Testosterone, growth and the evolution of sexual size dimorphism

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Abstract

The integration of macroevolutionary pattern with developmental mechanism presents an outstanding challenge for studies of phenotypic evolution. Here, we use a combination of experimental and comparative data to test whether evolutionary shifts in the direction of sexual size dimorphism (SSD) correspond to underlying changes in the endocrine regulation of growth. First, we combine captive breeding studies with mark-recapture data to show that male-biased SSD develops in the brown anole lizard (*Anolis sagrei*) because males grow significantly faster than females as juveniles and adults. We then use castration surgeries and testosterone implants to show that castration inhibits, and testosterone stimulates, male growth. We conclude by reviewing published testosterone manipulations in other squamate reptiles in the context of evolutionary patterns in SSD. Collectively, these studies reveal that the evolution of SSD has been accompanied by underlying changes in the effect of testosterone on male growth, potentially facilitating the rapid evolution of SSD.

Introduction

Biologists dating back to Darwin (1871) have sought to understand why males are larger than females in many species, yet females are the larger sex in numerous others (Fairbairn *et al.*, 2007). Evolutionary studies of sexual size dimorphism (SSD) have focused primarily on inferring the selective pressures responsible for its evolution using comparative approaches (Székely *et al.*, 2000; Pérez-Barbería *et al.*, 2002; Cox *et al.*, 2003) or measuring current selection on body size (Badyaev *et al.*, 2000; Preziosi & Fairbairn, 2000; Schulte-Hostedde *et al.*, 2002). Recently, physiologists, geneticists and developmental biologists have sought to integrate this ultimate perspective with an understanding of the proximate mechanisms that facilitate the expression of dimorphic phenotypes from a genome that is largely shared between the sexes (Rhen, 2007). A major goal of this integrative approach is to reconcile the observed phylogenetic lability of SSD with theoretical expectations that its evolution should be highly constrained because of inter-sexual genetic

correlations (Lande, 1980, 1987; Fairbairn & Roff, 2006; Bonduriansky, 2007; Delph, 2007; Fedorka *et al.*, 2007). One resolution to this paradox may be that males and females share most of the same genomic architecture for growth and body size, but that these shared genes are differentially regulated by sex-specific modifiers (Badyaev, 2002).

Sex steroids (i.e. androgens, estrogens and progestins) are excellent candidates for the regulation of sex differences in growth and body size because they are produced and secreted in sex-specific fashion by the gonads. However, if sex steroids are to account for divergent patterns of SSD across species, then either their patterns of secretion or their effects on growth must differ across species. This distinction bears on a fundamental question in evolutionary endocrinology. Are the pleiotropic effects of hormones on their target tissues evolutionary conserved across species, such that trait evolution proceeds primarily via changes to circulating hormone levels ('evolutionary constraint hypothesis', Hau, 2007)? Or does selection independently alter the responsiveness of various tissues to hormones, such that traits can evolve independently within a common hormonal milieu ('evolutionary potential hypothesis', Hau, 2007)? For example, testosterone is commonly regarded as an anabolic steroid that stimulates male growth. However, the

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presumed generality of this stimulatory effect may be an artefact of historical focus on the endocrinology of model species (primarily mammals, birds and fishes) in which males happen to be the larger sex (Cox & John-Alder, 2005). By contrast, most studies of reptiles have actually observed an inhibitory effect of testosterone on growth (Crews et al., 1985; Abell, 1998; Klukowski et al., 1998; Lerner & Mason, 2001; Cox & John-Alder, 2005; Cox et al., 2005a). However, the majority of these studies have been conducted on species in which females are the larger sex. Thus, a general problem plaguing this area of research is that observed differences in the effects of testosterone on growth cannot be unambiguously attributed to differences in SSD vs. phylogenetic conservatism within lineages.

Recent experiments on squamate reptiles have sought to disentangle these confounding factors by manipulating testosterone levels in closely-related species that differ in the direction of SSD (Cox & John-Alder, 2005; John-Alder & Cox, 2007; John-Alder et al., 2007). Squamates are ideally suited for such studies because they exhibit considerable phylogenetic lability in SSD (Fig. 1). In particular, studies of Sceloporus lizards suggest that

testosterone may act as a 'bipotential' regulator by stimulating growth in species with male-biased SSD, but inhibiting growth in species with female-biased SSD. Testosterone manipulations in mammals, fishes, birds and other reptiles hint at the generality of this 'bipotential regulation hypothesis' for SSD, but taxonomic disparity and differences in experimental methodology complicate direct comparisons (Cox & John-Alder, 2005; Sockman et al., 2008). Support for this hypothesis is tentative even within squamates, as only one study has found clear evidence that testosterone stimulates skeletal growth in this group (Cox & John-Alder, 2005). Moreover, this stimulatory effect was not observed when this same species was studied in captivity (Cox et al., 2006), raising questions as to whether testosterone is truly capable of stimulating growth in this lineage. Here, we address this deficiency by characterizing the effects of testosterone on male growth in a second lizard species with male-biased SSD.

The brown anole (Anolis sagrei) is a small, semi-arboreal lizard that is widely distributed throughout the West Indies. Similar to many other reptile lineages, the genus Anolis (Polychrotidae; Fig. 1) exhibits variation in
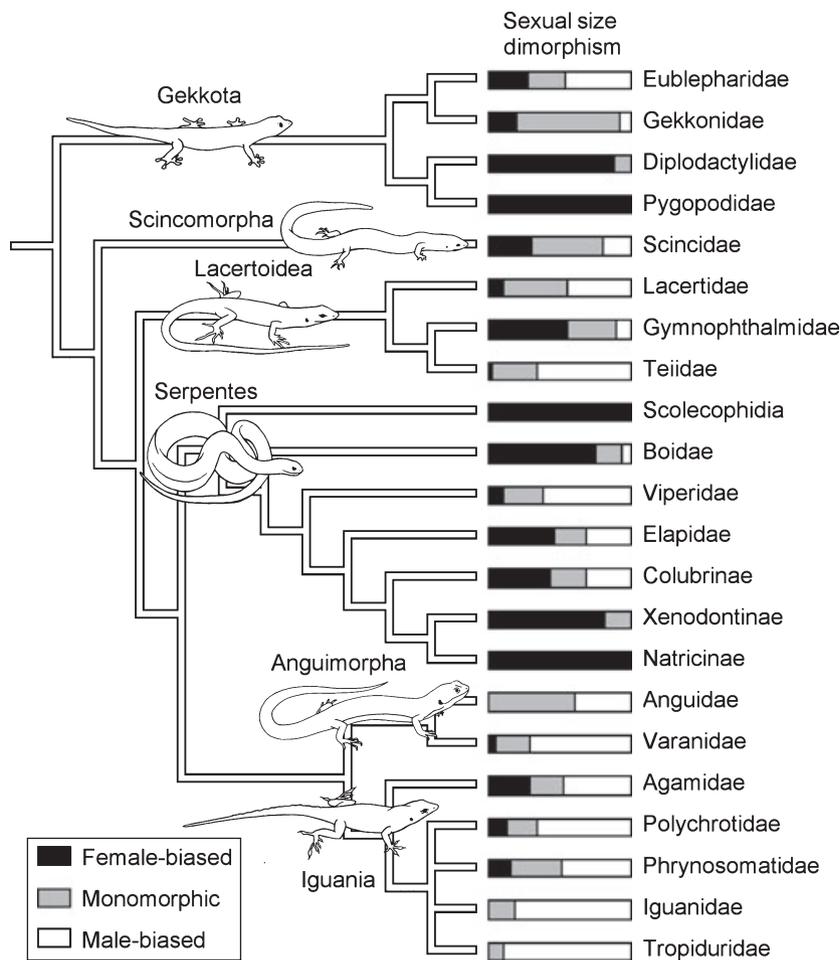
Fig. 1 Phylogenetic distribution of sexual size dimorphism (SSD) across major squamate lineages, illustrating the evolutionary lability of SSD. Bars indicate the relative frequency of male- and female-biased SSD [> 5% difference in mean adult snout-vent length (SVL)] and monomorphism (< 5% difference) within each lineage when counting each individual species as an observation. Squamate phylogeny is based on Vidal & Hedges (2005) and Brandley et al. (2008). SSD data are based on Cox et al. (2003) for lizards and Shine (1994) for snakes, with revisions by Cox et al. (2007). Multiple estimates of SSD per species were reduced to a single mean value to avoid multiple counting of species.

both the direction and the magnitude of SSD (Fitch, 1976; Butler *et al.*, 2000, 2007; Cox *et al.*, 2007). Although West Indian anoles typically exhibit male-biased SSD, the phylogeny of this group reveals repeated evolutionary transitions between relatively modest dimorphisms and extremes that are among the largest for any lizard (Butler *et al.*, 2000, 2007; Cox *et al.*, 2007). Similar to other 'trunk-ground' *Anolis* ecomorphs, *A. sagrei* exhibits extreme male-biased SSD (Butler *et al.*, 2000). Given the phylogenetic lability of SSD across *Anolis* and the extreme SSD of *A. sagrei*, it is likely that sex-specific growth modifiers underlie development and evolution of SSD in this genus in general, and this species in particular. Here, we provide a partial test of the bipotential regulation hypothesis as it applies to *A. sagrei*. We do this by (i) testing the underlying assumption that SSD reflects sex differences in growth, (ii) documenting the ontogenetic stages and environmental contexts in which growth divergence occurs and (iii) manipulating testosterone to determine its effect on male growth in this species. If the effect of testosterone is similar to that observed in the majority of reptiles studied to date, then testosterone should inhibit male growth. However, if the bipotential regulation hypothesis applies to *Anolis* lizards, then testosterone should stimulate male growth in this species with extreme male-biased SSD.

A complete test of the bipotential regulation hypothesis requires that the effects of testosterone be assessed in multiple species with divergent patterns of SSD (e.g. Cox & John-Alder, 2005). To complement our partial experimental test of this hypothesis in *A. sagrei*, we present a review of published studies measuring growth in response to castration and testosterone manipulation in squamate reptiles. We interpret these studies in light of phylogenetic relationships among species to determine whether the evolutionary lability of SSD in squamates (Fig. 1) corresponds to underlying lability in the effect of testosterone on growth.

Materials and methods

Growth and SSD in wild *Anolis sagrei*

We characterized SSD over five separate years (2003–2008) in a wild population of *A. sagrei* lizards on Kidd Cay near Georgetown (Great Exuma, Bahamas) (23°30′N, 75°45′W). Details regarding the ecology and demography of this population are available elsewhere (Calsbeek & Irschick, 2007; Calsbeek & Smith, 2008; Calsbeek *et al.*, 2008). In May of each year, we captured every adult lizard in the population with a hand-held noose and then measured its snout-vent length (SVL, to the nearest 0.5 mm using a ruler) and body mass (to the nearest 0.05 g using a 10-g Pesola® spring scale). To identify individuals, we permanently marked each lizard with a unique combination of coloured elastomer tags (Nauwelaerts *et al.*, 2000) that were injected subcutaneously

into the ventral surfaces of the limbs (Calsbeek & Marnocha, 2006). We used size measurements to calculate an index of SSD (Lovich & Gibbons, 1992) for SVL and body mass: SSD = (male size/female size) − 1. For consistency with conventional usage, and to avoid ambiguity when discussing comparative patterns in SSD, we assigned this index a negative value when males were the larger sex and a positive value when females were the larger sex. We calculated this index for all lizards above the minimal sizes of reproductive maturity for males (39 mm) and females (34 mm) (Lee *et al.*, 1989). In three of the 5 years of our study (2005, 2006 and 2007), we recaptured all surviving lizards in September and measured them to assess sex differences in adult growth (change in SVL) over the summer breeding season. We did not measure size and growth for juvenile lizards in the wild.

Ontogeny of SSD in captive *Anolis sagrei*

We characterized the ontogeny of SSD on the basis of 302 anoles (152 males and 150 females) that we hatched and raised to adult body sizes in captivity. We obtained these hatchlings from an initial sample of 69 gravid adult females that we collected near Georgetown (Great Exuma, Bahamas) (23°30′N, 75°45′W) and returned to our captive breeding facility at Dartmouth College. We housed females individually in 10-gal glass terraria (50 × 25 × 30 cm) containing pine mulch bedding and a potted plant in which to oviposit. We watered cages and plants daily and provided crickets *ad libitum* (dusted weekly with Fluker's Repta-Vitamin dietary supplement; Fluker Farms, Port Allen, LA, USA). 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 Inc., Montreal, Canada) for heat and ultraviolet radiation. Daytime temperatures within cages spanned a gradient from 26–35 °C, bracketing the mean body temperature (T_b = 29.2 °C) of active *A. sagrei* in the wild (Lee, 1980). Every 3 weeks, we searched cages for new hatchlings and measured their SVL (to the nearest 1 mm using a ruler) and body mass (to the nearest 0.01 g using an electronic balance). We assigned each hatchling a permanent identification number by clipping a unique combination of toes.

Female anoles store sperm for up to 3 months and lay a single egg at approximately 1- to 2-week intervals (Calsbeek *et al.*, 2007). Thus, hatchlings in this study were born continuously over a 3-month period, after which time females no longer produced fertilized eggs. For analytical convenience, we assigned each hatchling an age of zero on the first date at which it was measured, although these zero-age animals actually comprised a range of ages (0–21 days) owing to the 3-week census intervals. We housed hatchlings together with their siblings and dam for approximately 7 months until they attained sizes characteristic of reproductive adults, at

which point they were housed individually or, for brief intervals, in breeding male–female pairs as part of a separate experiment. As hatchlings matured, we conducted size measurements over less frequent intervals of 4 weeks. The resultant characterization of the ontogeny of SSD covers approximately 1 year of growth in captivity, at which point males and females were sexually mature and approaching mean adult sizes observed in the wild. In the Bahamian populations that we study, approximately 85–95% of all individuals die before their second year, so this captive measurement interval of 1 year encompasses most of the natural lifespan.

Testosterone experiment

We obtained adult *A. sagrei* males for our testosterone experiment from Carolina Biological Supply (Burlington, NC, USA). These males were collected from wild populations in Florida and thus represent a different genetic stock than the Bahamian population for which we characterized growth and SSD. However, *A. sagrei* exhibits extreme male-biased SSD throughout its range (Stamps, 1999; Butler *et al.*, 2000), including populations throughout Florida (Lee, 1987). Thus, we have no *a priori* reason to expect that overall sex differences in growth, or the responsiveness of male growth to testosterone, might differ in such a way that our use of these specimens would be problematic.

We measured each male for SVL and body mass and then assigned it to one of three, size-matched treatment groups (*n* = 14 males per treatment): castrated males receiving a placebo implant (CAST), castrated males receiving a testosterone implant (TEST), and intact control males receiving a placebo implant (CON). Males ranged in size from 52 to 67 mm (mean ± 1 SE = 57.9 ± 0.4 mm), all of which are within the size range for sexually mature adult males in the wild population. We housed males in glass terraria (described above) for 30 days prior to surgery. Following surgery, we housed three males (one per treatment) together in each cage and provided *ad libitum* food and water as described above. We measured SVL and body mass at 36 days post-treatment and calculated growth rate by dividing change in size by elapsed time.

We constructed tonic-release testosterone implants from 5 mm lengths of Silastic® tubing (1.47 mm i.d., 1.96 mm o.d.; Dow Corning, Midland, MI, USA). 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 Inc., St Louis, MO, USA) dissolved in dimethyl sulfoxide (DMSO, 100 μg T-1500 per μl of DMSO) into the open end of each implant. We then sealed each tubule with silicone adhesive and allowed the DMSO to evaporate and diffuse through the tubing over a period of 3 days. This left 300 μg of crystalline testosterone within the lumen (approximately 1.5 mm length) of each implant.

We constructed placebo implants in identical fashion, but injected them with pure DMSO, which left an empty tubule after evaporation and diffusion.

We fasted animals for 1 day prior to surgery and then applied local anaesthesia at the site of incision via intraperitoneal injection of lidocaine (2 μl of 2% lidocaine HCl; Phoenix Pharmaceutical Inc., St Joseph, MO, USA). We immobilized animals by placing them in a freezer at −20 °C for approximately 5 min before performing surgeries atop a slightly thawed chemical ice pack. We exposed the testes with a ventral incision and bilaterally castrated both CAST and TEST males via ligation of the spermatic cords and ablation of the testes. Spermatic cords were cauterized after removal of the testes. We conducted sham surgeries on CON males by making identical incisions to expose and manipulate the testes, which we left intact. Animals then received either a testosterone implant (TEST) or a placebo implant (CAST and CON) that was inserted into the coelomic cavity. We then closed the incisions with Nexaband® surgical glue (Veterinary Products Laboratories, Phoenix, AZ, USA) and allowed animals to recover in plastic containers overnight prior to being returned to their cages.

At the conclusion of the experiment (56 days post-treatment), we collected blood samples from the postorbital sinus of each animal using heparinized microhematocrit capillary tubes (cat. no. 22-362-566; Fisher Scientific, Pittsburgh, PA, USA). Samples were centrifuged and the separated plasma was stored at −20 °C until subsequent assays. Radioimmunoassays were performed by following methods reported elsewhere (Smith & John-Alder, 1999; Cox & John-Alder, 2005; Cox *et al.*, 2005a). Samples containing 20 μl of plasma were extracted twice in diethyl ether (mean 70.4% extraction efficiency), dried under a stream of ultra-filtered air, and reconstituted in phosphate-buffered saline with gelatin. Reconstituted samples were assayed with ^{3}H-testosterone as a radiolabel (PerkinElmer Life Sciences Inc., Boston, MA, USA) and testosterone antiserum (1 : 18 000 initial dilution) developed in rabbits by A.L. Johnson (The University of Notre Dame, IN). Samples were processed in a single assay with a limit of detection of 2.72 pg testosterone per assay tube. Typical intra-assay variation is 7% (Smith & John-Alder, 1999) and inter-assay variation compared to previous assays was 6.9%.

Statistical analyses

We compared body sizes and growth rates of wild adult males and females using ANOVA with sex and year as main effects with interaction. We also compared growth rates using ANCOVA with initial SVL as a covariate. Prior to employing ANCOVA, we verified homogeneity of slopes between sexes by testing for sex × size interactions. For captive animals, we calculated instantaneous growth rates over each individual measurement period (i.e. 0–3 and 3–6 weeks and so forth) by dividing change in body

size (mm) by elapsed time (days). We then compared body size and growth rate between males and females using repeated measures ANOVA with sex as a between-subjects effect, time as a within-subjects effect, and a sex × time interaction term. To account for the fact that offspring were not statistically independent because of shared genetic and/or maternal effects within families, we analysed body size and growth rate for each measurement interval using repeated measures ANOVA with mean values calculated across all progeny of the same sex from a given dam. We also assessed sex differences in growth over each individual measurement period using ANOVA with family mean weighted by the number of offspring contributing to the mean within each family.

We assessed the effects of testosterone on growth rate (SVL and mass) using ANOVA with treatment (CAST, CON and TEST) as the main effect and then compared treatment mean using Tukey's *post hoc* tests. When growth rate was related to initial size, we repeated these analyses using ANCOVA with size (SVL or mass) as a covariate. Prior to employing ANCOVA, we confirmed homogeneity of slopes across treatment groups by testing for treatment × size interactions. All statistical procedures were implemented in SAS (version 8.2; SAS Institute Inc., Cary, NC, USA) or JMP (version 6.0.2; SAS Institute Inc.).

Comparative evidence in squamates

We assessed current comparative evidence for the bipotential regulation hypothesis in squamate reptiles by reviewing published studies in which changes in SVL and/or body mass were assessed following surgical castration and/or treatment with exogenous testosterone. Differences in methodologies and analyses among studies prevented us from conducting formal meta-analysis, so we instead relied upon an assessment of whether castration and testosterone treatment had a significant effect on growth in a given study. For each study, we classified the effect of testosterone as stimulatory, inhibitory, non-significant or equivocal. We omitted experiments in which testosterone was manipulated in eggs or embryos because their relevance to the natural development of SSD is unknown. We classified each species as exhibiting male- or female-biased SSD if adults differed by more than 5% (i.e. SSD > 0.05) in mean SVL, using data reported in several recent reviews (Cox *et al.*, 2003, 2007). We then mapped these data onto a phylogeny to determine whether the evolution of SSD in squamates was accompanied by underlying changes in the effect of testosterone on growth.

Results

Growth and SSD in wild *Anolis sagrei*

Across 5 years, SSD in wild *A. sagrei* lizards ranged from −0.28 to −0.32 for mean adult SVL and −1.2 to −1.6 for

Table 1 Sexual size dimorphism (SSD) in snout-vent length (SVL) and body mass of wild *Anolis sagrei* from Kidd Cay near Georgetown (Exuma, Bahamas).

Year	Sex	N	SVL (mm), mean ± SD	Mass (g), mean ± SD
2003	Female	99	43.20 ± 2.25	1.86 ± 0.37
	Male	132	55.80 ± 5.60	4.41 ± 1.32
	SSD		−0.292	−1.37
2005	Female	127	42.73 ± 2.82	1.78 ± 0.44
	Male	111	54.85 ± 5.12	3.94 ± 1.19
	SSD		−0.284	−1.21
2006	Female	166	43.12 ± 2.45	1.89 ± 0.33
	Male	147	55.93 ± 6.2	4.47 ± 1.52
	SSD		−0.291	−1.37
2007	Female	169	43.12 ± 2.90	1.75 ± 0.40
	Male	119	56.77 ± 6.58	4.34 ± 1.50
	SSD		−0.317	−1.48
2008	Female	229	42.38 ± 2.47	1.60 ± 0.34
	Male	148	56.08 ± 6.43	4.19 ± 1.49
	SSD		−0.323	−1.62
All	Female	790	42.85 ± 2.61	1.77 ± 0.38
	Male	657	55.91 ± 6.05	4.28 ± 1.43
	SSD		−0.304	−1.42

SSD is calculated as (male size/female size) − 1, expressed as a negative number following the convention for species in which males are the larger sex (Lovich & Gibbons, 1992).

mean adult body mass (Table 1). Males averaged 55.91 mm in SVL and 4.28 g in body mass, compared to 42.85 mm and 1.77 g in females (Table 1). Sex differences in adult SVL and body mass were highly significant within each year ($P < 0.0001$ for all comparisons) and across all years (SVL: $F_{1,1447} = 2268.57$, $P < 0.0001$; mass: $F_{1,1440} = 1939.35$, $P < 0.0001$). Adult males also grew significantly more than females over the course of the breeding season (May–September; $F_{1,276} = 36.24$, $P < 0.0001$ for all years combined, Fig. 2a). Growth differed significantly across years ($F_{2,276} = 165.06$, $P = 0.0002$). A significant sex × year interaction ($F_{2,276} = 3.23$, $P = 0.041$) revealed that male growth was reduced to a greater extent than female growth in 2007 (Fig. 2a). Growth was negatively correlated with initial SVL in both sexes ($F_{1,279} = 479.13$, $P < 0.0001$; Fig. 2b) and the inclusion of SVL as a covariate strengthened the effect of sex on growth (ANCOVA $F_{1,279} = 574.71$, $P < 0.0001$). These data provide a benchmark for SSD in wild *A. sagrei* populations and show that sex differences in growth are present even in adult lizards nearing their asymptotic sizes.

Ontogeny of SSD in captive *Anolis sagrei*

Sexual size dimorphism developed rapidly as a result of highly divergent growth trajectories in captive *A. sagrei* males and females (Fig. 3). Males and females did not differ in size at hatching when treating all individuals as independent observations (SVL: $F_{1,272} = 0.09$, $P = 0.76$;
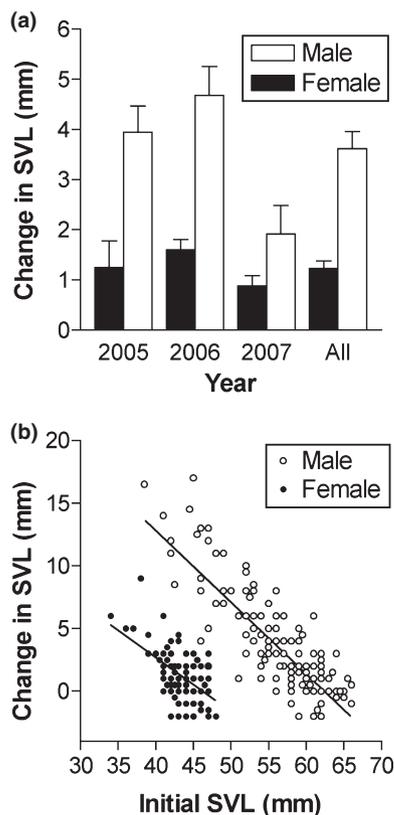
Fig. 2 (a) Mean (+ 1 SE) growth in SVL for adult *Anolis sagrei* males and females over three separate years in the wild. (b) Growth as a function of initial SVL for adult males and females. For any given initial size, males grow significantly more than females. Data are pooled across three years.

mass: $F_{1,272} = 1.24$, $P = 0.27$) or when using family means weighted by sample size (SVL: $F_{1,114} = 0.09$, $P = 0.77$; mass: $F_{1,114} = 1.17$, $P = 0.28$). However, males and females differed in growth rate immediately following hatching ($F_{1,171} = 9.12$, $P < 0.003$) and this difference remained significant when using weighted family means ($F_{1,45} = 7.14$, $P = 0.010$). By 6 weeks of age, males and females differed significantly in size when treating all individuals as independent observations (SVL: $F_{1,155} = 10.22$, $P < 0.002$; mass: $F_{1,155} = 8.65$, $P < 0.004$) and when using family means weighted by sample size (SVL: $F_{1,89} = 10.73$, $P < 0.002$; mass: $F_{1,89} = 9.86$, $P = 0.002$). Sex differences in growth persisted throughout postnatal ontogeny and were most pronounced from 9 to 30 weeks of age ($P < 0.0001$ for all comparisons; Fig. 3). The only time points for which males and females did not differ in growth rate were the final two measurement intervals of our study ($P > 0.05$ for all comparisons). Repeated measures analysis of SVL and mass over time confirmed sex differences in growth (i.e. sex × time interactions) when using individual animals as observations (SVL: $F_{1,89} = 10.73$, $P < 0.002$; mass: $F_{1,89} = 9.86$, $P = 0.002$) and when using family means (SVL: sex $F_{9,63} = 31.29$, $P < 0.0001$; mass: $F_{9,63} = 24.50$, $P < 0.0001$).

Testosterone experiment in *Anolis sagrei*

Prior to surgery, treatment groups did not differ in SVL ($F_{2,39} = 0.30$, $P = 0.74$) or body mass ($F_{2,39} = 0.06$, $P = 0.94$). Nor did these groups differ in pretreatment growth rates for SVL ($F_{2,39} = 1.01$, $P = 0.38$) or body mass ($F_{2,39} = 0.03$, $P = 0.97$). Pretreatment growth rates
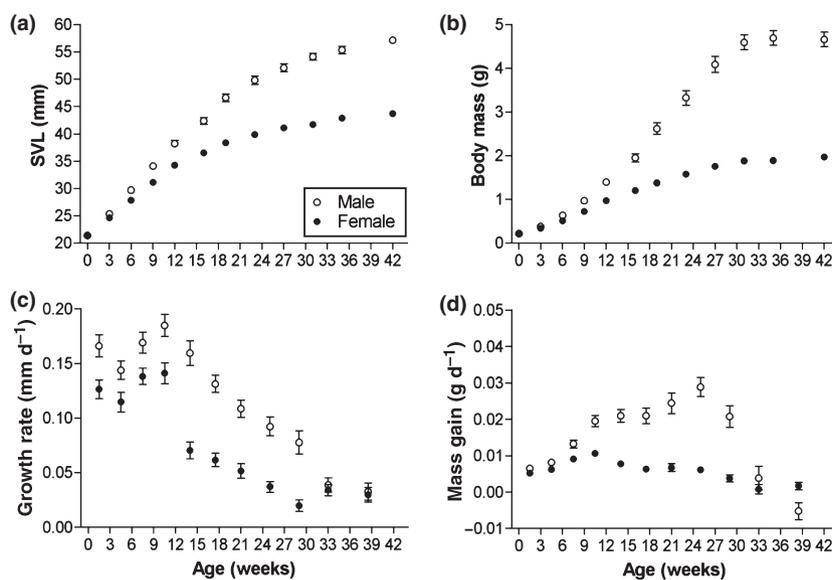
Fig. 3 Top panels report the mean (± 1 SD) SVL (a) and body mass (b) as a function of age for captive *Anolis sagrei* males and females. Bottom panels report mean (± 1 SD) rates of change in SVL (c) and body mass (d) for the intervals between successive size measurements in top panels.

were negatively correlated with initial SVL ($F_{1,38}$ = 18.39, $P < 0.0001$) but groups did not differ in pretreatment growth rate after controlling for SVL (ANCOVA: $F_{2,38}$ = 0.91, $P = 0.41$). Pretreatment rates of change in body mass were weakly related to initial mass ($F_{1,38}$ = 2.73, $P = 0.11$), but rates of mass gain did not differ across treatment groups after controlling for initial mass (ANCOVA: $F_{2,38}$ = 1.13, $P = 0.33$).

At 56 days post-treatment, plasma testosterone levels were low in CAST (mean ± 1 SE = 0.67 ± 0.11 ng mL^{-1}) and high in both CON (9.77 ± 2.01 ng mL^{-1}) and TEST (7.83 ± 0.91 ng mL^{-1}). Thus, castration reduced plasma testosterone of CAST males to basal levels that were significantly lower than either TEST or CON males, and hormone implants restored plasma testosterone of castrated TEST males to levels that were statistically indistinguishable from intact CON males ($F_{1,38}$ = 15.25, $P < 0.0001$).

Following treatment, TEST grew significantly more quickly in SVL than either CON or CAST ($F_{2,39}$ = 10.90, $P = 0.0002$; Fig. 4a). Growth rate was unrelated to initial SVL ($F_{1,38}$ = 2.63, $P = 0.11$). Treatment effects on growth in body mass were equally pronounced, with CON intermediate between the rapid mass gain of TEST and the zero mass gain of CAST ($F_{2,39}$ = 7.37, $P < 0.002$;
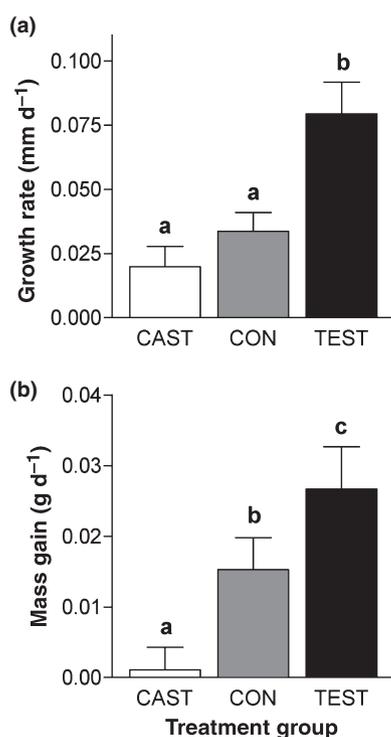
Fig. 4 Mean (+ 1 SE) rate of growth in SVL (a) and body mass (b) for *Anolis sagrei* males as a function of treatment group (CAST, CON and TEST). Lowercase letters denote statistical separation based on ANOVA with Tukey's *post hoc* tests.

Fig. 4b). Rate of mass gain was negatively related to initial body mass ($F_{1,38}$ = 6.98, $P = 0.012$), but the treatment effect on mass gain remained strong when including initial mass as a covariate ($F_{2,38}$ = 8.19, $P < 0.002$). By 36 days post-treatment, TEST males had grown an average of 2.9 mm and 1.0 g, compared to only 0.7 mm and 0.04 g in CAST males. These treatment groups both received castration surgeries and differed only in the implants they received.

Comparative evidence in squamates

Experimental data from castration and testosterone manipulation studies in squamate reptiles are summarized in Table 2. Aside from the present results for *A. sagrei*, experimental data are available for only one additional species with pronounced male-biased SSD. In a wild population of Yarrow's spiny lizards (*Sceloporus jarrovii*, SSD = −0.11), castration inhibited male growth and treatment of castrated males with exogenous testosterone restored growth to the level of intact controls (Cox & John-Alder, 2005). However, this clear stimulatory effect of testosterone was not observed in two analogous studies conducted in captivity (Cox *et al.*, 2006). Given that natural sex differences in growth were also abolished in captivity (Cox *et al.*, 2006, 2008), we conclude that testosterone stimulates growth in this species, but that this natural effect can be overridden by a surplus of available energy (i.e. *ad libitum* feeding in captivity).

Similar studies have been conducted in three species with pronounced female-biased SDD. In the garter snake (*Thamnophis sirtalis*, SSD = 0.18), castration dramatically increased mass gain in both neonatal and adult males (Crews *et al.*, 1985). Although exogenous testosterone reversed this effect in adult males, it had no effect on neonatal males or females. However, a subsequent study reported that exogenous testosterone inhibited skeletal growth in neonatal females, although effects could not be confirmed in males because of high mortality (Lerner & Mason, 2001). We tentatively conclude that testosterone inhibits growth in this species. Results are more straightforward for two lizards with female-biased SSD. In wild eastern fence lizards (*Sceloporus undulatus*, SSD = 0.13), castration of juvenile males had a weak stimulatory effect on growth and replacement of exogenous testosterone strongly inhibited growth (Cox *et al.*, 2005a). Testosterone also inhibited mass gain in wild adult males of this species (Klukowski *et al.*, 1998). In the striped plateau lizard (*Sceloporus virgatus*, SSD = 0.11), treatment with exogenous testosterone the onset of sexual maturation inhibited growth of wild males (Cox & John-Alder, 2005). Castration did not influence growth of small juvenile males, but it tended to increase the growth of larger, sexually mature males that presumably had higher endogenous testosterone levels (Cox & John-Alder, 2005). Exogenous testosterone also inhibited growth of juvenile males and females maintained in

Table 2 Support for the bipotential regulation hypothesis in squamate reptiles.

SSD and species	Effect of T	Treatment	Growth	Age	Sex	Site	Study
Female-biased SSD							
Sceloporus undulatus	–	C + T	L	J, A	M	Field	1
	–	T	L, M	A	M	Field	2
Sceloporus virgatus	–	C + T	L, M	J, A	M	Field	3
Thamnophis sirtalis	–	T	L, M	J	M, F	Lab	4
	–	C + T	L, M	N, A	M, F	Lab	5
	–	T	L	N	F	Lab	6
Male-biased SSD							
Anolis sagrei	+	C + T	L, M	A	M	Lab	7
Sceloporus jarrovii	+	C + T	L	J, A	M	Field	3
	NS	C + T	L	J, A	M	Lab	8
Monomorphic							
Psammodromus algirus	NS	T	M	A	M	Field	9
Urosaurus ornatus	+/–	C + T	L, M	N, A	M, F	Lab	10, 11

SSD, sexual size dimorphism; (+), testosterone-stimulated growth; (–), testosterone inhibited growth; NS, no significant (α = 0.05) effect on growth; (+/–) results were equivocal. In some instances, effects of testosterone are inferred indirectly from effects of castration. See text for details. Testosterone manipulated via (T) exogenous testosterone; (C + T) surgical castration and exogenous testosterone. Growth was measured as change in (L) snout-vent length; (M) body mass. Age at the time of manipulation (A) sexually mature adults; (J) sexually immature juveniles; N, neonates. Study conducted in (Field) natural environment or (Lab) captivity. (1) Cox *et al.*, 2005a; (2) Klukowski *et al.*, 1998; (3) Cox & John-Alder, 2005; (4) Abell, 1998; (5) Crews *et al.*, 1985; (6) Lerner & Mason, 2001; (7) this study; (8) Cox *et al.*, 2006; (9) Salvador & Veiga, 2000; (10) Hews *et al.*, 1994; (11) Hews & Moore, 1995.

captivity (Abell, 1998). There was no indication that testosterone stimulated growth in any study of these three species with female-biased SSD.

Comparable data are also available for two squamates in which SSD is weak or absent. In a lacertid lizard (*Psammodromus algirus*, SSD = 0.01), exogenous testosterone had no effect on growth in SVL or mass of wild adult males (Salvador & Veiga, 2000). In a phrynosomatid lizard (*Urosaurus ornatus*, SSD = –0.05), exogenous testosterone inhibited growth in SVL, but not body mass, of captive male and female neonates. However, castration alone also inhibited growth, so these results are somewhat equivocal (Hews *et al.*, 1994; Hews & Moore, 1995). Moreover, exogenous testosterone resulted in high mortality, leading these authors to suggest that it was elevated to pharmacological levels. We therefore regard the effect of testosterone on growth as equivocal in *U. ornatus* and conclude that testosterone has no effect on growth in *P. algirus*.

Placing the comparative data from these seven species in a phylogenetic context reveals a strong association between the evolution of SSD and the effect of testosterone on growth (Fig. 5). Testosterone stimulates growth in two lizards from separate lineages with male-biased SSD, whereas testosterone inhibits growth in three species from two phylogenetically independent lineages with female-biased SSD. Effects of testosterone on growth are absent or equivocal for two lizards from separate lineages in which SSD is minor or absent.

Discussion

Growth and SSD in *Anolis sagrei*

The brown anole (*A. sagrei*) exhibits extreme male-biased SSD, as illustrated by the 30% sex difference adult SVL and the 140% difference in adult body mass that we consistently documented across 5 years in a wild Bahamian population (Table 1). Collectively, our results from studies in the wild and in a laboratory common garden environment clearly demonstrate that this extreme SSD develops because of pronounced sex differences in growth rate that are present immediately after hatching (Fig. 3) and persist through adulthood (Fig. 2). Although it is not intuitively surprising that extreme SSD is associated with underlying sex differences in growth, our results actually differ substantially from those observed in several other reptiles, where sex differences that give rise to SSD in the wild are greatly reduced or absent in captivity (Haenel & John-Alder, 2002; Taylor & DeNardo, 2005; Cox *et al.*, 2006; John-Alder *et al.*, 2007). We detected modest interannual variation in growth and SSD in the wild (Table 1, Fig 1), potentially because of corresponding variation in environmental factors (e.g. temperature, rainfall and food availability) that could influence relevant demographic parameters such as growth rate, survival and hatching date. Despite this variation, the overall pattern of extreme male-biased SSD and underlying sex differences in growth remained constant across years. Thus, our growth studies confirm
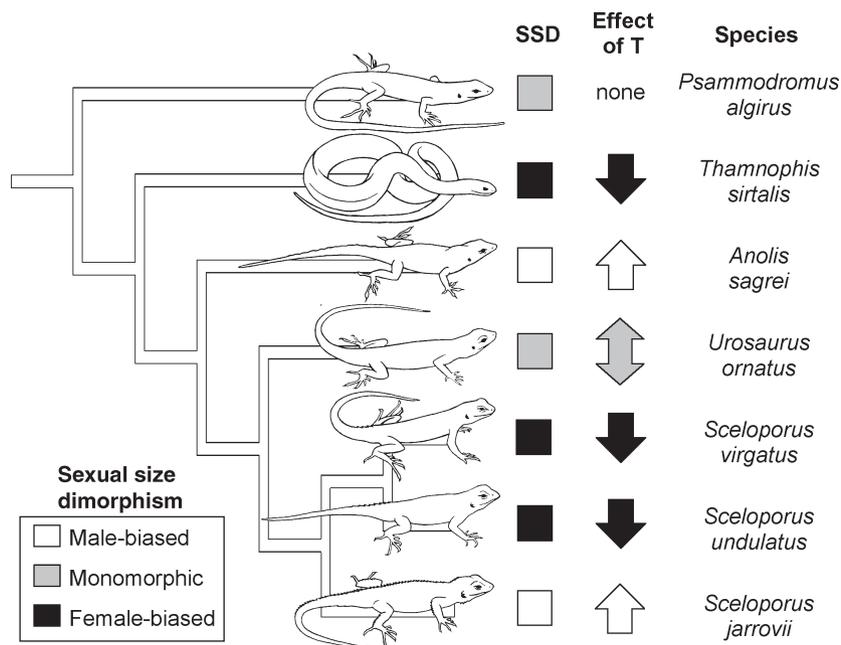
Fig. 5 Evolutionary changes in SSD are accompanied by changes in the effect of testosterone on male growth. SSD is categorized as male- or female-biased (> 5% difference in mean adult SVL) or monomorphic (< 0.05 difference). Arrows indicate the effect of testosterone (T) on growth in each species (⇧ = testosterone stimulates growth; ⬇ = testosterone inhibits growth; ⇕ = results are equivocal; none = no effect). See text for further discussion and Table 2 for a summary of each study.

that SSD develops in *A. sagrei* because of sex differences in juvenile and adult growth, as assumed by the bipotential regulation hypothesis.

In the majority of squamate reptiles studied to date, testosterone has a clear inhibitory effect on growth (Crews *et al.*, 1985; Abell, 1998; Klukowski *et al.*, 1998; Lerner & Mason, 2001; Cox & John-Alder, 2005; Cox *et al.*, 2005a). However, on the basis of the bipotential regulation hypothesis, we predicted that testosterone would stimulate growth in *A. sagrei*. Our results clearly show that castration inhibits and exogenous testosterone stimulates growth in both length and mass of adult *A. sagrei* males (Fig. 4). Given that adult *A. sagrei* males and females differ in growth rates (Fig. 1) and in circulating testosterone levels (Tokarz *et al.*, 1997), our experimental testosterone manipulation in adult males is biologically relevant to the issue of sex-specific growth regulation. Moreover, our manipulations are physiologically relevant in the sense that castration surgeries reduced plasma testosterone to basal levels below those of intact males, while exogenous hormone implants restored plasma testosterone to levels identical to those of intact males.

Although our manipulations were conducted at an ontogenetic stage when the sexes are known to differ in both growth rate (Fig. 2) and circulating testosterone levels (Tokarz *et al.*, 1997), it is important to note that SSD begins to develop immediately following hatching (Fig. 3). Thus, our experimental results do not directly address the initial development of SSD in *A. sagrei*. Given that males and females diverge in growth at such an early age, it would be informative to document the post-natal ontogeny of circulating testosterone levels in each sex. It would also be informative to manipulate testosterone in juvenile males and females or in eggs prior to hatching, as studies of other lizards suggest that experimental elevation of testosterone in the pre-natal environment can influence post-natal growth (Uller & Olsson, 2003; Uller *et al.*, 2007). Males and females of *A. sagrei* are sexually dimorphic in colour pattern at hatching, but other dimorphisms (e.g. males develop dorsal crests and orange dewlaps) become pronounced only upon maturation. By analogy, sex differences in growth that give rise to SSD may also depend on a combination of early organizational and late activational effects of testosterone (Hews *et al.*, 1994; Hews & Moore, 1995; Hews & Quinn, 2003).

Testosterone clearly stimulates growth in *A. sagrei*, but the precise physiological and/or behavioural mechanisms underlying this effect are unclear. One important caveat to our study is that males were housed together with one other size-matched male from each treatment. Thus, we cannot distinguish between direct effects of testosterone on behaviour and physiology (e.g. increased feeding and/or energy allocation to skeletal and muscular growth) vs. indirect effects mediated by social interactions (e.g. behavioral dominance of castrated males by testosterone males, resulting in differences in feeding, basking and/or stress). However, much of the existing evidence for growth regulation by testosterone comes from studies of free-living animals in which similar behavioural interactions are likely (Klukowski *et al.*, 1998; Cox & John-Alder, 2005; Cox *et al.*, 2005a). Interestingly, the inhibitory effects of castration on growth of free-living *S. jarrovii* lizards were absent when animals were held in social isolation in captivity (Cox *et al.*, 2006).

Bipotential regulation hypothesis

When our results from *A. sagrei* are considered alongside those of previous experiments on squamate reptiles, an intriguing pattern emerges. As predicted by the bipotential regulation hypothesis (John-Alder & Cox, 2007; John-Alder *et al.*, 2007), evolutionary changes in the direction of SSD correspond to shifts in the effect of testosterone on male growth (Fig. 5). Testosterone inhibits growth in three separate species representing two phylogenetically independent occurrences of female-biased SSD, whereas testosterone stimulates growth in two species from separate lineages exhibiting male-biased SSD. In each of these five species, males and females differ by at least 10% in mean adult SVL and sex differences in growth are known to underlie the development of SSD. In two species with slight or absent SSD, testosterone either has no effect on growth, or results from castration and testosterone addition treatments give equivocal results. Because males are known to exceed females in circulating testosterone levels in each of these seven species, differences in growth regulation across species likely reflect differences in the effect of testosterone on growth, rather than differences in circulating testosterone.

Two studies of squamates have also reported increased post-natal mass gain following elevation of prenatal testosterone levels, one in a species with moderate female-biased SSD (*Lacerta vivipara*, Lacertidae, Uller & Olsson, 2003), the other in a species with moderate male-biased SSD (*Ctenophorus fordi*, Agamidae, Uller *et al.*, 2007). However, in the former case, this stimulatory effect of testosterone was only observed in the absence of tick parasitism (Uller & Olsson, 2003). The relevance of prenatal testosterone to natural post-natal or adult patterns of sex-specific growth and SSD is unknown for either species, so implications with respect to the bipotential regulation hypothesis are limited. However, these studies do provide some additional evidence that testosterone can stimulate growth in squamates.

The novelty of our findings stem largely from comparison to other reptiles, but the resultant inference that evolutionary shifts in SSD can be achieved by underlying changes in the effect of testosterone on growth is potentially general across vertebrates. Although testosterone is commonly regarded as an anabolic steroid that promotes muscular and skeletal growth, most of the evidence supporting this generalization comes from studies of mammals, birds and fishes with male-biased SSD (reviewed by Cox & John-Alder, 2005; John-Alder & Cox, 2007; John-Alder *et al.*, 2007). Interestingly, several studies of birds and mammals with atypical female-biased SSD suggest that testosterone actually inhibits growth in these species (Swanson, 1967; Sockman *et al.*, 2008). Thus, the bipotential nature of testosterone may be general across vertebrates, providing an elegant regulatory mechanism for sex-specific phenotypic development

from a genome that is largely shared between the sexes. Given that SSD is also common among invertebrates (Fairbairn *et al.*, 2007), which lack testosterone and many other components of the vertebrate endocrine system, the generality of testosterone as a proximate mechanism for SSD has limits. However, other endocrine messengers (e.g. insulin, juvenile hormone and ecdysteroids) are known to influence growth in arthropods and may also underlie the development of within- and between-sex dimorphisms (Nijhout, 2003; Emlen *et al.*, 2005, 2006). Thus, an analogous version of the bipotential regulation hypothesis could, at least in principle, be extended to invertebrates. However, the evolution of sexual dimorphism in invertebrates may generally involve changes in threshold mechanisms regulating developmental responsiveness to hormones, rather than bipotentiality of the hormones themselves (Emlen *et al.*, 2005).

Although we have focused our discussion on testosterone, the bipotential regulation hypothesis can also extend to other androgens that may influence male growth (Hews *et al.*, 1994; Hews & Moore, 1995). Even in species where androgens are known to influence male growth, sexual dimorphism may reflect additional effects of estrogens and progestins on female growth (Holloway & Leatherland, 1997; Lerner & Mason, 2001). Moreover, our present experiment does not address the effects of testosterone on growth of females. Whereas some male traits can be induced in females treated with exogenous testosterone (Cox *et al.*, 2005b; Zysling *et al.*, 2006), other studies reveal that effects of sex steroids can differ between males and females (Holloway & Leatherland, 1997; Lerner & Mason, 2001). However, because males and females of *A. sagrei* and other squamates differ markedly in circulating testosterone levels, experimental manipulation in males is presumably more relevant to the natural role of testosterone in sex-specific development. Moreover, given that evolutionary patterns in SSD are often achieved primarily by interspecific changes in male size (Abouheif & Fairbairn, 1997; Fairbairn, 1997; Fairbairn *et al.*, 2007), it may often be appropriate to focus on underlying changes in the regulation of male growth.

Evolutionary implications

Our results illustrate an intriguing endocrine mechanism that may help to explain the phylogenetic lability of SSD across reptiles in general (Cox *et al.*, 2007; Fig. 1) and within *Anolis* lizards in particular (Butler *et al.*, 2000, 2007). West Indian *Anolis* lizards comprise one of the most spectacular examples of adaptive radiation on the planet, and the repeated evolution of convergent ecological and morphological specialists (termed 'ecomorphs') is mirrored by convergent patterns in SSD (Butler *et al.*, 2000, 2007; Butler, 2007). For example, although *A. sagrei* and other 'trunk

ground' ecomorphs are phylogenetically independent species, they have convergently evolved the same pattern of extreme male-biased SSD. It is tempting to speculate that this phylogenetic lability in SSD is facilitated by the bipotential role of testosterone in growth regulation, such that evolutionary changes in SSD could occur simply by altering the direction and/or extent to which growth responds to testosterone. Alterations to the interaction between endocrine modifiers and their genetic targets could present a more expeditious evolutionary pathway than the perpetual erosion and reformation of intersexual genetic correlations underlying complex polygenic traits such as body size (Lande, 1980; Badyaev, 2002; Fairbairn & Roff, 2006; Rhen, 2007). Future work on *Anolis* should attempt to identify the sexually antagonistic selection pressures that maintain SSD in wild populations (Calsbeek & Bonneaud, 2008; Cox & Calsbeek, 2009) and employ a comparative framework to test the prediction that effects of testosterone on growth will be absent in sexually monomorphic species and reversed in mainland species with female-biased SSD.

In a more general context, the bipotential nature of testosterone as a growth regulator illustrates how macro-evolutionary patterns can be linked to the evolution of underlying developmental mechanisms. Evolutionary changes to the interaction between hormones and their target tissues may explain phylogenetic diversity in other sexually dimorphic traits, such as the exaggerated horns of male beetles (Emlen *et al.*, 2006) and aggression and paternal behaviour in male birds (Lynn *et al.*, 2005; Lynn, 2008). The role of testosterone in regulation of squamate growth stands in contrast to the classic view that its phenotypic effects are evolutionarily conserved, which has been termed the 'evolutionary constraint hypothesis' (Hau, 2007). Rather, our results support the emerging perspective that selection can alter the linkage between testosterone and male traits, such that individual traits can independently evolve differential responsiveness to testosterone. This 'evolutionary potential hypothesis' is consistent with recent evidence on the hormonal regulation of life history trade-offs (Hau, 2007). Although our results provide a promising proximate context for the evolution of SSD, further work is required to determine whether the bipotential regulation hypothesis provides a general explanation for SSD. We emphasize that such studies should be guided by an explicit consideration of evolutionary patterns in SSD within and among lineages.

Acknowledgments

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