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Phenotypic Plasticity in the Relative Hind-Limb Growth of Lab-Reared *Anolis sagrei*: Replication of Experimental Results and a Test of Perch Diameter Preference

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Abstract.—Several observational and experimental studies have shown that perch diameter has an impact on the development of hind-limb length (HL) in *Anolis* species. This "phenotypic plasticity" in relative hind-limb growth (RHG) has implications for short-term and long-term adaptation to different structural habitats. Our study is the first to replicate research in which hatchling/juvenile *Anolis sagrei* were reared on narrow-diameter or broad-diameter dowels in a laboratory setting. Although subjects reared on different dowel diameters did not differ significantly in RHG at 5 weeks into the experiment, results at 15 weeks revealed a significant effect of treatment but not of sex: subjects in the broad ($N = 69$) treatment group exhibited significantly greater RHG than did subjects in the narrow ($N = 61$) treatment group. We extended this research with a novel follow-up study: we placed our lab-reared subjects into outdoor enclosures where they had a choice of narrow- or broad-diameter dowels on which to perch. Results showed that subjects in both treatment groups chose broad-diameter dowels as perches more often than narrow-diameter dowels. We offer several potential explanations for the strong preference of our subjects for broad-diameter dowels irrespective of the dowel diameter on which they were reared.

A hallmark characteristic of Caribbean *Anolis* lizards is the parallel evolution of "ecomorphs"—microhabitat specialists that resemble each other physically and behaviorally to an astonishing degree, despite having independent phylogenetic origins (Williams, 1983; Losos et al., 1998). One way in which ecomorphs differ anatomically is in limb length. For example, "trunk-ground" anoles exhibit relatively long hind limbs that increase sprint speed when chasing insect prey and avoiding predators, whereas "twig" anoles possess short hind limbs that allow them to maneuver effectively through the extensive branch networks of trees (e.g., Losos and Sinervo, 1989; Losos, 1990). Although natural selection can explain evolutionary differences in the limb lengths of *Anolis* ecomophs, variation in limb length also can arise developmentally through "phenotypic plasticity." Experiments in the field and in the lab have shown that the average diameter of substrates on which anoles perch can have a discernable impact on the length of their limbs (e.g., Losos et al., 1997, 2000; Kolbe and Losos, 2005; Dill et al., 2013).

In a seminal experiment by Losos et al. (1997), Cuban Brown Anoles (*Anolis sagrei*) were introduced onto small islands in the Bahamas that contained primarily shrubs (small-diameter substrates) or trees (large-diameter substrates). Between 10 and 14 yr later measurements of the founding anoles' descendants showed that hind-limb lengths adjusted for body size were correlated positively across populations with perch diameter. Although the authors favored an interpretation of rapid divergence in limb length attributable to natural selection (Losos et al., 1997), they proposed an alternative hypothesis that phenotypic plasticity might explain their results. To test this possibility, Losos et al. (2000) conducted a laboratory experiment in which juvenile *A. sagrei* were reared in cages that contained either small-diameter or large-diameter substrates. Results at the end of the study (approximately 16 weeks later) indicated that hind-limb growth is highly malleable during development: subjects reared on narrow substrates had significantly shorter hind limbs relative to body size than those reared on broad substrates. The adaptive implication of these findings is clear: when hunting prey or escaping predators, longer limbs facilitate greater sprint speed on broad substrates, whereas short limbs enable better maneuvering capability on narrow substrates (Losos et al., 1997, 2000).

This laboratory experiment was replicated later in *Anolis carolinensis* (Kolbe and Losos, 2005). Findings were similar to those for *Anolis sagrei*: *A. carolinensis* hatchlings reared on narrow perches exhibited significantly shorter hind limbs than did those reared on broad perches. The magnitude of the within-sex in hind-limb growth differences between the two treatments was less than had been observed in *A. sagrei*, because male *A. carolinensis* hind limbs were only slightly longer in the broad treatment than in the narrow treatment (Kolbe and Losos, 2005, fig. 2).

In this study, we had two goals. First, we wished to replicate the laboratory experiment of Losos et al. (2000) to assess the generality of their findings for *A. sagrei*. We tested the hypothesis that subjects reared in a broad-dowel treatment would exhibit significantly longer hind limbs proportional to body length (i.e., relative hind-limb growth: RHG) than would subjects reared in a narrow-dowel treatment. Second, we wished to extend prior laboratory experiments (Losos et al., 2000; Kolbe and Losos, 2005; Rosier and Langkilde, 2012) by determining whether RHG differences between broad- and narrow-dowel treatment groups translate into differences in perch preference beyond the laboratory environment. We used an outdoor experimental enclosure to test the hypothesis that subjects from our rearing experiment would spend more time perched on dowels of the same diameter as those on which they were reared (i.e., narrow or broad), than on dowels of a different diameter.

Materials and Methods

Subjects and Housing.—During September 2011, 160 hatchling and young juvenile *A. sagrei* were collected on the campus of Florida Southern College (Lakeland, FL). Subjects ranged in snout–vent length (SVL) from 17.2–30.4 mm at the start of the experiment (Table 1). The lizards were divided into two groups: 80 subjects were reared on small-diameter dowels ("narrow treatment": 8 mm) and 80 were reared on wide-diameter dowels

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TABLE 1. Snout–vent length (SVL), hind-limb length (HL), and relative hind-limb growth (RHG) of *Anolis sagrei* hatchlings at the start (initial), 5 weeks, and 15 weeks into the experiment. Sample sizes (N) are shown by sex and treatment. Values (mm) are mean ± 1 SE (range).

Sex	Treatment	SVL initial	HL initial	SVL 5 weeks
Males (N = 41)	Broad	23.1 ± 0.58 (17.2–30.4)	18.2 ± 0.48 (12.9–24.4)	25.3 ± 0.51 (18.7–32.2)
Males (N = 44)	Narrow	24.1 ± 0.40 (18.9–29.4)	19.4 ± 0.35 (15.6–23.5)	26.5 ± 0.41 (21.3–32.0)
Females (N = 20)	Broad	22.6 ± 0.60 (17.7–26.9)	17.8 ± 0.52 (13.9–21.8)	24.6 ± 0.47 (20.1–28.2)
Females (N = 25)	Narrow	23.6 ± 0.54 (19.5–30.3)	19.1 ± 0.48 (15.7–23.8)	25.9 ± 0.50 (21.5–31.1)

("broad treatment": 38 mm). Subjects were housed in pairs in plastic cages designed for small pets (Petco® Pet Keeper: 27 cm L × 17 cm W × 16 cm H) that contained two identical perches (narrow or broad depending on the treatment) and mulch flooring for moisture retention. The interior walls of the plastic cages were painted with Fluon® to prevent the lizards from climbing. The temperature in the laboratory was maintained at 27–30°C in a room with a large skylight that provided basking opportunities and supplemental fluorescent lighting.

Subjects were misted with water and fed two calcium-powder-dusted crickets daily. Cricket size was constant across the treatment groups, but crickets varied from 1–3 weeks of age throughout the experiment. Crickets were watered with cricket gel and were maintained with a diet of dry dog food, carrots, and spinach.

For the perch diameter preference experiment, four outdoor enclosures ("quadrants") were constructed using a wooden frame (2.13 m L × 2.13 m W × 0.91 m H). Metal flashing was secured to the wooden frame approximately 0.6 m from the base, and a 7.6-cm horizontal lip was constructed at the top of the flashing. Fluon® was painted on the interior portion of the flashing to prevent the lizards from escaping. Netting was secured to the top and sides of the frame to exclude potential predators, and a securable flap was constructed in the netting of each quadrant to allow for feeding and enclosure maintenance. Lizards were hydrated daily by spraying water on top of the ceiling netting. The sprayed water then dripped onto the dowel preference platforms (see below). Subjects were provided with crickets every 2–3 days throughout the experiment.

Sixteen large-diameter and 16 small-diameter dowels were attached with hot glue to each of four 1.22-m × 1.22-m plywood platforms. A platform was placed in each quadrant. Two quadrants held subjects that had been reared on small-diameter dowels for 15 weeks; the remaining two quadrants held lizards that had been reared on large-diameter dowels for 15 weeks. Subjects were marked with a treatment-identifying color pattern using Sharpie® permanent markers. Marking allowed observers to verify that no subject from one treatment had entered a quadrant containing members of the other treatment group, but subjects were not reliably individually identifiable.

Data Collection.—Subjects were uniquely toe clipped for future identification by removing the distal portion of 1–2 toes on the forelimbs. Measurements of hatchling hind-limb length (HL: insertion of femoral head in body to tip of metatarsal IV claw) and snout–vent length (SVL) were made using digital calipers (Mitutoyo, Absolute Digimatic) at three time points: A) initial measurements (just prior to being placed into plastic cages with dowel perches); B) at 5 weeks into the experiment; and C) at 15 weeks into the experiment. Although the hatchlings/young juveniles were captured and handled as gently as possible, 30 of the 160 subjects died between the time of their initial measurements and before their 5-week measurements could be made. Of these 30 individuals, 28 had a cage mate that survived through the end of the study; in one case, neither cage mate survived. Following other published laboratory studies of *Anolis* hind-limb phenotypic plasticity (Losos et al., 2000; Kolbe and Losos, 2005), subjects were not sexed until the end of the experiment. The sex of nonsurvivors in our experiment, therefore, was unknown. The remaining 130 subjects survived at least through the 15-week endpoint in the laboratory part of our study. Sample sizes for each treatment were: females, narrow (N = 25); females, broad (N = 20); males, narrow (N = 44); and males, broad (N = 41). Although our sample exhibits a male sex bias, we have no evidence that it arose from reduced female survivorship early in the experiment. A male bias in sex ratio has been observed previously in *A. sagrei*, where males in good condition produce proportionally more male than female progeny (e.g., Cox et al., 2010). We cannot rule out that a similar male bias exists in our study population.

Observations in the outdoor dowel-preference enclosure were made for 30 min per quadrant, 5 days per week for 6 weeks. Every 3 min, the number of lizards perched on each of the two dowel sizes was recorded. We had planned to recapture and measure all subjects after they had spent 6 weeks in the enclosures, to determine whether RHG had changed since their 15-week measurements in the laboratory. Unfortunately, just prior to the 6-week end point of the perch choice experiment, strong winds damaged the enclosures, and most of the lizards escaped, thus preventing a final comparison.

Data Analysis.—We used SPSS (v.20 Macintosh) for our statistical analyses. Measurements were natural log transformed prior to analysis (Table 1). Previous studies (Losos et al., 2000; Kolbe and Losos, 2005) used the mean of cage-mate subjects as units of analysis to avoid pseudoreplication. In those studies, when two subjects in a given cage were determined to be opposite sexes, those cages were eliminated from the analysis. Assessment of sex at the end of our experiment revealed that too few cages contained same-sex individuals to analyze cage-mate means statistically (males: narrow: N = 7, broad: N = 7; females: narrow: N = 2, broad: N = 5). Therefore, we ran two sets of analyses: one in which we chose one subject per cage randomly, and one in which we calculated cage-mate means while ignoring subject sex. In cases where cages contained only one subject (attributable to cage-mate death), data from these individuals constituted the "random subject" and "cage-mate means" for their cages.

We used analysis of covariance (ANCOVA) to determine whether HL differed between treatment groups at the outset of the experiment. We tested for differences in initial HL using treatment and sex as fixed factors and initial SVL as the covariate. Next, ANOVAs were used to test for effects of treatment and sex on RHG. Following Losos et al. (2000), RHG was calculated as follows: ([final HL − initial HL] / [final SVL − initial SVL]). RHG was determined at 5- and 15-week intervals from the start of the laboratory experiment to ascertain how

TABLE 1. Extended.

HL 5 weeks	RHG 5 weeks	SVL 15 weeks	HL 15 weeks	RHG 15 weeks
20.3 ± 0.44 (14.5–25.7)	1.2 ± 0.11 (0.2–2.4)	27.3 ± 0.43 (20.5 – 32.8)	22.5 ± 0.41 (15.6–27.1)	1.1 ± 0.06 (0.7–2.0)
21.8 ± 0.36 (18.0–26.4)	1.0 ± 0.08 (0.4–1.0)	29.3 ± 0.39 (23.7 – 34.0)	24.2 ± 0.37 (20.1–30.1)	0.9 ± 0.03 (0.5–1.5)
19.7 ± 0.45 (16.4–22.9)	1.1 ± 0.12 (0.5–1.8)	26.3 ± 0.45 (22.4 – 30.4)	21.5 ± 0.40 (18.5–24.9)	1.1 ± 0.10 (0.6–1.4)
21.1 ± 0.45 (17.2–25.3)	1.0 ± 0.11 (0.2–1.9)	28.2 ± 0.49 (23.4 – 33.0)	23.1 ± 0.45 (18.8–27.2)	0.9 ± 0.05 (0.5–1.7)

quickly differences in RHG developed between treatment groups. Last, we qualitatively tested the hypothesis that subjects reared on broad-diameter dowels would exhibit proportionally greater RHG than subjects reared on narrow-diameter dowels. We did not analyze our perch preference data statistically, however, because we could not identify subjects individually during observations.

RESULTS

Using one randomly selected subject from each cage and controlling for initial SVL as a covariate, ANCOVA results showed that initial HL did not differ between treatments ($F_{1,74} = 3.168$, $P = 0.079$) or sexes ($F_{1,74} = 0.015$, $P = 0.904$), nor was there a significant treatment × sex interaction ($F_{1,74} = 0.024$, $P = 0.878$; slopes were homologous for all factors, smallest $P = 0.371$). When using cage-mate subject means as data points, however, initial HL did differ between treatments ($F_{1,76} = 8.065$, $P = 0.006$), with subjects in the narrow treatment exhibiting longer HL (mean = 19.3 mm, SE = 0.28, $N = 69$) than subjects in the broad treatment (mean = 18.1 mm, SE = 0.36, $N = 61$). Note that longer initial HL in the narrow treatment biases outcomes against our hypothesis, which is that subjects reared on narrow dowels should exhibit shorter hind limbs relative to body size than subjects in the broad dowel treatment group.

When using one randomly selected subject from each cage, ANOVA results at 5 weeks failed to show a significant influence of treatment (2-way ANOVA: $F_{1,75} = 1.506$, $P = 0.224$), sex ($F_{1,75} = 0.442$, $P = 0.508$), or a treatment × sex interaction ($F_{1,75} = 0.001$, $P = 0.969$) on RHG. However, comparison of RHG at 15 weeks with the same subjects revealed a main effect of treatment (2-way ANOVA: $F_{1,75} = 4.171$, $P = 0.045$) but no effect of sex ($F_{1,75} = 0.538$, $P = 0.465$) or treatment × sex interaction ($F_{1,75} = 0.008$, $P = 0.929$). Similarly, when we used the mean RHG from both subjects in each cage, results at 5 weeks failed to show an effect of treatment (1-way ANOVA: $F_{1,77} = 1.449$, $P = 0.232$) but at 15 weeks revealed a treatment effect (1-way ANOVA: $F_{1,77} = 7.260$, $P = 0.009$). We note parenthetically that using all subjects ($N = 130$) in our analysis of RHG produced the same results as when using a randomly chosen subject per cage or cage-mate subject means: no effect of treatment or sex was observed at 5 weeks (Fig. 1a), and only a treatment effect was seen at 15 weeks (Fig. 1b). Thus, the results of our RHG analyses support our hypothesis that subjects in the broad-treatment group would exhibit significantly longer hind limbs proportional to body size (i.e., RHG) than subjects in the narrow treatment. Results from our perch preference experiment were only partly consistent with this hypothesis. Subjects reared on broad dowels were observed on broad dowels more often than on narrow dowels, but subjects reared on narrow dowels also were observed more frequently on broad than on narrow dowels (Fig. 2).

DISCUSSION

Previous studies have shown that phenotypic plasticity in the hind limbs of *Anolis* lizards can be induced during ontogeny by rearing different treatment groups on perches of different diameters (Losos et al., 2000; Kolbe and Losos, 2005). Results from our laboratory study likewise indicate that hind-limb

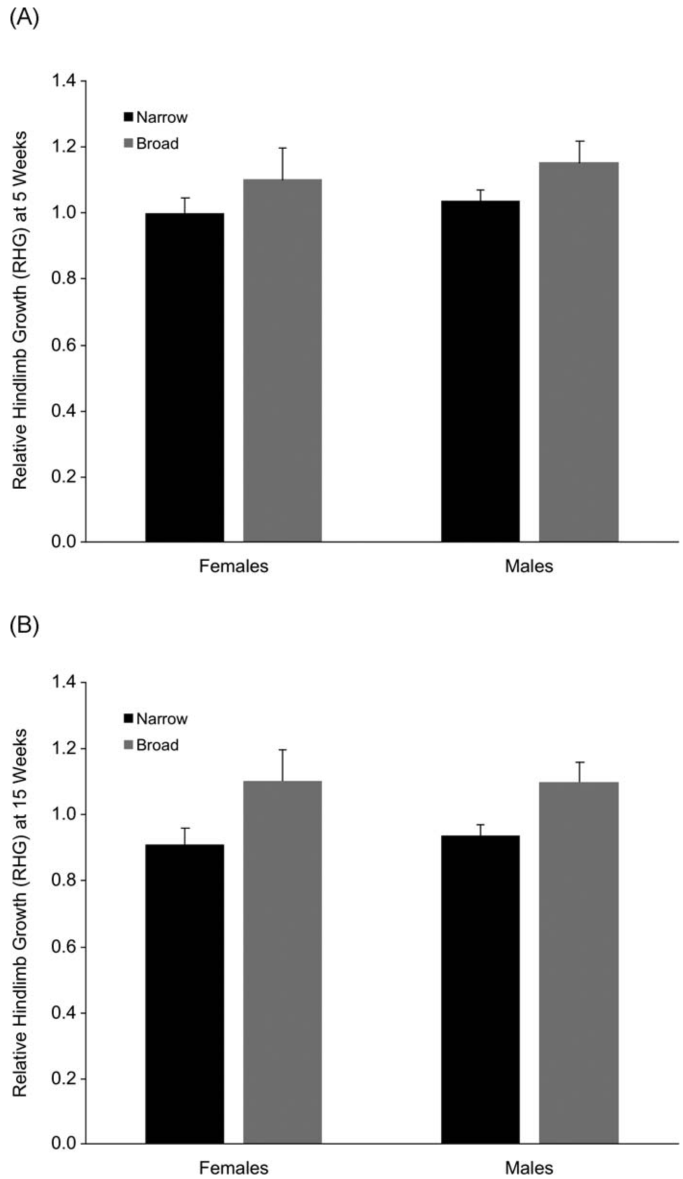

FIG. 1. Relative hindlimb growth (RHG) of young female and male *Anolis sagrei* reared on narrow or broad dowel perches. (a) RHG at 5 weeks; (b) RHG at 15 weeks. Bars show means and SEs for all subjects included in the study. Sample sizes: females, narrow ($N = 25$); females, broad ($N = 20$); males, narrow ($N = 44$); males, broad ($N = 41$).

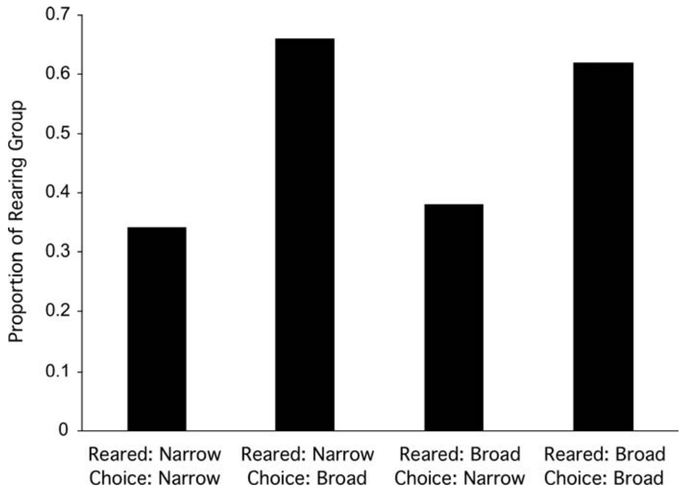

FIG. 2. Proportions of dowel size preferences in an outdoor enclosure by subjects reared on narrow or broad dowels. Proportion in each treatment (narrow or broad) sums to 100%. Sample sizes: reared on narrow, observed on narrow, $N = 2{,}225$; reared on narrow, observed on broad, $N = 3{,}627$; reared on broad, observed on narrow, $N = 2{,}225$; reared on broad, observed on broad, $N = 3{,}445$.

growth in juvenile *A. sagrei* is malleable and can be altered by the diameter of perches on which the lizards spend their time. A treatment effect was already evident at 5 weeks, although the effect was not statistically significant (Fig. 1a). Although RHG changed little between 5 and 15 weeks in the broad-dowel treatment, it decreased during the same time period in the narrow dowel treatment (Fig. 1b). Therefore, it appears that between 5 and 15 weeks, hind-limb growth relative to growth of the rest of the body slowed in the narrow-dowel treatment group, in comparison to the broad-dowel treatment group. Our finding that the two treatment groups differed significantly in RHG at 15 weeks replicates that of Losos et al. (2000), where at roughly 16 weeks subjects reared on broad perches exhibited significantly greater RHG than subjects reared on narrow perches. Losos et al. (2000) also detected a sex effect on RHG: males exhibited longer hind limbs relative to SVL than did females. Our study did not reveal a similar sex effect on RHG (Fig. 1). This difference may be attributable to the difference in ages of subjects in the two studies. Subjects in Losos et al. (2002) were substantially larger at the start of their experiment (mean $= 33.1$ mm $\pm$ 0.1 SE, $N = 116$) than were subjects in the present study (mean $=$ 23.5 mm $\pm$ 0.3 SE, $N = 130$), and sexual dimorphism was still small at the end of our study.

Results of the work reported here, together with those of prior laboratory (Losos et al., 2000; Kolbe et al., 2005) and field (Dill et al., 2013) studies indicate that phenotypic plasticity in leg length is a real and replicable phenomenon in *Anolis*. Losos et al. (2000) suggested that phenotypic plasticity in limb length may be an important component underlying the adaptive radiation of
?2 anoles into diverse structural habitats. Although differences in hind-limb length among *Anolis* "ecomorphs" (for a review, see Losos, 2009) are considerably greater than experimentally induced intraspecific RHG (i.e., Losos et al. 1997, 2000; Kolbe et al., 2005; present study), natural selection foreseeably could shift a species' RHG over time to a degree that might account for differences among ecomorphs in hind-limb length (see Kolbe et al., 2012).

In this study, we extended prior experimental research on phenotypic plasticity of limb growth in *Anolis* by testing whether differences in juvenile *A. sagrei* hind-limb growth had a detectable effect on perch preference in an outdoor enclosure. We found that, regardless of rearing conditions (i.e., treatment), broad dowels were preferred strongly over narrow dowels as perches in our sampling regimen. Interestingly, this outcome appears to be consistent with a preference observed in free-ranging adult *A. carolinensis* for perches with larger diameters than those available randomly (Dill et al., 2013). This bias toward using perch sizes larger than those found in random samples was also observed for juveniles in two of the three plots sampled by Dill et al. (2013).

A number of nonmutually exclusive explanations seem possible for the results from our investigation of perch preference. One explanation could be, if female subjects tended to perch on narrow dowels and males tended to perch on broad dowels, our finding of a preference for broad dowels could reflect our sample size bias toward males (i.e., 85 males vs. 45 females). Then again, perhaps some subjects in the narrow treatment simply had "outgrown" the narrow dowels by the time they were placed in the outdoor enclosures. Another possibility could be that RHG in our subjects, although significantly different between treatments at 15 weeks, is not biologically meaningful under the conditions we imposed. Also, we consider the prospect that a dichotomous choice test paradigm may not be an ideal or even relevant means to assess the functional significance of hind-limb plasticity in anoles. Some of these possibilities seem more readily amenable to experimentation than do others.

Finally, it has been pointed out that phenotypic plasticity in RHG could be particularly advantageous in the contexts of colonization or invasion, because experimental studies have demonstrated structural niche shifts in response to congeneric competition (e.g., Pacala and Roughgarden, 1982; Rummel and Roughgarden, 1985). One potentially fruitful avenue for future research might be best framed as comparative studies of different *Anolis* ecomorphs. As suggested by Kolbe and Losos (2005), experiments conducted on highly divergent habitat specialists (e.g., "twig" anoles vs. "crown giants") may provide important insights for understanding the long-term impact of substrate-induced limb growth plasticity in anoles and in other lizards.

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