0.38$; Fig. 2*B*). The time-by-treatment interaction for sprint speed was not significant using repeated-measures ANOVA ($F_{2,35} = 0.72$, $P = 0.50$). Moreover, sprint speed was independent of circulating plasma testosterone levels ($F_{1,37} = 0.71$, $P = 0.40$, $r^2 = 0.02$; Fig. 2*B*). Sprint speed was not correlated with SVL at any of the three measurement points ($P > 0.1$ for all comparisons; Fig. 2*B*). Similar to stamina, our data do not support the prediction that testosterone increases sprint speed in *A. sagrei*

Bite Force

Bite force did not differ among groups before treatment ($F_{2,30} = 0.45$, $P = 0.64$). However, posttreatment bite force was positively correlated with circulating plasma testosterone levels ($F_{1,39} = 8.76$, $P < 0.006$, $r^2 = 0.18$; Fig. 2*C*). Following treatment, TEST exerted significantly greater bite forces than CAST, with CON intermediate and statistically indistinguishable from either group ($F_{2,38} = 4.46$, $P = 0.018$; Fig. 2*C*). Repeated-measures ANOVA revealed a significant decrease in bite force from pre- to posttreatment ($F_{1,30} = 205.83$, $P < 0.0001$), but the time-by-treatment interaction was weak and nonsignificant ($F_{2,30} = 2.22$, $P = 0.13$). Moreover, bite force was strongly correlated with SVL ($F_{1,39} = 51.36$, $P < 0.0001$; Fig. 2*C*), and posttreatment differences in bite force among treatments were not significant after including SVL as a covariate ($F_{2,37} = 0.87$, $P = 0.43$). Thus, the significantly greater posttreatment bite force exhibited by TEST males appears primarily to reflect their growth to greater absolute sizes.

Dewlap Area

Dewlap area did not differ among groups before treatment ($F_{2,30} = 0.93$, $P = 0.41$), but repeated-measures ANOVA revealed a significant time-by-treatment interaction ($F_{2,30} = 5.74$, $P = 0.008$). Dewlap area increased slightly in CON and TEST males but decreased substantially in CAST males (Fig. 2*D*). Posttreatment dewlap area was positively correlated with SVL ($F_{1,29} = 10.47$, $P < 0.003$; Fig. 2*D*), and the time-by-treatment interaction remained significant when including SVL as a covariate ($F_{2,29} = 4.61$, $P = 0.018$). Posttreatment dewlap area was positively correlated with circulating plasma testosterone level ($F_{1,39} = 10.39$, $P < 0.003$, $r^2 = 0.21$; Fig. 2*D*). While the slight increase in dewlap area of CON and TEST can be explained simply by their overall growth in body size during the experiment, the decrease observed in CAST suggests that elevated testosterone levels are required to maintain the large size of the dewlap.

Dewlap area and bite force both increased with body size, and, consequently, dewlap area predicted bite force before treatment ($r^2 = 0.23$, $P = 0.005$; Fig. 3*A*) and following treatment ($r^2 = 0.20$, $P < 0.004$; Fig. 3*B*). The positive correlation for pretreatment values was not simply the result of underlying allometry because we found a strong relationship between bite force and dewlap area even after including SVL as a covariate ($F_{1,29} = 7.04$, $P = 0.013$; Fig. 3*C*). However, we found no evidence that animals with relatively larger dewlaps had relatively greater bite forces when including SVL as a covariate using posttreatment measures ($F_{1,39} = 1.12$, $P = 0.30$; Fig. 3*D*).

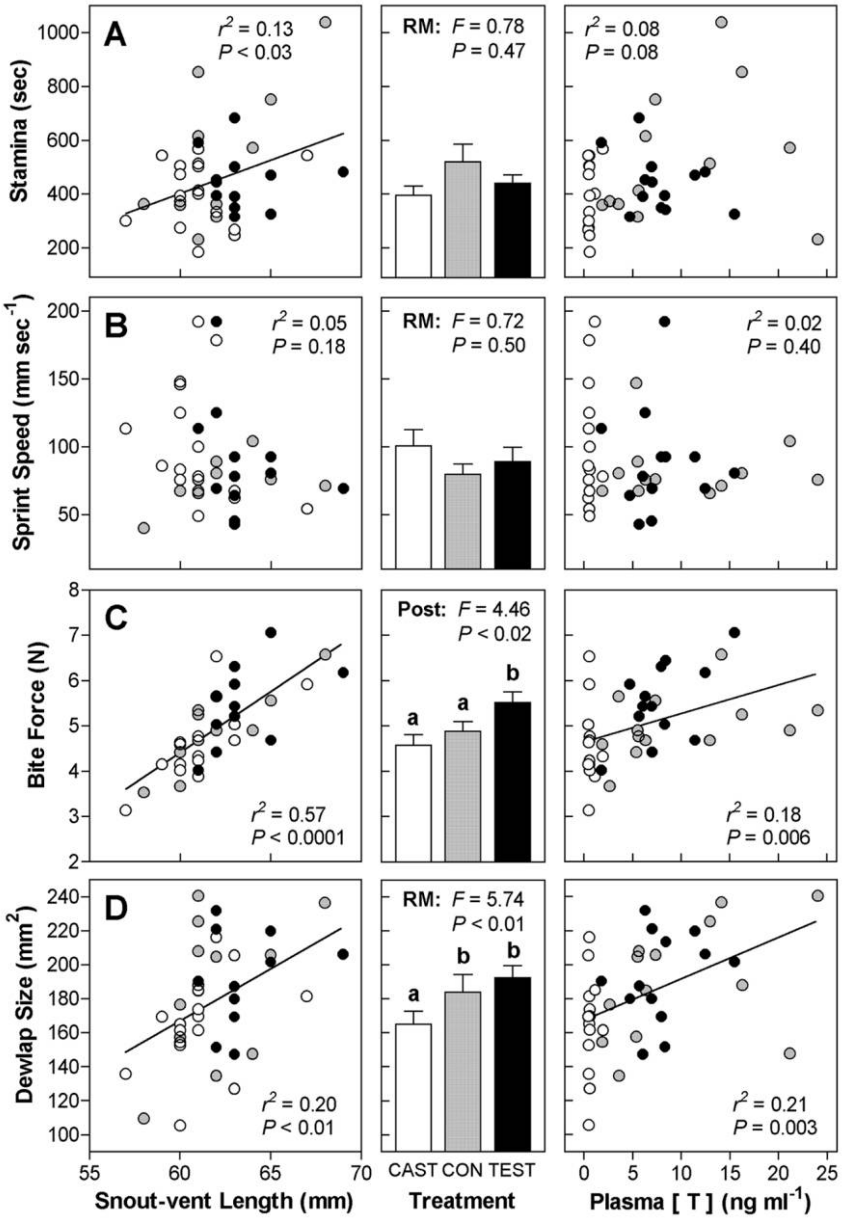

Figure 2. Treatment effects on (*A*) stamina, (*B*) sprint speed, (*C*) bite force, and (*D*) dewlap area. *Left*, scaling of each trait with body size (snout-vent length [SVL]). *Center*, mean (+1 SE) posttreatment values for each trait. Statistics are reported for repeated-measures analyses including pretreatment values, except *C*, which reports results of one-way ANOVA using only posttreatment values. Lowercase letters denote statistical separation among groups on the basis of Tukey's post hoc tests. *Right*, relationship between each trait and circulating plasma testosterone levels measured at 56 d posttreatment.

Immune Function

Although TEST males exhibited the lowest mean swelling response (Fig. 4*A*), this measure of immune function did not differ significantly among treatment groups ($F_{2,38} = 0.83$, $P = 0.44$). Swelling response to PHA was unrelated to SVL ($F_{1,39} = 0.80$, $P = 0.38$) and body mass ($F_{1,39} = 3.22$, $P = 0.08$). However, swelling response was negatively correlated with growth in body mass ($F_{1,36} = 6.69$, $P = 0.014$), and this effect differed significantly across treatments ($F_{2,39} = 5.61$, $P = 0.008$; Fig. 4*B*). Whereas CAST and CON exhibited no relationship between growth and swelling response, these variables were negatively correlated in TEST males ($r^2 = 0.63$, $P < 0.001$; Fig. 4*B*). Thus, although we found no overall treatment effect on swelling response to PHA, this latter result is consistent with the hypothesis that testosterone stimulates growth at the expense of immune function.

Behavior

Before treatment, groups did not differ in their overall frequency of dewlap extensions (Kruskal-Wallis: $\chi^2 = 2.22$, $P =$

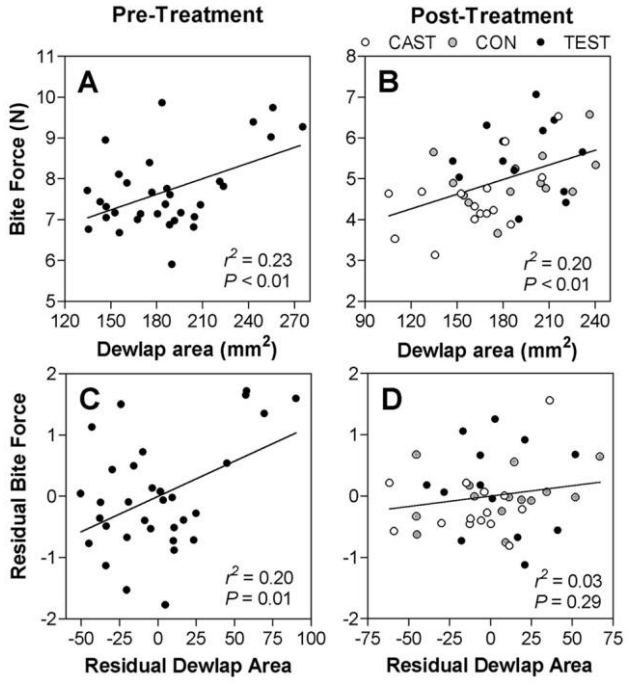

Figure 3. Bite force as a function of dewlap size before treatment (*left*) and at 66 d posttreatment (*right*). *Top*, positive correlations between bite force and dewlap size; *bottom*, residual values from each measure regressed against snout-vent length to remove the effects of body size.

0.33), head bobs ($\chi^2 = 0.05$, $P = 0.98$), or push-ups ($\chi^2 = 0.11$, $P = 0.95$). Following treatment, TEST tended to exhibit higher mean frequencies of dewlap extensions and push-up displays than CAST and CON (Fig. 5), but these effects were never significant when using nonparametric Kruskal-Wallis analysis to account for skewed distributions of behavioral data (dewlap extensions: $\chi^2 = 1.52$, $P = 0.47$; push-up displays: $\chi^2 = 2.43$, $P = 0.30$). Treatment groups did not differ in head bob displays ($\chi^2 = 3.51$, $P = 0.17$), but this analysis was heavily influence by one CAST male who performed 63 head bobs, more than any other animal in the study. No other CAST male performed even a single head bob, and with this one outlying male excluded, TEST and CON exceeded CAST in frequency of head bobs ($\chi^2 = 6.28$, $P = 0.04$; Fig. 5). Plasma testosterone levels for this animal were extremely low at both 55 d (0.56 ng/mL) and 108 d (0.47 ng/mL) posttreatment, so exclusion of this animal is justified only by its atypical behavior, not by any apparent failure of the castration surgery.

Discussion

Our experiment revealed effects of castration and testosterone replacement on several traits (i.e., growth rate, dewlap size, bite force) but provided no evidence for similar effects on other traits (i.e., stamina, sprint speed). Several other results were suggestive but failed to provide definitive support for an activational role of testosterone in trait expression (i.e., immune function, behavioral displays). Our castration surgeries reduced plasma testosterone to basal levels well below those of wild breeding males (Tokarz et al. 1998) and intact control males (Fig. 1). Moreover, our hormone implants restored plasma testosterone to physiologically realistic levels similar to those of intact males (Fig. 1). This supports the biological relevance of our observed treatment effects (or lack thereof) on behavior, morphology, physiology, and performance. Given that we focus solely on testosterone, our results must be interpreted with the caveat that many other factors could influence the traits we measured. This list includes but is not limited to (1) the metabolism of testosterone to other behaviorally relevant hormones (e.g., estradiol, dihydrotestosterone; Winkler and Wade 1998; Rosen and Wade 2001), (2) the importance of tissue sensitivity (e.g., androgen receptor expression) rather than circulating androgen levels per se (Holmes and Wade 2005), and (3) interactions with other signaling pathways, such as the endocrine stress axis the or somatotrophic axis. However, to the extent that testosterone varies in a seasonal and sex-specific fashion and is likely to interact with these factors, it remains a promising mechanism for the integration of this complexity.

The most dramatic effect of testosterone that we observed was its stimulatory effect on growth in SVL and body mass. This result is analyzed and discussed in detail elsewhere in the context of the extreme male-biased sexual size dimorphism (SSD) exhibited by *Anolis sagrei* (Cox et al. 2009). However, because these data are directly relevant to the allometry of

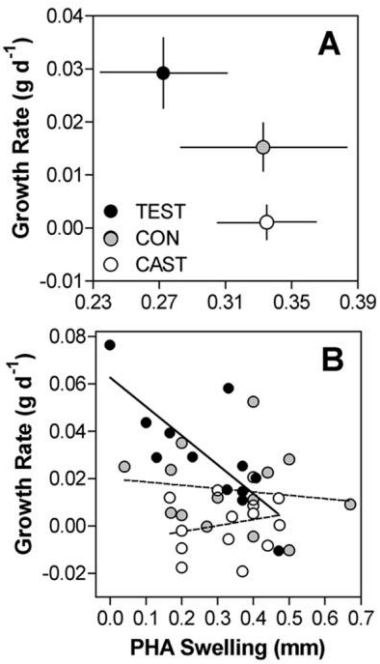

Figure 4. Treatment effects on immune function (phytohemagglutinin swelling) and growth rate, illustrating the negative relationship between these traits (*A*) across the mean (+1 SE) values for each treatment group and (*B*) within the TEST group. The solid regression line in *B* illustrates the negative relationship within TEST. Dashed lines illustrate the lack of such a relationship in CON (*upper line*) and CAST (*lower line*).

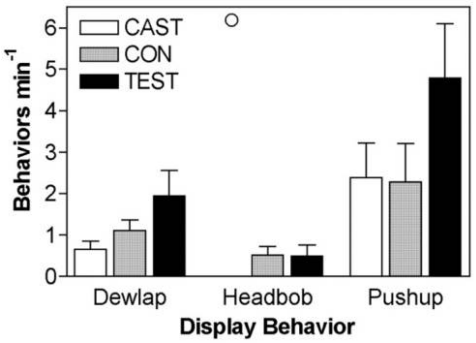

Figure 5. Mean (+1 SE) frequency of dewlap extensions, head bobs, and push-up displays during 10-min focal trails in which lizards were stimulated by their own image in a mirror. The circle indicates a single CAST male that performed 63 head bobs. This male was excluded from the mean calculated for this group because no other CAST male performed a single head bob.

morphology and performance (Fig. 2) and potential energetic trade-offs with immune function (Fig. 4), we briefly summarize them here. TEST grew more than twice as fast as CON and exceeded CAST fourfold in rate of SVL growth. TEST also gained mass more than twice as fast as CON, whereas CAST exhibited no change in mass (Cox et al. 2009). Across treatment groups, growth rate increased as a function of circulating plasma testosterone level ($F_{1,39} = 5.32$, $r^2 = 0.12$, $P = 0.027$). These results are consistent with unpublished data from *A. sagrei* suggesting that castration inhibits growth in this species (H. B. John-Alder, personal communication) and with comparative data from other lizard species indicating that testosterone acts as a "bipotential" regulator by stimulating growth in species with male-biased SSD and inhibiting growth in species with female-biased SSD (Cox and John-Alder 2005; Cox et al. 2005*a*, 2009; John-Alder and Cox 2007; John-Alder et al. 2007). These growth effects also provide indirect evidence for dramatic physiological differences among treatment groups, thus corroborating our measures of plasma testosterone as evidence that our manipulations induced their intended physiological effects.

The stimulatory effect of testosterone on growth may help explain why TEST exerted absolute bite forces stronger than those of CON and CAST males (Fig. 2*C*). Bite force was strongly and positively correlated with body size (Fig. 2*C*), but we found no difference in size-specific bite force when including SVL as a covariate. Thus, the significantly greater bite forces exerted by TEST appear simply to reflect their growth to greater absolute sizes rather than increased bite force for a given body size (e.g., by hypertrophy of the jaw musculature). This may not be surprising given that in the related species *Anolis carolinensis*, bite force of males is actually greater in the winter when testosterone levels are basal than in the spring when testosterone levels peak (Irschick et al. 2006). Thus, bite force of anoles may be largely independent of direct activational effects of testosterone. This natural decrease in bite force from winter to spring may also help explain the sharp overall decrease in bite force that we observed from pretreatment (winter) to posttreatment (spring), which is otherwise puzzling given that body size increased over this same interval. The fact that we observed greater growth and bite force in TEST relative to CON males is also somewhat puzzling given that plasma testosterone levels did not differ between these groups (Fig. 1). One explanation may be that testosterone levels of TEST were higher immediately following implantation but had declined to stable levels at 56 and 108 d posttreatment. Despite the overall similarity of mean testosterone levels in CON and TEST, the positive correlations between circulating testosterone levels and both growth rate and bite force across individuals further support the conclusion that these traits are influenced by circulating testosterone.

Dewlap area also increased with body size (Fig. 2*D*), but unlike bite force, treatment differences in dewlap area do not appear to result simply from differences in overall body size. Although increased growth of CON and TEST may explain the slight increase in dewlap area in each of these groups (mean 6% and 2% increases, respectively), the overall treatment effect that we observed is driven primarily by a large (15%) decrease in dewlap area of CAST males. This suggests that testosterone is required to maintain the expression of large dewlaps. Consistent with this implied activational role of testosterone, dewlap area decreases from breeding to nonbreeding seasons in a related species, *A. carolinensis* (Irschick et al. 2006). A subsequent experiment found no effect of castration and testosterone replacement on dewlap area (J. P. Henningsen, J. F. Husak, R. M. Cox, I. T. Moore, and D. J. Irschick, unpublished data), but other studies have shown that testosterone influences cartilage and muscle fibers in the dewlap during development (Lovern et al. 2004; Holmes et al. 2007). Our results for dewlap area are consistent with those of numerous other studies that have demonstrated androgen-mediated seasonal and sexual variation in colorful structures used in social signaling (Kimball and Erpino 1971; Rand 1992; Hews and Moore 1995; Salvador et al. 1996; Hews and Quinn 2003; Quinn and Hews 2003; Cox et al. 2005*b*, 2008).

We predicted that testosterone would exert pleiotropic effects on the traits that we studied because many of these traits interact to determine survival and reproductive success. For example, dewlap area has been proposed as an honest signal that conveys information about bite force (Vanhooydonck et al. 2005; Irschick et al. 2006). In anoles and other lizards, bite force determines dominance in male-male contests to establish territories and acquire access to females (Lailvaux et al. 2004; Lappin and Husak 2005; Husak et al. 2006). Because contests that escalate to biting can often result in serious injury or death of the participants (Calsbeek and Marnocha 2006), honest signaling can provide a mutually beneficial means of pacific resolution when contestants are unevenly matched in performance. We found that absolute measures of bite force were positively correlated with dewlap area and that relative measures of size-specific bite force were positively correlated with relative dewlap area for pretreatment (but not posttreatment) mea-

surements (Fig. 4). Similar results have been reported for other sexually dimorphic anoles with territorial mating systems, suggesting that one function of the dewlap is the transmission of reliable information on fighting performance mediated through bite force (Vanhooydonck et al. 2005; Irschick et al. 2006).

Structures such as the dewlap require appropriate behavioral displays to function as social signals, so it is intuitive to hypothesize that these interactive morphological and behavioral traits are regulated by common endocrine mechanisms (Hews and Quinn 2003). In *A. sagrei* males, the frequency of dewlap displays is significantly greater in the breeding season than in the nonbreeding season, a behavioral shift that mirrors seasonal changes in circulating testosterone levels (Tokarz et al. 1998, 2002). A previous experiment in *A. sagrei* reported that castration reduces and exogenous testosterone restores the rate of dewlap extension in response to conspecifics (Tokarz et al. 2002). Our results are qualitatively consistent with this pattern (Fig. 5), although our use of a single posttreatment behavioral assay per animal may have contributed to the considerable variation within treatments that prevented us from statistically confirming this result. Moreover, our use of a mirror, rather than a conspecific male, created a situation in which there was no potential for resolution of aggressive interactions. This could potentially magnify individual variation by generating increased frequency of aggression in those animals predisposed to fight while providing no stimulus whatsoever for those animals not inclined to fight. This could explain why a previous study using conspecific males as a stimulus found stronger evidence for a stimulatory effect of testosterone on dewlap display (Tokarz et al. 2002). However, studies of a related species, *A. carolinensis*, have shown that testosterone has a much stronger effect on dewlap extensions in the context of courtship displays to females than in the context of aggressive displays to males (Winkler and Wade 1998; O'Bryant and Wade 2002).

We also observed a reduction in the frequency of head bob displays following castration, as only one CAST male was observed performing this behavior (Fig. 5). However, this individual displayed the greatest number of head bobs observed in the experiment. Given that this male was found to have low plasma testosterone levels and no visible gonadal tissue upon necropsy, it is clear that head bob displays are not entirely dependent on circulating testosterone. It is perhaps noteworthy that this individual was also the largest male in the experiment, suggesting that he may have been socially dominant both before and during the experiment. Studies of a related species, *A. carolinensis*, have shown that exogenous testosterone implants dramatically increase the frequency of head bob displays in juvenile males and females (Lovern et al. 2001), but our results were equivocal. Finally, although TEST exhibited the greatest mean frequency of push-up displays (Fig. 5), the considerable variance among individuals overwhelmed any potential treatment effects. Overall, our results provide only weak support for an activational role of testosterone in stimulating aggressive behavioral displays. However, numerous other studies have demonstrated that testosterone regulates seasonal and sexual differences in lizard aggression (Moore and Marler 1987; Moore 1988; Moore and Lindzey 1992; Winkler and Wade 1998; Smith and John-Alder 1999; Lovern et al. 2001; Hews and Quinn 2003; Weiss and Moore 2004). Our inability to detect robust treatment effects also indicates that social context and other factors are likely to interact with testosterone to influence aggressive behavior.

Testosterone-mediated increases in traits such as growth rate, bite force, dewlap area, and aggressive displays are presumably adaptive in the sense that they facilitate intrasexual dominance and thereby increase reproductive success. However, elevated testosterone levels may also incur fitness costs. For example, in lizards, elevated testosterone levels are often associated with increased susceptibility to parasites (Salvador et al. 1996; Olsson et al. 2000; Klukowski and Nelson 2001; Uller and Olsson 2003; Cox et al. 2005*a*; Cox and John-Alder 2007). One potential explanation for these effects is that testosterone increases susceptibility to infestation via suppression of the immune system (Folstad and Karter 1992; Klein 2004). Immunosuppressive effects of testosterone have been observed in numerous vertebrate taxa (Roberts et al. 2004) and are particularly prominent in studies of lizards (Saad et al. 1990; Veiga et al. 1998; Olsson et al. 2000; Belliure et al. 2004; Oppliger et al. 2004). Although we observed a trend toward suppressed immune function following treatment with exogenous testosterone (Fig. 4*A*), we did not detect a significant overall treatment effect. However, we did find a strong negative relationship between immune function and growth rate both across treatments (Fig. 4*A*) and within the TEST group (Fig. 4*B*), providing some support for the hypothesis that testosterone mediates an energetic tradeoff between growth and immune function. Although our measure of immune function via PHA swelling response is difficult to interpret with respect to immunocompetence and fitness per se (Kennedy and Nager 2006; Martin et al. 2006), previous studies of wild *A. sagrei* populations have shown that this measure correlates with viability in the wild, often in a sex-specific fashion (Calsbeek and Bonneaud 2008; Calsbeek et al. 2008).

Evolutionary biologists often focus on measuring selection on individual morphological, physiological, and behavioral traits, but it is widely acknowledged that fitness is often more directly influenced by aspects of whole-organism performance that reflect the interaction of these underlying traits (Arnold 1983; Lappin and Husak 2005; Calsbeek and Irschick 2007; Calsbeek et al. 2008). For example, in *A. sagrei*, morphological traits such as body size and limb length influence survival by virtue of their contributions to ecologically relevant aspects of whole-organism performance such as stamina and sprint speed (Calsbeek and Irschick 2007; Calsbeek 2008). In an ecologically similar species, *Anolis cristatellus*, locomotor performance also correlates with assertive behavioral displays and social dominance, suggesting the involvement of a common regulatory mechanism such as testosterone (Perry et al. 2004). Although we did not assess social dominance in our study, we found no evidence that testosterone influences either sprint speed or stamina in *A. sagrei* (Fig. 2). However, a previous study did report a stimulatory effect of testosterone on stamina in this species (John-Alder 1994), and studies of other lizards have

revealed similar effects (John-Alder et al. 1996; Klukowski et al. 1998; Sinervo et al. 2000). Thus, a link between testosterone, locomotor performance, and social dominance seems likely (Sinervo et al. 2000; Perry et al. 2004), although our present results do not support this scenario.

One important aspect of our experimental design that bears on the interpretation of our results is that males were housed in size-matched groups of three, with one male of each treatment per cage. In such conditions, anoles can form dominance hierarchies that could then influence their subsequent behavior and performance (Greenberg and Crews 1990). For example, CON and TEST males may have exhibited greater growth rates because of an inherent stimulatory effect of testosterone on the somatotrophic axis or because behavioral subordination of CAST males resulted in lower feeding rates despite ad lib. food availability. Similarly, the propensity of individual males to display in a mirror could reflect their current social status. If testosterone influences social status, which in turn influences behavior, this could effectively magnify any direct effect of testosterone on behavior. However, if the formation of dominance hierarchies is independent of circulating testosterone levels, then the effects of social status on behavior could obscure any direct effects of testosterone, perhaps explaining the large variance in behavior that we observed within treatments. While this caveat complicates interpretation of our results, the fact remains that all animals were housed in identical social environments. Thus, any observed differences among treatment groups reflect either direct or indirect effects of testosterone.

In summary, we have shown that testosterone influences several traits that have previously been shown to influence survival and reproductive success in lizards. However, we did not find any effect of testosterone on other ecologically relevant performance measures such as sprint speed and stamina. Finally, we have provided indirect evidence that elevated testosterone levels may incur fitness costs in the form of energy allocation trade-offs between growth and immune function. Our results provide some support for the hypothesis that testosterone acts pleiotropically to coordinate the expression of diverse phenotypic traits (e.g., growth, bite force, dewlap size, aggressive behavior) that interact to influence fitness in a sex-specific and seasonal fashion. Because our results are entirely based on captive animals, future studies will benefit from simultaneously linking testosterone to trait expression and resultant fitness (i.e., survival and reproductive success) in natural populations. This approach will help to simultaneously elucidate the counterbalancing fitness costs and benefits of elevated plasma testosterone levels and provide an explicit analytical framework in which to consider how traits with common endocrine regulatory mechanisms interact to influence fitness. Moreover, because hormonal pleiotropy involves tissue-specific responses to a single endocrine messenger, future studies would benefit from a mechanistic investigation of androgen receptor expression and cellular response to testosterone on a tissue-specific basis. Previous research has clarified much of the molecular endocrine basis for effects of testosterone on the behavior of *Anolis* lizards (Gans and Crews 1992), but few data exist to integrate these findings with its regulatory effects on traits such as growth, performance, and immune function.

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