

Developmental processes underlying the evolution of a derived foot morphology in salamanders

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Interdigital webbing has evolved repeatedly in tropical salamanders (*Bolitoglossines*). This derived foot morphology is only one of many homoplastic traits in this diverse amphibian clade. Indeed, few if any morphological traits sort lineages within this clade. We investigate the processes underlying the homoplastic evolution of morphological characters in these salamanders by analyzing selective and developmental processes that generate interdigital webbing. We show that a pedomorphic developmental change generates the new foot morphology and that pedomorphosis affects a number of morphological traits, thus creating a developmental correlation among them. This correlation among traits is maintained across most species, thus facilitating the repeated evolution of traits. Although we find evidence that the changes in foot morphology are adaptive in one species, the evolution of webbing in all other species does not carry an adaptive signature. The new foot morphology therefore evolves repeatedly, even in the apparent absence of a direct selective advantage.

geometric morphometrics | homoplasy | limb evolution | pedomorphosis | *Bolitoglossa*

In tropical salamanders, a wide variety of morphological traits evolves repeatedly in separate phylogenetic lineages. In the extreme case, two morphologically indistinguishable species have evolved independently from different ancestors (1). The ubiquity and extent of repeated evolution demand an analysis of the underlying mechanisms. Traditionally, the repeated evolution of characters has been interpreted as *prima facie* evidence for adaptive processes. Adaptation does lead to similar phenotypes in similar environments and can thus account for the repeated evolution of traits. However, developmental processes, which bias the range of phenotypes, can also explain the repeated evolution of morphological traits (2, 3).

We investigate selective and developmental processes underlying the repeated evolution of webbed feet in tropical salamanders. The genus *Bolitoglossa* is composed of seven geographically delimited, monophyletic subgenera, four including only webbed species and three displaying a range of webbing (4). Webbing is a derived trait within Plethodontidae and the clade of tropical salamanders, supergenus *Bolitoglossa* (5), and because of its repeated evolution it was thought to be adaptive. Extensive webbing of hands and feet has been viewed as an adaptation to a new arboreal lifestyle that evolved in these tropical salamanders. In particular, webbing was hypothesized to improve attachment to smooth plant surfaces (6). Webbing in miniaturized salamander species, however, has been recognized to be the consequence of truncated limb development rather than an adaptation for climbing (6, 7). Our morphometrical analyses now show that all webbed *Bolitoglossa* species, miniaturized as well as others, share a juvenile foot morphology. A pedomorphic developmental change thus gives rise to the webbed foot morphology.

We show that webbing itself is an adaptation for climbing in only a single species under the current hypothesis. However, through pedomorphic changes, webbing is accompanied by the appearance of other morphological characters, any one of which, or all, may be under selection. The repeated evolution of these

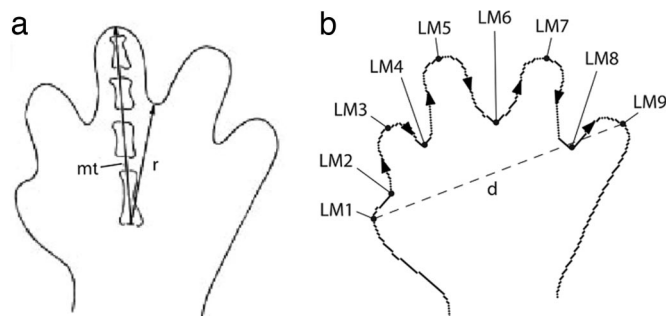


Fig. 1. Two measures to quantify the amount of interdigital webbing. (a) Amount of webbing measured as a ratio of two distances mt and r . mt is taken from the base of the metatarsal to the tip of digit 3 (dt3) and r from the metatarsal to the edge of the skin between dt2 and dt3 (8). (b) Amount of webbing measured as the sinuosity of the foot. The outline of the foot from the tip of dt1 to dt5 (arrowheads) is measured as linear distance. This distance is divided by d , the diameter or width of the foot, to yield the sinuosity. The positions of the landmarks used for foot-shape analysis are labeled LM1–LM9. The sinuosity is more efficient at discriminating between different foot morphologies than the previous measure.

suites of pedomorphic traits in the genus *Bolitoglossa* is evidence that a degree of developmental integration is stable during the evolution of this clade. This integration may explain the repeated evolution of similar or identical phenotypes found in nature.

Results

We characterized interdigital webbing in 31 species of salamanders sampled from four families and seven genera [supporting information (SI) Table 2]. To quantify webbing, we devised a measure that is highly efficient at discriminating foot morphologies of different species (Fig. 1). This measure uses the sinuosity of the foot to determine the amount of webbing. The sinuosity is defined as the length of the outline of the distal part of the foot [from the tip of digit one (dt1) to digit five (dt5)] divided by the width of the foot. This dimensionless ratio allows discriminating three morphotypes with significant statistical support (data not shown). Species differences were difficult to capture with previously used measures (ref. 8 and Fig. 1).

The *Bolitoglossa* Lineage Has More Webbing than Outgroups. A comparison of sinuosity among different salamander families, genera, and species reveals a phylogenetic trend for increased

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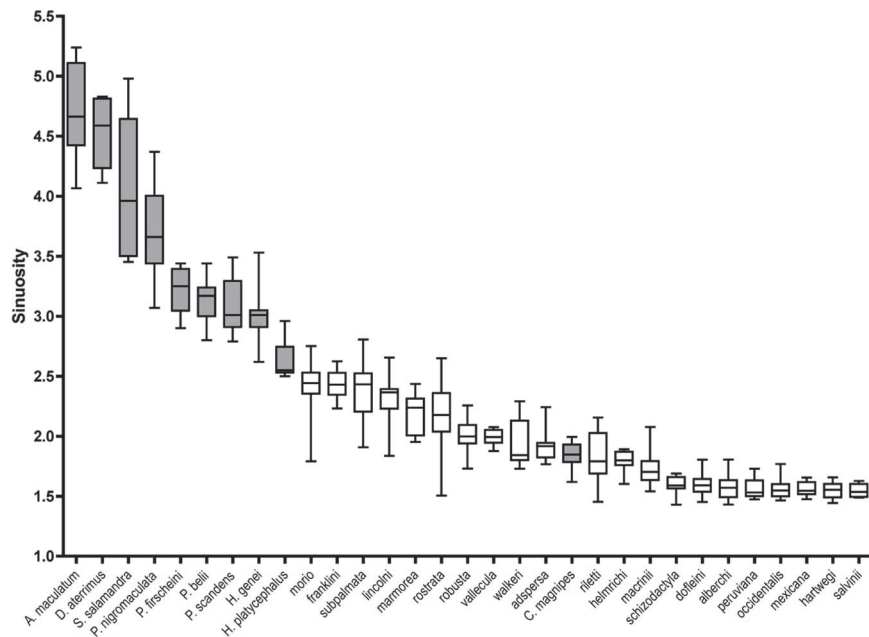


Fig. 2. Degree of webbing in *Bolitoglossa* and outgroup taxa. Each box plot shows the median, interquartile range, and total range of the degree of webbing for a given species. In total, 31 species are analyzed. The white boxes belong to members of *Bolitoglossa*, the gray boxes to outgroup taxa. All *Bolitoglossa*, irrespective of their ecology, have more webbing (i.e., lower sinuosity) than outgroup taxa (with the exception of *C. magnipes*). The most (Ambystoma, Dicamptodon, Salamandra, Pseudoeurycea, Hydromantes, Chiropoterotriton) webbed species all reach very similar degrees of webbing (sinuosity ≈ 1.56).

webbing within the tropical genus *Bolitoglossa* (ref. 9 and Fig. 2). Even fully terrestrial species of *Bolitoglossa* have significantly more webbing than almost all species outside the genus, the exception being the cave-dwelling Mexican bolitoglossine *Chiropoterotriton magnipes* (ref. 10 and Fig. 2).

Webbed Species Lack Developmental Change in the Amount of Webbing. The most webbed species have remarkably similar degrees of webbing close to $\pi/2$ (sinuosity ≈ 1.56) and little intraspecific variation in the degree of webbing (Fig. 3*a*; *B. schizodactyla* through *B. salvinii*). This diminished intraspecific variation has a telling developmental origin: in webbed species the sinuosity remains constant over the whole course of development, whereas in unwebbed species the sinuosity progressively diminishes as development proceeds (Fig. 3*b*). In species such as *B. rostrata* or *B. lincolni*, for example, the degree of webbing decreases (i.e., the sinuosity increases) during growth, whereas in species like *B. salvinii* and *B. alberchi* there is no change in sinuosity. Consequently, the reduced amount of intraspecific variation in webbed species is caused by the lack of ontogenetic change in the degree of webbing.

Ontogenetic Shape Change Occurs in the Distal Part of the Foot Only in Unwebbed Species. To understand better the changes in foot shape that occur over the course of development, we used a morphometrical method that compares juvenile and adult foot shapes, based on a set of landmarks. Specimens of different developmental stages were used to trace ontogenetic shape change in six species: three unwebbed (*B. lincolni*, *B. franklini*, *B. rostrata*) and three webbed (*B. mexicana*, *B. alberchi*, *B. occidentalis*). The shape change is represented in a grid plot (Fig. 4*a* and *b*). The undistorted grid represents the position of landmarks in a juvenile salamander. As the salamander matures, the position of landmarks changes, and the grid becomes deformed. The grid plot of the unwebbed species *B. rostrata* (Fig. 4*a*) is much more deformed than that of the fully webbed species *B. mexicana* (Fig. 4*b*), indicating more extensive shape change over the course of development. Regressing the procrustes distance

(representing the total amount of shape change of all landmarks) onto the centroid size (a size measure of the specimen calculated as the summed squared distances of all landmarks from the centroid) shows that the shape change in the analyzed unwebbed species is significant at the 5% level, whereas no significant change is found in webbed species. Fully webbed species thus indeed show less ontogenetic shape change. The grids suggest that shape change in unwebbed species occurs via outgrowth of toe tips and perhaps through loss or differential growth of skin tissue between digits.

A Change in Ossification Mediates the Change in Foot Morphology. The lack of ontogenetic change in sinuosity and shape suggests that a webbed foot is the consequence of retention of a juvenile morphology. A pedomorphic developmental change may thus give rise to the new foot morphology. A comparison of the foot skeleton between webbed and unwebbed species demonstrates this point. We took x-rays of species of *Bolitoglossa* and other genera to measure the relative lengths of bony elements in the third toe. Over the course of salamander development, bony elements in the toes (phalanges) ossify and lengthen. In webbed species (*B. alberchi*, *B. platydactyla*, and *B. dofleini*) phalangeal lengthening does not occur. All phalanges (ph1, ph2, ph3) are reduced in length and ossification compared with unwebbed species (Fig. 5*a* and *b*, *Inset*). The same reduction of distal bony elements in the foot is found in miniaturized species (*B. altamazonica*), which also maintain juvenile morphologies into adulthood (ref. 8 and Fig. 5*a*). Hence, pedomorphosis mediated by the reduced ossification of ph2 and ph3 underlies the evolution of webbed feet (Fig. 5*b*). In other organisms, connecting tissue between digits is usually maintained through the down-regulation of apoptosis (11). The developmental mechanism in salamanders is therefore different: it adjusts the degree of ossification and hence does not depend solely on the regulation of apoptosis.

Growth Trajectories Are Conserved in *Bolitoglossa* and Have Not Adapted. Using a mathematical model, we tested whether the evolution of interdigital webbing is adaptive. The model uses the

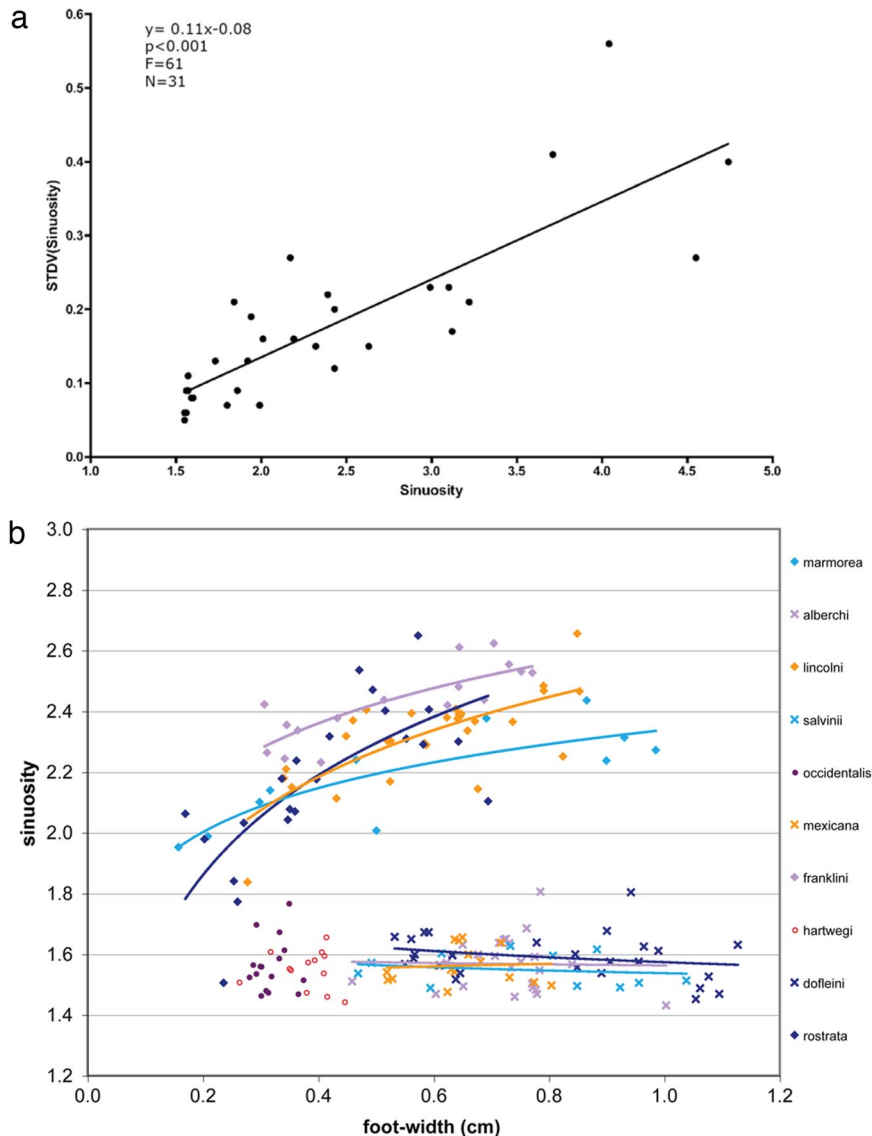


Fig. 3. Diminished variation in sinuosity in webbed species. (a) A linear least-squares regression shows that the variation in webbing found within a species depends on their average sinuosity ($P < 0.001$). Webbed species vary less in their sinuosity than unwebbed species. The standard deviation is used to measure the spread in sinuosity in 31 species. This analysis uses the same species as Fig. 2. (b) The ontogenetic change in the degree of webbing is shown for 10 species of *Bolitoglossa*. In webbed species (crosses), the sinuosity does not change over the course of development. It stays at the same level as in the miniaturized species *B. hartwegi* and *B. occidentalis* (circles). In unwebbed species (diamonds), the sinuosity increases as development proceeds from juvenile to adult. Foot width increases over the course of development (data not shown) and is taken here as a proxy for age. Nonlinear regressions were used to calculate the trend lines.

allometric equation $A = bW^\alpha$ to make specific predictions with respect to the evolution and scaling of body weight W and foot surface area A (see *Materials and Methods*). The allometric growth trajectory relating A to W , for example, is expected to evolve if foot webbing constitutes an adaptation to an arboreal lifestyle. Specifically, given that W in webbed and unwebbed species is distributed over the same range, the growth trajectory for webbed species should be closer to isometry ($\alpha = 1$) than for unwebbed species. However, the growth trajectories of webbed and unwebbed species in the genus *Bolitoglossa* are identical (Fig. 6). The residuals from a pooled nonlinear regression between webbed and unwebbed species are not significantly different confirming this result (data not shown). These results show that interdigital webbing does not increase A relative to W and that webbing thus does not constitute an adaptation according to the current hypothesis.

Species-specific estimates of the parameters b and α were

subsequently obtained for six webbed and four unwebbed *Bolitoglossa* species as well as for *C. magnipes* (Table 1). All *Bolitoglossa* species, webbed and unwebbed, share similar parameter values ($\alpha \approx 0.66$, as expected for an area to volume ratio; $b \approx 0.11$). The trajectory of *C. magnipes*, however, is very distinct (Fig. 6). The change in *C. magnipes* is the result of an increase in parameter b (Table 1). Because *C. magnipes* is known to climb on smooth surfaces on the walls of caves, this change may therefore indeed be adaptive. However, no similar change in growth trajectories is found in species of *Bolitoglossa*, webbed or unwebbed. Accordingly, the parameters α , b , A , and W have not been optimized by selection for better climbing performance in webbed species of *Bolitoglossa*.

Discussion

Increased foot webbing occurs in bolitoglossine salamanders irrespective of their lifestyle: terrestrial as well as arboreal

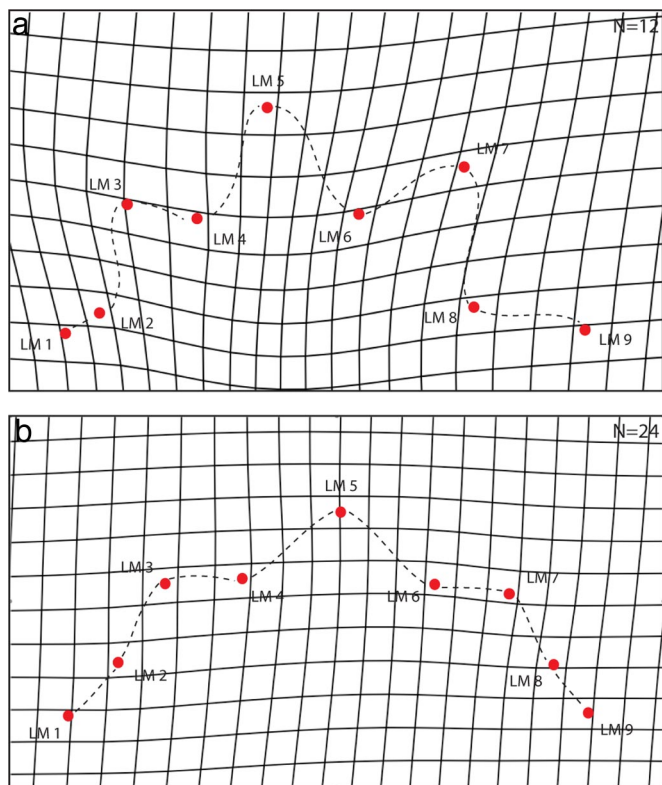


Fig. 4. Ontogenetic shape change in foot morphology. Grid deformation plots visualize shape changes over the course of development in the webbed species *B. mexicana* (a) and the unwebbed species *B. rostrata* (b) (landmarks LM1–LM9 are shown in red). The average landmark positions of the two smallest individuals in a sample represent the undistorted grid. (a) In *B. rostrata*, landmarks at the tips of toes move distally and away from the center (e.g., LM3 and LM7), whereas landmarks between toes move proximally and toward the center of the palm (e.g., LM4 and LM6) as development proceeds. This finding accounts for the observed increase in sinuosity in unwebbed species (Fig. 3b). (b) The grid of *B. mexicana* is less deformed than the grid of *B. rostrata*. Therefore, the shape of webbed feet changes less than the shape of unwebbed feet over the course of development. A statistical analysis of shape change supports this visual result ($P_{mexicana} = 0.1$; $P_{rostrata} = 0.03$; see Results).

species have more webbing than species outside the genus *Bolitoglossa*. We show that a pedomorphic developmental change leads to this increased degree of webbing, just as in

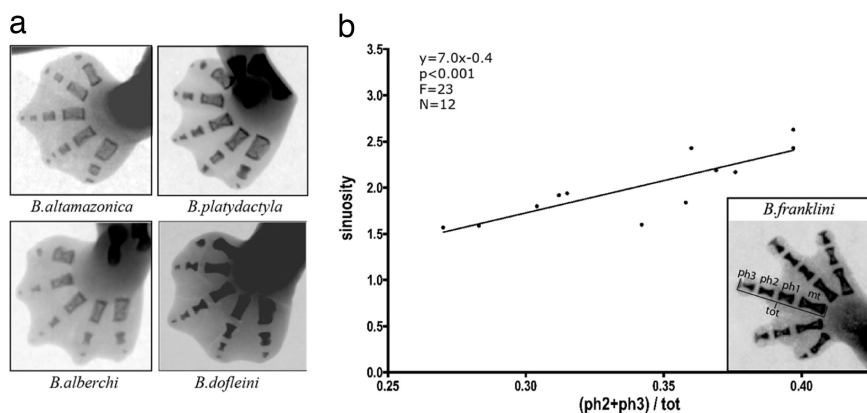


Fig. 5. Pedomorphosis in limb development. (a) In fully webbed species (*B. altamazonica*, *B. platydactyla*, *B. alberchi*, and *B. dofleini*) phalanges (ph1–ph3) do not ossify as much as in unwebbed species (*B. franklini*; see Inset in b). *B. altamazonica* is a miniaturized species that is truncated in development (8). It is indistinguishable from larger, webbed species. (b) The reduction of phalanges causes the webbed foot morphology. The contribution of ph2+ph3 to total toe length shrinks from 40% in unwebbed to 27% in webbed species, which causes a steep decrease in sinuosity ($y = 7x - 0.4$). (Inset) Bony elements in the third toe.

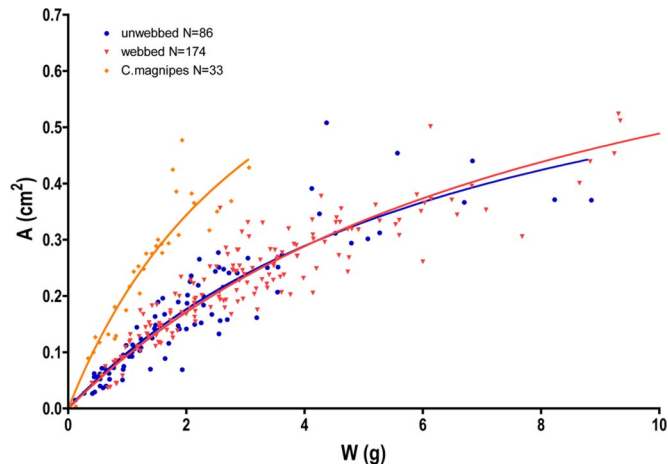


Fig. 6. Growth trajectories in webbed and unwebbed salamanders. The foot surface area, A , increases nonlinearly with body weight, W . Webbed (red) and unwebbed (blue) species of *Bolitoglossa* share the same trajectory. Data from six webbed and four unwebbed species were pooled to calculate least-squares nonlinear regressions. Regressions using datasets from individual species show the same results (Table 1). The foot area relative to body weight is therefore identical in webbed and unwebbed species. *C. magnipes*, however, has a distinct growth trajectory closer to isometry. The mechanism underlying limb development in *C. magnipes* evolves in accordance with our mathematical model and is different from the mechanism found in *Bolitoglossa* (see Results).

miniaturized species. Thus, webbing in *Bolitoglossa* does not improve attachment (as illustrated by our mathematical model) and may have evolved alongside another trait affected by pedomorphosis. Consequently, the new foot morphology correlates with other morphological traits: (i) foot surface area scales with body weight; (ii) foot morphology correlates with skull ossification (8, 12); (iii) the developmental correlation between individual toe bones is maintained, i.e., phalanges ossify and lengthen in a concerted fashion over the course of development in unwebbed species (and fail to do so in webbed species); (iv) all toes are affected in a correlated way. These developmental correlations are stable in the genus *Bolitoglossa* and hence affect phenotypic evolution in this clade by facilitating the repeated evolution of similar or identical phenotypes.

There is one interesting exception: *C. magnipes* not only has evolved interdigital webbing but also has changed its growth

Table 1. Growth trajectories in individual species

Species	<i>n</i>	Sinuosity	Avg. <i>W</i> , g	α	<i>b</i>	$b \cdot \alpha \cdot 100$
<i>B. subpalmata</i>	24	2.39	1.6	0.78	0.08	6.2
<i>B. dofleini</i>	26	1.59	9.0	0.68	0.10	6.8
<i>B. salvinii</i>	13	1.55	7.3	0.66	0.11	7.3
<i>B. alberchi</i>	55	1.57	4.3	0.68	0.11	7.5
<i>B. lincolni</i>	24	2.32	2.8	0.71	0.11	7.8
<i>B. schizodactyla</i>	9	1.60	2.5	0.87	0.09	7.8
<i>B. franklini</i>	27	2.43	2.0	0.66	0.12	7.9
<i>B. mexicana</i>	56	1.56	2.7	0.66	0.12	7.9
<i>B. helmrichi</i>	15	1.80	1.3	0.68	0.12	8.2
<i>B. marmorea</i>	11	2.19	2.5	0.71	0.13	9.2
<i>C. magnipes</i>	33	1.86	1.4	0.73	0.21	15.3

Nonlinear least-squares regression was used to estimate α and *b* in 10 *Bolitoglossa* species and in *C. magnipes*. α and *b* are similar in all *Bolitoglossa* species (see rightmost column). Webbed and unwebbed *Bolitoglossa* species therefore share the same growth trajectory. The increase in the *B. schizodactyla* α value may be influenced by the small sample size; however, *C. magnipes* shows an increase in its *b* value, explaining the increase in relative foot surface area.

trajectory. As a consequence of these changes, this species has very large feet compared with its body size, suggesting adaptation to its lifestyle. The increase in parameter *b* suggests that the number of cells specified as part of the foot primordium is higher than in other salamanders. The growth rate (i.e., the rate of division) of these cells at the tip of the limb bud remains unaffected because α remains constant.

Pedomorphic changes initially might have been selected to allow diversification of body size: both the largest and the smallest terrestrial salamander species occur in the diverse tropical clade, supergenus *Bolitoglossa*. These changes might then have affected other traits as well (such as skull and foot ossification). Widespread pedomorphic tendencies in salamanders suggest a developmental switch that triggers pedomorphosis. A major quantitative trait locus triggering pedomorphic changes indeed has been identified in *Ambystoma* (13). *Bolitoglossa* development bypasses the larval stage that characterizes *Ambystoma* development. Pedomorphosis in *Bolitoglossa* may therefore employ a different genetic mechanism. However, evidence that aquatic larvae have reevolved in lineages of direct-developing plethodontid salamanders (14, 15) raises the possibility that genes coordinating metamorphosis from larva to adult may provide such a switch.

Bolitoglossa contains more species than any other salamander genus. Seven clades have been recognized as subgenera, based on analyses of mitochondrial DNA (4). A high degree of overall similarity is evident, and few if any morphological traits sort the clades. Webbed species are found in all seven clades. Using out-group comparisons, one must conclude that ancestral *Bolitoglossa* arose from unwebbed ancestors. The sister clade of *Bolitoglossa* is the *Pseudoeurycea* (sensu lato; 5) clade, all members of which, even miniaturized species, have unwebbed feet. The sister clade to the entire *Bolitoglossa*–*Pseudoeurycea* combined clade is *Chiropterotriton*, and only the derived *C. magnipes* is fully webbed. However, the parallelism in foot morphology and the increased webbing in all species of *Bolitoglossa* raise the possibility that pedomorphosis might have been present in the common ancestor of *Bolitoglossa* before it began diversifying. In this scenario, reduced webbing appears repeatedly in the genus *Bolitoglossa* as a derived trait. The underlying developmental mechanism of such repeated evolution would remain the same (i.e., ossification of phalangeal elements) but would evolve in an opposite direction. All members of *Bolitoglossa* display traits that have been interpreted as pedomorphic: incomplete skull ossification with loss of septomaxilla, frequent loss of prefrontals, the usual presence of a cranial fontanelle, and failure of distal tarsals to individuate (10).

We believe that these results foster deeper insight into phenotypic evolution: phenotypic patterns are used to infer processes in evolutionary biology. The repeated evolution of a morphological character in particular has been used to infer adaptive processes. Our results show that this inference may not be robust for individual traits but needs to be considered in a wider context of morphological characters. We conclude that without understanding the developmental mechanisms underlying character evolution it will remain difficult to infer process from pattern.

Materials and Methods

Acquisition of Morphological Data. Alcohol-preserved specimens from several museums were measured for foot surface area (*A*), snout–vent length, body weight (*W*), length of digits, length of individual phalanges and metatarsals, foot width, degree of webbing, and foot shape. Specimens were photographed with a digital camera (Nikon Coolpix 995) and x-rayed for 25 s at 25 kV. The digital photographs and digitized x-rays were archived on a Dell PC. Enlargement of these digitized, high-resolution pictures allowed the precise measurement of morphological structures with the help of Scion Image (www.scioncorp.com). Weights were measured on a digital scale. The Integrated Morphometrics Package (IMP) (www2.canisius.edu/~sheets/morphsoft.html) and tpsdig (<http://life.bio.sunysb.edu/ee/rohlf/software.html>) were used for shape analysis. The statistics programs JMP and Prism 4.0 (GraphPad) were used to perform statistical analyses of morphological data. A list of species studied is presented in [SI Table 2](#). Sample sizes differed among species. Not all species were included in all analyses because different analyses required different minimal sample sizes. We sampled several species from each of the seven subgenera of the genus *Bolitoglossa* (4) wherever possible.

Measuring the Allometric Growth of Foot Surface Area. We measured the increase in foot surface area *A* relative to body weight *W* over the course of development in 10 species. Preserved specimens that differed with respect to their developmental stage were measured for *A* and *W*. A least-squares nonlinear regression of *A* on *W* was used to estimate the growth trajectory of *A*.

Analysis of Foot Shape. We used geometric morphometric techniques to compare foot shapes over the course of development. The comparison is based on a choice of nine landmarks set at the tip of each toe of the right foot of salamander specimens and at the lowest point between toes (ref. 16 and Fig. 1*b*). Differences in shape are defined here as differences that are not caused by scaling, translation, or rotation of feet (17). The shape difference is measured as a linear approximation of the procrustes distance, which is the square root of the summed squared distances between homologous landmarks when configurations are in procrusted superimposition (18). This linear approximation is the quantity used by morphometrical software to compare shapes and perform statistical tests. A thin-plate spline is used to locate shape differences between two objects. Grid plots visualize this shape change.

Mathematical Model. A mechanical model was used to study the adaptive value of webbing to climbing on smooth surfaces. This model tests whether A and W have evolved to optimize attachment. Initially, the forces acting on a salamander hanging upside down on a smooth surface are calculated. Subsequently, given a set of species-specific parameters (A , W , α , and b), the ability of the salamander to attach is assessed. Eqs. 1–3 show that relationships of A and W resulting in optimal attachment can be predicted (SI Fig. 7).

$$A = bW^\alpha \quad [1]$$

is the allometric equation describing the relationship of A and W as the organism grows.

$$(\Delta P + \nu)A - gW = 0 \quad [2]$$

describes the balance of forces acting on a salamander hanging upside down on a smooth surface, where ΔP is the amount of suction and ν the viscosity of

the secreted mucus. g is the acceleration of gravity. By setting this equation to zero, one describes the boundary condition where the salamander reaches its maximal weight with zero force acting on it in an upward or downward direction (6).

$$\text{Eq. 1} - \text{Eq. 2} = bW^\alpha - gW/(\Delta P + \nu). \quad [3]$$

Eq. 2 solved for A and then subtracted from Eq. 1 yields Eq. 3. Optimizing Eq. 3 identifies the optimal weight W^* for a given species with a given set of parameters b , α , ΔP , and ν . Alternatively, given the average W of a species, Eq. 3 predicts the shape of Eq. 1, resulting in optimal attachment.

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Supporting Information

Files in this Data Supplement:

SI Table 2

SI Figure 7

SI Figure 7

Fig. 7. Optimizing foot morphology for arboreal life. The red and blue curves represent growth trajectories in two different, hypothetical species. The trajectories relate foot area (A) and body weight (W) over the course of development. Both curves are described by $A = bWa$ using different values for the parameter a . Any combination of W and A determines whether a salamander species is able to climb or whether it is too heavy relative to its A . The boundary condition $F = 0$ is described by Eq. 2 in the main text: along this line, no force is acting in an upward or downward direction on a salamander hanging upside down on a smooth surface. Below this line, salamanders will detach and fall; above the line they remain attached. Given a curvilinear relationship of A and W , there is a point along the growth trajectory where attachment is optimized. Optimizing the difference of Eq. 1 and Eq. 2 yields this point of optimal attachment (denoted by $W1^*$ and $W2^*$).

