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R. Gillespie: "There are a number of reasons to recommend this paper—the most obvious is that it makes a first important step towards integrating developmental biology into the study of one of the most famous study systems in evolutionary ecology {2}. The reason we choose to emphasize this paper, however, is, for lack of a better term, its pedagogical value." (http://f1000.com/717548108)

Repeated modification of early limb morphogenesis programmes underlies the convergence of relative limb length in *Anolis* lizards

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The independent evolution of similar morphologies has long been a subject of considerable interest to biologists. Does phenotypic convergence reflect the primacy of natural selection, or does development set the course of evolution by channelling variation in certain directions? Here, we examine the ontogenetic origins of relative limb length variation among *Anolis* lizard habitat specialists to address whether convergent phenotypes have arisen through convergent developmental trajectories. Despite the numerous developmental processes that could potentially contribute to variation in adult limb length, our analyses reveal that, in *Anolis* lizards, such variation is repeatedly the result of changes occurring very early in development, prior to formation of the cartilaginous long bone anlagen.

1. INTRODUCTION

From some of the earliest evolutionary speculation to the present day, developmental drivers of evolution have been viewed as an alternative to adaptationist explanations for evolutionary patterns (e.g. [1–3]; reviewed in [4,5]). The basic argument has revolved around the issue of which is more important in determining evolutionary patterns: developmental factors that constrain the variation that is produced, or selective factors that determine how genetic variation is selected across generations (e.g. [6–9])?

A particular case of contention is that of convergent evolution. The independent evolution of similar phenotypes (morphologies) in similar environments has long been taken as evidence of the primacy of natural selection (reviewed in [8]). However, in recent years, workers have argued that convergence—and, in particular, what is sometimes called 'parallel evolution', in which convergent patterns are produced through similar developmental changes—is evident for the biasing role of development in shaping evolutionary patterns [7,10–15]. But these views are not necessarily mutually exclusive. On one hand, convergence *per se* does not necessarily imply the similar action of natural selection; only when convergence occurs in similar selective environments is adaptive evolution implied [8]. Even in such cases, however, the channelling effects of shared developmental programmes may still be important by limiting the universe of possible variation upon which selection can operate. In this view, a key question is whether convergent phenotypes are produced by convergent developmental changes.

For most of the 20th century, the fields of evolutionary biology and developmental biology progressed in parallel, acquiring independent research objectives and experimental techniques [5,16]. But renewed interest in the developmental mechanisms of morphological evolution in the late 1970s and 1980s—stimulated, in part, by the works of Gould [17] and the discovery of pan-metazoan Hox gene conservation [18]—galvanized the field of evolutionary developmental biology. Recently, much attention in evolutionary developmental biology has been given to uncovering the molecular bases of morphological evolution and whether changes in the action of the same genes underlie phenotypic convergence [19–22]. But phenotypic convergence can result from non-convergent genotypic evolution (reviewed in [23–25]). To fully understand the developmental bases of convergent phenotypes, it is therefore critical that detailed analyses of developmental mechanisms be placed within the appropriate phenotypic context.

Evolutionary developmental biology has predominantly focused on studying dramatic morphological changes where the scoring of discrete phenotypes rarely requires detailed measurement [26,27]. By contrast, much of the study of natural selection and evolutionary diversification focuses on traits that vary quantitatively rather than qualitatively, such as body size, limb length and head shape [28–30]. While many of these qualitative shifts in morphology have been linked to changes in early development [19–21,31], variation in quantitative traits
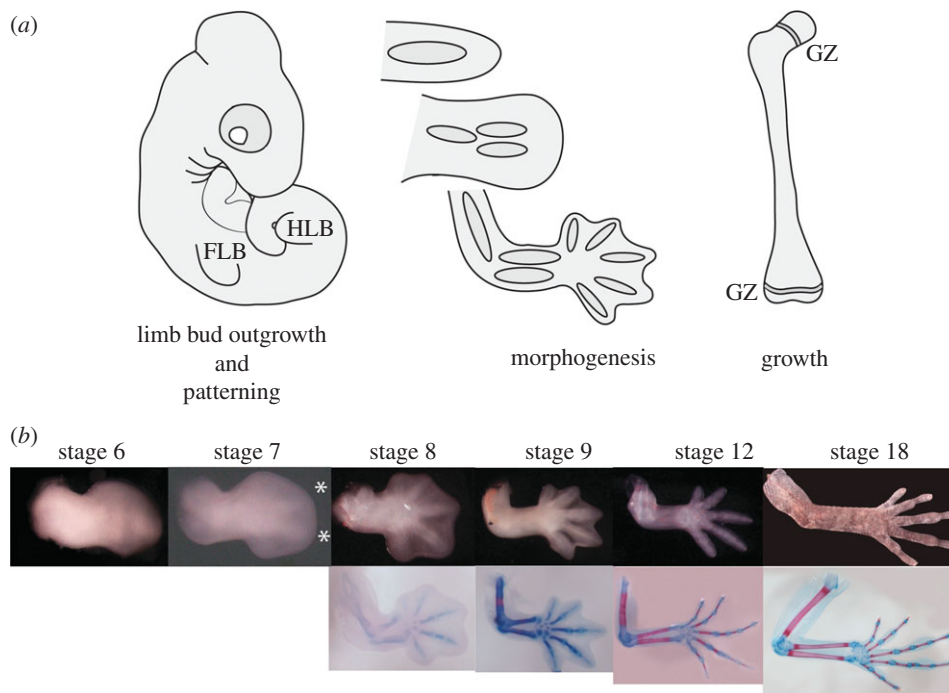
Figure 1. (*a*) Summary of limb and long bone development. Limb development proceeds through several stages: limb bud outgrowth and patterning, when the limb bud is only filled with undifferentiated mesenchyme; morphogenesis, where the cartilaginous anlagen of the limb bones form; and growth, mediated from epiphyseal growth zones. FLB, forelimb bub; HLB, hindlimb bud; and GZ, growth zone. (*b*) Limb development for *Anolis sagrei*. Asterisks denote visible condensations in the digital plate of stage 7 embryos. The earliest samples in our study were collected during stage 9, the earliest time when cartilage stains with Alcian blue.

often has complex developmental origins: variation can arise through changes in morphogenesis, differential growth rates or both [32–37]. The most critical question to address first when studying the developmental bases of convergent quantitative phenotypes is whether the morphologies arise through convergent developmental trajectories representing the full developmental history of a structure [38].

Caribbean *Anolis* lizards provide an excellent and extensively studied group for investigating the repeated, independent evolution of similar morphologies (reviewed in [30]). Anoles have radiated, for the most part, independently on each island in the Greater Antilles (Cuba, Hispaniola, Jamaica and Puerto Rico), producing essentially the same set of habitat specialists—termed 'ecomorphs'—on each island. Of particular importance in this repeated ecomorphological specialization is variation in relative limb length (i.e. limb length controlled for body size), which is known to play a significant role in adaptation to different parts of the structural habitat (e.g. tree trunks, twigs and grass blades). Despite many years of ecological and evolutionary research on this genus, the developmental bases of limb length variation have not yet been examined. Just how many different ways has variation in relative limb length evolved in this genus? Discovering that species converge morphologically via similar developmental trajectories might suggest that anoles have inherited common patterns of variation from their ancestor, potentially biasing the response to selection. But finding that different lineages converge morphologically using different developmental mechanisms would indicate that natural selection was the primary factor determining evolutionary trajectories. The

goal of this work was to test the hypothesis that species possessing similar limb morphologies have converged on these morphologies through similar alterations to their growth trajectories.

2. METHODS
(a) *Overview*
Predicting potential mechanisms of divergence first requires a general understanding of limb development (figure 1). During embryonic development, limb buds irrupt from the flank of the animal and are filled with an undifferentiated mesenchyme. During patterning, mesenchymal cells aggregate and differentiate to form a single cartilaginous anlagen, the precursor to the limb long bones. This cartilaginous rod then grows and bifurcates to form the limb skeleton. Ossification begins as a bony collar around each anlage, which expands towards the ends of the bone, establishing a growth zone. From this time on, proximodistal extension occurs solely at the growth zone, although the precise mechanism is unclear in reptiles (T. J. Sanger & C. E. Farnum 2004, personal observation). Variation in adult long bone length can therefore either result from variation generated during limb bud patterning, influencing the initial dimensions of the anlagen, or through differential rates of long bone elongation via the action of growth plates at either end of the bone.

Interpreting evolutionary and developmental changes in relative size is best done in the context of allometry. Ontogenetic allometry, in particular, examines the relationship between local versus global rates of growth [39]. Several alternative allometric hypotheses could account for the patterns of limb length variation observed in adult *Anolis* lizards (figure 2). Variation in limb length may be due to localized
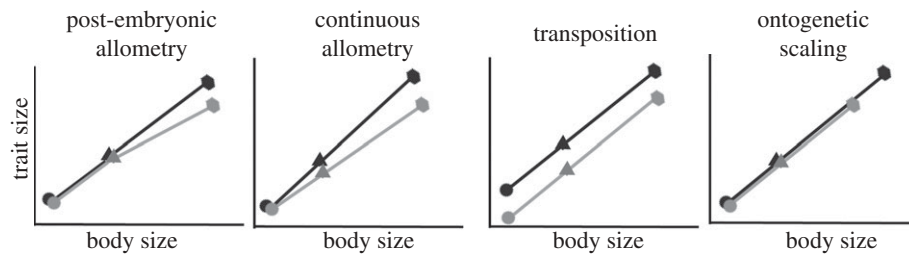
Figure 2. Suite of alternative allometric hypotheses contrasting hypothetical species pairs. During post-embryonic allometry, species share similar embryonic trajectories, but diverge in relative size after hatching. Continuous allometry refers to a pattern in which species appear similar following morphogenesis, but subsequently diverge. A transposition of the allometric regressions reflects that a change has occurred prior to the earliest developmental stages measured. During scaling, the absolute size of the trait changes, but in proportion to body size. Circles, morphogenesis; triangles, hatching; hexagons, adulthood.

changes in the rate of limb growth, either from the initial stages of morphogenesis (continuous allometry) or through stage-specific changes that occur later in ontogeny (post-embryonic allometry). These are both represented by a change in the slope of the allometric regression. Variation in adult morphology may also be the result of changes that occur during morphogenesis, represented by a transposition, or a shift in the intercept of the allometric regressions. Variation in adult morphology can also arise by scaling (extending or compressing) an allometric trajectory. However, in this case, we compare species that are roughly the same adult body size, indicating that this explanation cannot account for differences in relative limb length in these species. Long bone development is a complex process and, as a result, limb length variation can arise through many processes, whether by changing the patterning of the early limb bud or through time-specific changes in growth rate (e.g. [34,35,37,40–44]).

Detailed description of our methodology is included in the electronic supplementary material, appendix S1. Here, we briefly describe our study species, specimen preparation and statistical analyses.

(b) *Study species and specimen preparation*
We collected growth series for eight Caribbean *Anolis* species representing one trunk-ground and one trunk-crown eco-morph from each island in the Greater Antilles (figure 3). Trunk-ground species have relatively longer limbs than trunk-crown species [30]. Limb patterning occurs early in development, and establishes the initial size and shape of the skeletal anlagen (figure 1). To extend juvenile patterns of allometric long bone growth closer to the period of morphogenesis, we also collected embryological series for four species—the trunk-crown and trunk-ground specialists from Cuba and Jamaica. These species were chosen because of their relative ease of care in captivity and the rate at which they lay eggs. Detailed methods of embryo collection have been described elsewhere [45,46].

As is common in herpetological studies in general, and previous studies of anoles in particular (e.g. [47,48]), we used snout-to-vent length (SVL) as a standard measure of body size. For embryological comparisons, we collected one to three individuals per stage for each of the four species examined, from the early stages of digital webbing reduction (stage 9/10) through hatching (stage 19 [45]). Combined with the post-hatching series, this represents nearly the entire period of long bone skeletal development and growth (figure 1).

We prepared post-hatching skeletons with standard clearing and staining protocols [49–51]. Scaled digital photographs of
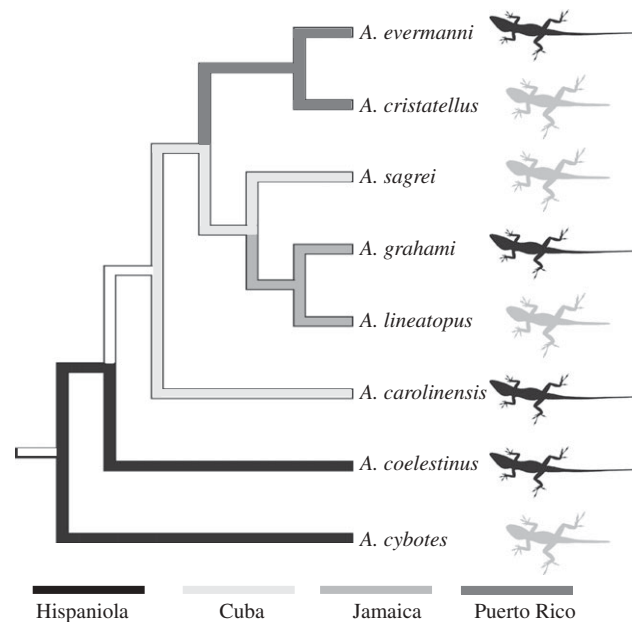

Figure 3. Phylogeny of eight anole species used in this study depicting independence of comparisons between island habitat specialists. Cartoons in the margin depict habitat specialist classification of each species (grey, trunk-ground; black, trunk-crown). Note that habitat specialists have evolved independently on each island.

the limb elements were obtained using a Nikon DN100 camera mounted on a Nikon SMZ1000 dissecting microscope. We obtained individual long bone measurements with IMAGEJ using a single linear measurement [52,53].

(c) *Statistical analysis*
Log-transformed limb data for each species were best fit using a linear regression [54]. For each island pair, we tested for differences in slope and intercept between the two species with analysis of covariance (ANCOVA). To test for deviations from isometry (slope of the log–log regression = 1), we used a *t*-test for differences in slope for each species [55]. Embryonic and post-hatching data were treated independently.

To verify the results of this analysis using a second method, we also performed an ANCOVA using all post-hatching data, testing whether species across islands differed in slope. Because no significant differences in slope were found (see below), we then performed a phylogenetic ANOVA on the mean of the residuals for each species obtained from a single linear regression through all data. Residuals indicate the extent to which each species's limb

elements, relative to body size, are larger or smaller than expected based on the data from all the species in the study; these residuals can thus be used to determine whether the ecomorphs differ in relative limb length. A phylogenetic ANOVA [56] controls for the relatedness of the taxa [57,58]. To perform the phylogenetic ANOVA, we first obtained an unbiased estimate of the evolutionary variance–covariance matrix [59]. Using multivariate Brownian motion simulations on a phylogeny of the eight species (figure 3) [60], we then generated 999 simulated datasets. The probability value for this analysis is the proportion of the simulations in which the simulated F-statistic is greater than the empirical F-statistic. All statistical procedures were run using SYSTAT v. 10.2 (Systat Software Inc., San Jose, CA, USA) or C code written expressly for this study and available from one of the authors (L.J.R.) upon request.

3. RESULTS

(a) *Relative growth of post-hatching limbs*
Variation in relative hindlimb length in adults is associated solely with changes that occur before hatching. This is clearly illustrated by significant differences in intercept, represented by a transposition in the post-hatching allometric regression (figure 4; table 1; electronic supplementary material, appendix S2). Both hindlimb elements examined (femur and tibia) exhibit the same pattern of post-hatching isometric growth. In no instance did we find significant differences in slope between species from the same island. Therefore, differences in relative hindlimb length *cannot* be attributed to differences in post-hatching allometric growth.

Forelimb elements exhibit different patterns of divergence on different islands. Significant differences in slope were never found between species from the same island. Only the forelimb elements from Cuban and Hispaniolan species have statistically distinguishable intercepts (figure 4; table 1; electronic supplementary material, appendix S2). Jamaican and Puerto Rican species vary neither in slope nor intercept, and the allometric trajectories vary only by ontogenetic scaling. Similar to the patterns shown by the hindlimbs, divergence of forelimb elements for Cuban and Hispaniolan species cannot be attributed to post-hatching allometric growth, but rather to a process that occurs prior to hatching.

Phylogenetic ANOVAs using species residuals support these conclusions (figure 5; electronic supplementary material, appendix S3). Trunk-ground anoles have relatively longer hindlimb elements than trunk-crown anoles, but the relative size of the forelimb elements does not differ.

(b) *Relative growth of embryonic limbs*
By stage 10, 'early digital web reduction' [45] (figure 1), the relative size of the long bones is well established. Significant differences in intercept occur between the Cuban species' forelimbs and hindlimbs, and among the hindlimbs of Jamaican species (figure 6; table 1). Forelimbs are indistinguishable between the two Jamaican species, just as they are after hatching. Significant positive allometry exists for many of the embryonic long bones in these species (electronic supplementary material, appendix S4). But despite the decreased rate of long bone growth after hatching, these results indicate that the

differences between ecomorphs have already been determined by the earliest stage at which repeatable measurements are possible.

4. DISCUSSION
Do properties of development affect the rate or direction of morphological evolution, or does natural selection simply remould variation to match current ecological conditions? Phenotypic convergence provides us with the opportunity to test this long-standing question by comparing the effects of natural selection and developmental biases during morphological evolution. If development were, in fact, channelling evolution along particular paths, one would expect that lineages converging phenotypically would use the same developmental trajectories.

Our analyses of post-hatching growth trajectories for *Anolis* trunk-ground and trunk-crown habitat specialists show that adult limb length variation is consistently the result of changes that occur prior to hatching (figures 4 and 5): differences in limb length are apparent at hatching, and limb long bones elongate in parallel relative to body size in different species. Examination of pre-hatching embryonic growth shows that species-specific morphologies are the result of changes that occur very early in limb development, prior to formation of the cartilaginous anlagen (figure 6). These results therefore suggest roles for both natural selection and the generative mechanisms regulating limb length variation during the diversification of this genus.

Several evolutionary scenarios can explain the repeated recruitment of early limb morphogenesis programmes for the generation of interspecific variation in adult limb length. Vavilov [61] and Haldane [62] observed that closely related species tend to vary in similar directions, suggesting commonalities in their developmental-genetic architecture. Such common genetic architectures might predispose evolutionary changes to occur along similar lines, following what has been termed 'genetic lines of least resistance' [63]. Under this scenario, while different developmental trajectories are possible, development serves as a channelling force by making some outcomes more easily attained by selection than others. Within a theoretical context, developmental constraints must align with genetic constraints to have a lasting evolutionary impact [64]. Although certainly possible in theory, *Anolis* is a very old genus (estimated age is at least 40 million years [30]); finding conserved genetic architectures over such great periods of time might be considered surprising ([65–68] and references therein; but see [69] for evidence of conserved phenotypic integration over long evolutionary timescales in anoles).

Alternatively, it may well be that, owing to the developmental architecture of the limb, there are only a limited number of ways that variation in adult morphology can be accomplished developmentally; in other words, the only way to modify an anole's limb morphology might be through changes in the size of the cartilaginous anlagen [44]. This would represent a form of absolute or global constraint, whereby some possibilities cannot be attained owing to the constraints of limb development. However, limbs are complex structures, and processes active during later development and growth are known to contribute to limb length variation, both within species
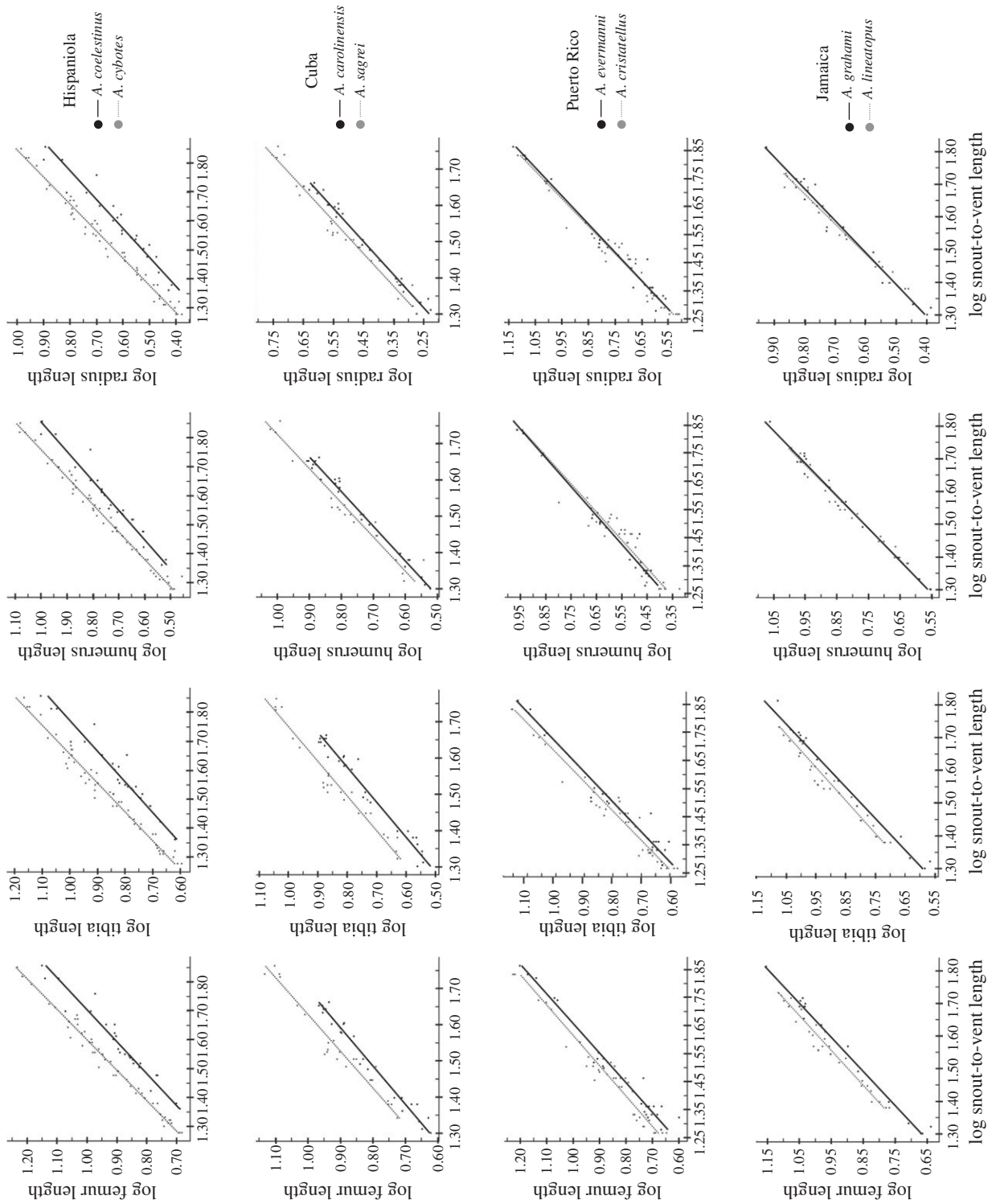
Figure 4. Allometric plots of post-hatching growth series summarized by island and limb element. Note the parallel lines in all cases except for Jamaican and Puerto Rican forelimb elements.

Table 1. Comparisons of relative limb growth between species from the same island. We found no significant differences in slope for any species pairs. Notice that post-hatching allometric trajectories for hindlimb elements show significant differences in intercept on all islands, while only Cuban and Hispaniolan forelimb elements show significant differences. Similar patterns were recovered in the embryonic analysis. Significant comparisons are highlighted in bold.

	slopes			intercepts		
	d.f.	*F*	*p*	d.f.	*F*	*p*
post-hatching allometry						
Cuba						
A. sagrei versus *A. carolinensis*						
femur	1,42	1.257	0.269	1,43	72.278	**$p < 0.0001$**
tibia	1,42	0.34	0.563	1,43	94.614	**$p < 0.001$**
humerus	1,42	0.361	0.551	1,43	17.427	**$p < 0.0001$**
radius	1,42	0.417	0.522	1,43	27.108	**$p < 0.0001$**
Jamaica						
A. lineatopus versus *A. grahami*						
femur	1,38	0.029	0.866	1,39	26.346	**$p < 0.001$**
tibia	1,38	0.145	0.705	1,39	19.807	**$p < 0.001$**
humerus	1,38	0.371	0.546	1,39	0.177	0.677
radius	1,38	1.685	0.202	1,39	0.449	0.507
Hispaniola						
A. cybotes versus *A. coelestinus*						
femur	1,70	1.098	0.298	1,71	248.036	**$p < 0.0001$**
tibia	1,70	1.213	0.275	1,71	203.093	**$p < 0.0001$**
humerus	1,70	2.54	0.115	1,71	158.09	**$p < 0.0001$**
radius	1,70	1.638	0.205	1,71	231.776	**$p < 0.0001$**
Puerto Rico						
A. cristatellus versus *A. evermanni*						
femur	1,57	0.178	0.675	1,58	21.87	**$p < 0.001$**
tibia	1,57	0.147	0.703	1,58	11.226	**$p < 0.001$**
humerus	1,57	0.233	0.631	1,58	0.292	0.591
radius	1,57	0.206	0.651	1,58	2.028	0.16
embryonic limb allometry						
Cuba						
A. sagrei versus *A. carolinensis*						
femur	1,34	0.087	0.77	1,35	31.137	**$p < 0.001$**
tibia	1,34	1.937	0.173	1,35	39.659	**$p < 0.001$**
humerus	1,34	0.006	0.937	1,35	16.99	**$p < 0.001$**
radius	1,34	0.782	0.383	1,35	8.884	**$p < 0.001$**
Jamaica						
A. lineatopus versus *A. grahami*						
femur	1,31	0.394	0.535	1,32	5.515	**0.025**
tibia	1,31	0.003	0.956	1,32	5.694	**0.023**
humerus	1,31	0.591	0.448	1,32	0.993	0.326
radius	1,31	0.123	0.728	1,32	0.007	0.934

(e.g. [34,35,37]) and among species (e.g. [41]; reviewed in [69]). In addition, forelimb and hindlimb elements appear to respond to selection independently on Jamaica (figures 3 and 4), and other lizard species exhibit non-isometric growth trajectories [70,71], which further indicates that no global constraints preclude allometric changes in limb dimensions.

A third possibility is that selection favouring differences in limb length between species in different habitats operates throughout their life histories. This would create strong functional and genetic correlations between early and late developmental stages [72]. If selection favours differences in limb length among juveniles, then it is possible that such differences might most easily be accomplished evolutionarily through changes in pre-hatching development, explaining why similar patterns recur repeatedly among species. Consistent

selection throughout life would explain why there is no positive limb allometry in juvenile lizards; but because selection presumably does not act directly on limb length in developing embryos, this cannot explain why differences are repeatedly accomplished through early changes in limb morphogenesis time and time again.

At this time, it is not possible to distinguish between these competing evolutionary hypotheses. As mentioned above, deeply conserved developmental constraints may have channelled variation along certain dimensions, but the age and history of anoles make conserved genetic constraints seem unlikely. In addition, limb morphogenesis is highly variable in other vertebrate species, indicating that the overall developmental architecture of the limb is unlikely to channel the response to selection to particular developmental processes. Is it possible that the radiation of *Anolis* lizards used similar 'genetic lines of least
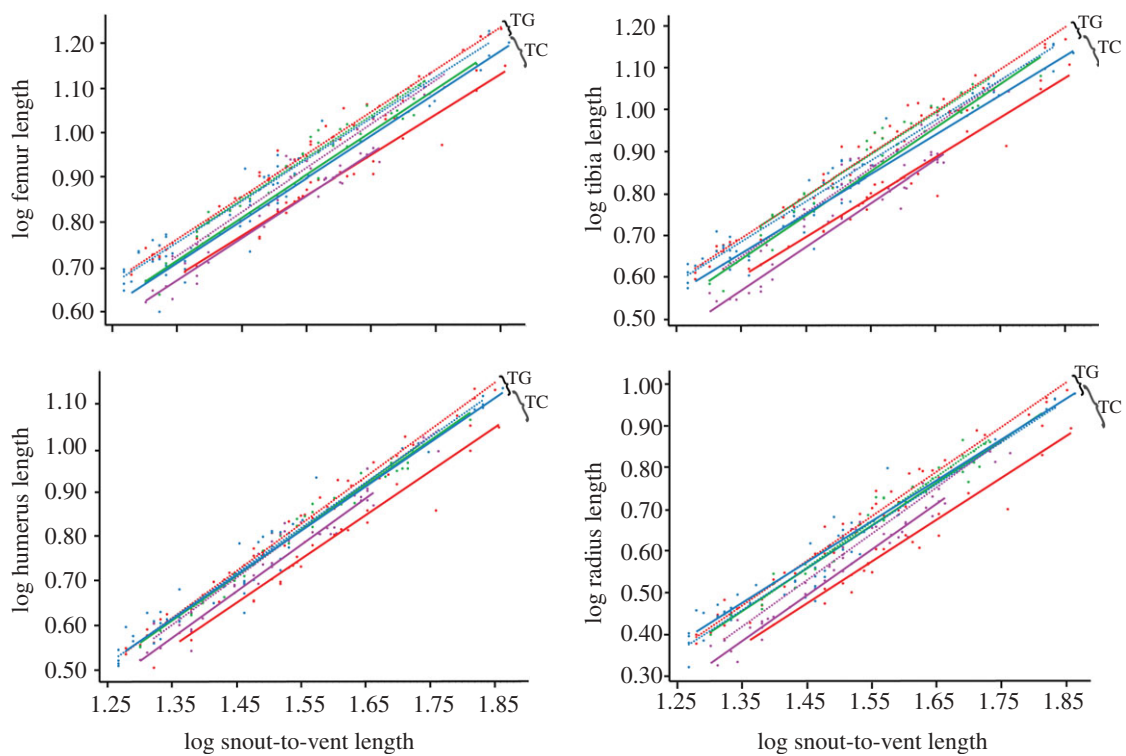
Figure 5. Summary of allometric plots of post-hatching growth series organized by island and habitat. Despite variation in the degree of divergence in limb proportions on each island, trunk-ground anoles consistently have relatively longer hindlimbs compared with the trunk-crown anole from the same island. Ecomorph: solid line, trunk-crown; dotted line, trunk-ground. Islands: red lines, Hispaniola; purple lines, Cuba; blue lines, Puerto Rico; green line, Jamaica.
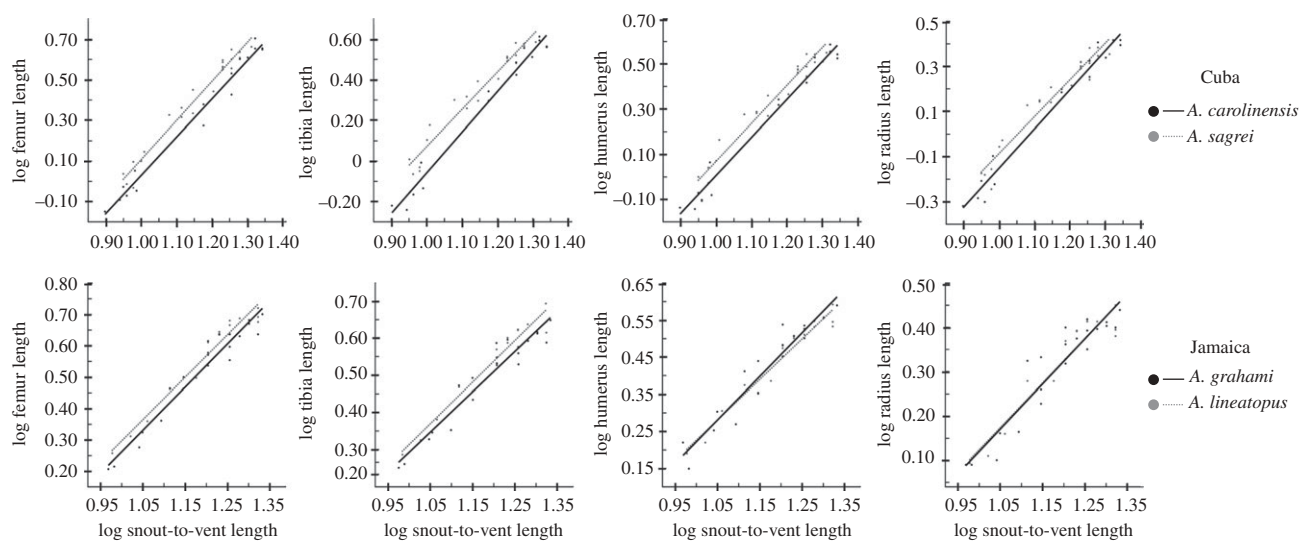

Figure 6. Plots of the relative growth of embryonic hindlimb elements highlighting the developmental origin of the ecomorphological variation early in development. Note the parallel allometric trajectories arising at the earliest stages of long bone development.

resistance' to rapidly diversify? Could the genetic architecture of early limb patterning actually channel the response to selection? addressing these questions will lead to a more thorough understanding rsof the relative roles of selection and constraint in shaping the radiation of *Anolis* lizards, but will require further data on the underlying genetics of limb development among anole species, a greater understanding of the molecular underpinnings of limb variation within species, and further data on the habitat preferences of hatchlings and juvenile lizards.

Have anoles diversified in limb length through convergence or in parallel? While the distinction between these terms is contentious [8,23], a key factor often used to distinguish parallelism from convergence is whether the same developmental mechanisms underlie convergent phenotypes. In this light, parallelisms are a special case of convergence: parallelisms represent the interaction between selection and shared developmental constraints, while convergence is a more general phenomenon that can arise through multiple, unrelated evolutionary mechanisms (reviewed in [8]). Based on our results, we cannot

rule out the possibility that anoles have evolved in parallel, by selection acting on a common set of developmental parameters controlling the size of the long bone anlagen. However, it is also possible that anoles have converged both morphologically and developmentally for reasons other than developmental constraint, as discussed above. The ultimate test of the parallel evolution hypothesis will be finding similarities in the genomic signature of natural selection to determine whether changes to the same loci have been independently selected on each island. Exploitation of the soon-to-be-published *Anolis carolinensis* genome, along with more detailed studies of natural history and development, should provide greater clarity regarding the relative importance of natural selection versus developmental constraint in guiding the evolutionary trajectory of *Anolis*.

Biologists are only just starting to bridge the divide between studies of natural selection and molecular genetics, providing a more detailed mechanistic understanding of the adaptive process [73]. But many of the best-studied examples of developmental evolution—where the specific genetic loci involved have been identified—have focused on discrete traits such as the presence or absence of specific characters (e.g. pelvic spines [19,74], wing spots [21] or vertebrate limbs [31]). Most studies of adaptation and divergence, however, focus on subtler, quantitative changes [75,76]. Thus, to connect studies of developmental evolution with the vast majority of studies of selection in nature, we need to shift our focus to quantitatively varying traits, the genetic basis of which is probably considerably more complicated than that in many of the model systems currently studied in evolutionary developmental biology.

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