Metamorphosis shapes cranial diversity and rate of evolution in salamanders

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Abstract

Metamorphosis is widespread across the animal kingdom and induces fundamental changes in the morphology, habitat, and resources used by an organism during its lifetime. Metamorphic species are likely to experience more dynamic selective pressures through ontogeny compared to those with single-phase life cycles, which may drive divergent evolutionary dynamics. Here, we reconstruct the cranial evolution of the salamander using geometric morphometric data from 148 species spanning their full phylogenetic, developmental, and ecological diversity. We demonstrate that life cycle influences cranial shape diversity and rate of evolution. Shifts in rate of cranial evolution are consistently associated with transitions from biphasic to either direct-developing or paedomorphic life cycle strategies. Direct-developers exhibit the slowest rates of evolution and lowest disparity, and paedomorphic species the highest. Species undergoing complete metamorphosis (biphasic and direct-developing) exhibit greater cranial modularity (evolutionary independence among regions) than do paedomorphic species, which undergo differential metamorphosis. Biphasic and direct-developing species also display elevated disparity relative to evolutionary rate for bones associated with feeding, whereas this is not the case for paedomorphic species. Metamorphosis has profoundly influenced salamander cranial evolution, requiring greater autonomy of cranial elements and facilitating the rapid evolution of regions that are remodelled through ontogeny. Rather than compounding functional constraints on variation, metamorphosis appears to have promoted salamander morphological evolution over 180 million years, which may explain the ubiquity of this complex life cycle strategy across disparate organisms.

Main

Developmental processes play a fundamental role in structuring the morphological diversity of organisms[1-3], being both a driver of, and a constraint on, phenotypic change[1,4,5]. As such, shifts in development and life history can have profound impacts on the evolutionary trajectories of lineages. Early attempts to delineate these effects resulted in the recapitulationist doctrine, stating that ontogeny replicates phylogeny[6]. However, studies of groups such as amphibians have shown that shifts in developmental strategies (e.g., biphasic, direct-development, paedomorphy, and viviparity) have occurred many times. In some cases, metamorphic species can even eliminate later ontogenetic stages and mature with larval

characteristics (a form of paedomorphosis), demonstrating that the relationship between ontogenetic and evolutionary dynamics is often complicated[7]. Biphasic development with a complex life cycle is a common developmental strategy[8] and has resulted in much of the exceptional diversity that is evident today [majority of all animals, with most of the successful and speciose groups of insects (over 80%) and vertebrates (~ 50%); e.g., [8-14]]. This distribution indicates that metamorphosis may be an important driver of biodiversity overall, but why? Previous studies have suggested that larval stages may not overlap in resource use with adults[15], meaning that intraspecific competition between larvae and adult stages is reduced, which may favor population growth, but this does not necessarily translate into phenotypic diversity[16]. Importantly, biphasic species typically undergo a change in environment alongside metamorphosis from the larval to the adult stage, which is coupled with distinct physiological, morphological and functional changes. Such complex life cycles offer a conceptual framework to test the capacity of an organism to cope with environmental changes by producing morphological variation during their life span[17,18].

One way for a lineage to evolve extreme phenotypic modifications is by having aspects of its morphology vary independently from others (also defined as modularity), allowing each module (quasi-autonomous subsets of highly-correlated traits) to vary and evolve independently. This modularity may increase the overall capacity of a species to generate heritable morphological variation and novel form as well as potentially facilitating greater morphological diversification[19,20]. Life cycle complexity offers a unique perspective on these fundamental biological concepts, as radical transitions in form occur at multiple ontogenetic and evolutionary scales[18]. For example, the existence of a modular life cycle with compartmentalized developmental stages has led researchers to suggest the "adaptive decoupling hypothesis"[21-24], where antagonistic selection pressures occur at each life-history stage, maintaining low genetic correlations between larval and adult traits. However if stages are not autonomous, structures that are required for different functions at different ontogenetic stages could be constrained in terms of their morphological evolution[25]. Thus, metamorphosis may either increase developmental canalization, leading to reduced morphological diversity in metamorphic forms, or it could reset the pattern of variation between larval and adult stages and allow greater morphological diversity across species[26].

Among the organisms experiencing metamorphosis, salamanders (Amphibia: Caudata, ~ 700 species[27,28]) display a tremendous diversity of species, ecologies and life cycles with multiple independent evolutions thereof[25,29-33]. Thus, salamanders provide an excellent framework to study how developmental processes can produce morphological diversity. Interestingly, contrasting results have been found so far for salamanders depending on the morphological structure of interest. Bonett and Blair [25] found accelerated rates of body form/ vertebral column evolution in species with a simple life cycle (paedomorphic, aquatic species and direct-developing, terrestrial species). They also found that constraints on body form evolution are stage-specific and that shifts in life cycle complexity can alter the dynamics of morphological evolution in salamanders. Ledbetter and Bonett [33] showed that limbs evolve faster and are less constrained for aquatic species (mostly paedomorphic) than terrestrial species (mostly direct-developing). Vučić, et al. [26] demonstrated that metamorphosis cannot

be regarded as a developmental constraint on the overall external head in a newt. Finally, Blankers, et al. [34] hypothesized that developmental constraints on phenotype may limit morphological evolution to different microhabitats in plethodontids based on analysis of body and appendage lengths.

Here, we assess the role of metamorphosis and the associated changes of environment on the evolution of morphological diversity and evolutionary modularity of the skull across salamanders displaying a diversity of life cycles. To do so, we generated a novel dataset describing cranial shape in salamanders at unprecedented scales, spanning the full phylogenetic, ecological, and developmental breadth of Caudata, with species belonging to all families and representing nearly all genus-level diversity (>95% of extant genera, Fig. 1, Extended Data Figs. 1-3 and Supplementary Figs. 1-3). Changes in salamander skull shape during metamorphosis relate to changes in both diet and environment[35], with musculoskeletal (bone and muscle) remodeling leading to the modification of certain bones (mostly bones involved in food processing including the pterygoid and vomer), while others disappear entirely (e.g. the palatine portion of the palatopterygoid), or first appear after metamorphosis (such as the maxilla or the prefrontal)[29,36]. Consequently, assessing every cranial element is critical for uncovering the complexity of salamander cranial evolution. We test the prediction that adult phenotypes in biphasic and direct-developing species have a lower disparity and evolutionary rate than paedomorphic species, as developmental canalization has been hypothesized to constrain developmental processes during metamorphosis[26]. In addition, we investigate whether complexity of life cycle or extent of metamorphosis influence evolutionary modularity. Specifically, we hypothesize that biphasic species transitioning between environments and diets may exhibit increased fragmentation of phenotypic traits into evolutionary modules, compared with direct-developing and paedomorphic species experiencing just one environment. Alternatively, we hypothesize that species undergoing complete metamorphosis (biphasic and direct-developing species) may display more modular evolution than those experiencing no or differential metamorphosis (paedomorphic species). Finally, we explore the relationship between evolutionary rates and morphological variation for each cranial bone depending on life cycle. We predict that bones involved in food processing or those remodeled during metamorphosis have an elevated morphological diversity and rate of evolution and thus an increased ability to evolve.

Results

Life cycle and associated habitat differentiates adult cranial morphology. There is a significant but low impact of size (centroid size) on salamander cranial shape evolution (PGLS R^2= 0.063, *P*=0.001; Supplementary Table 1 and Fig. 4). The adult cranial morphological space (morphospace) of all 148 species can be summarized by thirty-two principal components (PC) [and 42 phylogenetic PCs] explaining 95% percent of the shape variation. Life cycle has a highly significant influence on shape of all cranial regions (Supplementary Table 2), with species sharing the same life cycle clustering together in cranial morphospace (Fig. 2 and Extended Data Fig. 4 for results of fine-grained classifications). In contrast, microhabitat (e.g. see method) is a significant influence on only four individual elements and overall cranial shape, at much lower significance levels

(Supplementary Table 3) and does not clearly discriminate species in cranial morphospace beyond its interaction with life cycle (Supplementary Fig. 5). PC1 (>31% of overall shape variation) segregated species with a fully aquatic life cycle (paedomorphic) from species with either a fully (viviparous, direct-developing) or largely (biphasic) terrestrial life cycle. Variation along this axis also corresponds to the degree of metamorphosis, from differential to complete metamorphosis. Aquatic paedomorphic species are elongated with a reduced number of bones whereas more terrestrial species have a complete and more robust skull (Fig. 2). PC2 (>19% of overall shape variation) described differences between species with an aquatic and terrestrial life cycle, with fully terrestrial direct-developing plethodontids clearly differentiated from other species that spend some or all of their lives in water, including a number of troglodytic plethodontids. These differences between fully aquatic species versus fully or primarily terrestrial species are even more evident in the phylogenetic PCA (Extended Data Fig. 5). Shape differences along PC2 are mainly concentrated on the pterygoid and vomer with terrestrial direct-developing species showing a more gracile cranium without a pterygoid and with a vomer lacking the transverse series of teeth. Paedomorphic species occupy the largest area of morphospace followed by biphasic species. Notably, the direct-developing plethodontids, the most species-rich clade of salamanders, occupy only a small area of the morphospace described by the first two PC axes.

Cranial diversity is influenced by life cycle. We tested for differences in morphological diversity depending on life cycle (Extended Data Fig. 6 and Supplementary Data 1) to explore the overall morphological pattern. Aquatic paedomorphic species show a higher disparity of cranial shape compared to species with other types of life cycles (as well as relative to other more fine-grained categories, Extended Data Fig. 6) and associated habitats. Direct-developers exhibit low disparity whereas biphasic species are intermediate between direct-developers and paedomorphic taxa (Extended Data Fig. 6).

Transitions in life cycle influence rate of cranial evolution. We performed an ancestral reconstruction of the different life history strategies (Fig. 3, Extended Data Fig. 7 and Supplementary Tables 4-5) and estimated rates of cranial evolution on the Caudata phylogeny (Fig. 4). Our results support a biphasic life cycle as the ancestral condition for Caudata (Fig. 3 and Extended Data Fig. 7) with paedomorphosis evolving independently in several lineages. Furthermore, the biphasic life cycle re-evolved several times in lungless salamanders (plethodontids, Fig. 3). Fast evolution of cranial shape occurred in lineages leading to paedomorphic species and biphasic species (Fig. 4 and Extended Data Figs. 8-9). Direct-developers have a relatively lower rate of cranial evolution. Major shifts in the rates of cranial evolution occurred early in the evolution of salamanders and were followed by transitions in life cycle strategies. For example, during the Mesozoic, major shifts in cranial rate occurred at the transition between the paedomorphic Sirenoidea and the species-rich Salamandroidea, as well as within the families of the latter clade, which vary considerably in life history. Another major shift in the rate of cranial evolution occurred between the paedomorphic Cryptobranchidae and the biphasic Hynobiidae. The relationship between rates of morphological evolution and life history transition is particularly evident within two genera, the plethodontid *Eurycea* and the ambystomatid *Ambystoma* (Fig. 3-4), that both

include metamorphosing and paedomorphic species. In both genera, the shift to a paedomorphic life cycle, which is accompanied by a reversal to an aquatic environment, is associated with an increase in the rate of cranial evolution.

Species with complete metamorphosis are more modular than paedomorphic species. We found that species exhibiting complete metamorphosis (biphasic with 12 modules and CR=0.61; direct-developing species with 13 modules and CR =0.54, respectively Fig. 5a,d and b,e) exhibit more modular skull evolution than paedomorphic species (11 modules and CR =0.71, Fig. 5c,f and Supplementary Data 2-4). These results hold after subsampling our samples of biphasic and direct-developing species to match the sample size of paedomorphic species (CR after subsampling: CR_{bi}= 0.63; CR_{dd}=0.56; Supplementary Data 5-6). Our analyses further demonstrate that the suspensorium (composed of the squamosal, quadrate, and pterygoid) and the maxilla have high within-region evolutionary integration across caudates.

Per-module rate and disparity are correlated only in completely metamorphic species. We tested the tendency to evolve disparate morphologies depending on life cycle by assessing the relationship between the rate of evolution and disparity for each module. Our results show a positive correlation between the rate of cranial evolution and disparity in both biphasic (Rho_S=0.72; *P*=0.01, Fig. 5g) and direct-developing species (Rho_S=0.7; *P*=0.009, Fig. 5h), with the suspensorium and the maxilla evolving at the highest rates of any cranial region. No correlation was found in paedomorphic forms (Fig. 5i).

Per-landmark relationship between rate and disparity differs between completely metamorphic and paedomorphic species. Comparing the relationship between disparity and evolutionary rate to the relationship expected under a constant rate of Brownian motion evolution can reveal instances of facilitation (disparity is higher than expected) or constraint (disparity is lower than expected). In biphasic species, disparity is lower than expected for nearly all cranial bones except for the vomer and the pterygoid which show instead a relatively higher disparity (Fig.6). Direct-developing species show a similar pattern to biphasic species, with higher disparity than expected given the estimated rate of evolution for the vomer; while nearly all the other elements (maxilla, nasal and parietal) fall within the expectation of a Brownian motion process (Fig. 6). In contrast, in paedomorphic species, some cranial elements such as the parietal, frontal, vomer, orbitosphenoid, occipital, and parasphenoid display lower disparity than expected given the estimated rate of evolution. Only the maxilla and nasal show a high disparity relative to the estimated rate, and all other cranial bones in paedomorphic species follow the expectation of a Brownian motion model of evolution (Fig. 6).

Discussion

Metamorphosis requires substantial ecological, functional, and morphological transformations through ontogeny, and yet is ubiquitous across animals[8,37], including half of the living vertebrates[8]. However, its impact on morphological diversity remains poorly understood. Our results indicate that species with different life cycles have distinct

evolutionary patterns for adult cranial morphology. Cranial shape changes along the main axis of variation followed a gradient of metamorphosis associated with habitat use as well as feeding mode. The cranial shape of paedomorphic species with differential metamorphosis is streamlined with a reduced number of bones, likely related to both aquatic and suction-feeding adaptations[38-40]. In addition, aquatic species with more complete differential metamorphosis (cryptobranchids, some ambystomatids) tend to have wider heads, likely related to suction-feeding as wider heads allow for greater volume expansion during jaw opening. In contrast, species exhibiting complete metamorphosis (biphasic and direct-developing species, including most salamandrids, plethodontids, rhyacotritonids, hynobiids, and ambystomatids) have a more complete and robust cranium, as well as a more terrestrial habitat and tongue-based feeding strategy. Among these terrestrial feeders, most biphasic species using tongue protrusion coupled with jaw prehension display a more robust suspensorium (composed of the pterygoid, quadrate, and squamosal) compared to many of the direct-developing species with a long-tongued ballistic feeding mode. Paedomorphic species occupy a large area of morphospace and exhibit the highest disparity, suggesting multiple routes to their simplified and "larval-like" morphology. These include taxa that undergo differential metamorphosis (e.g., Amphiumidae, Cryptobranchidae, and Ambystomatidae) that are similar in shape to fully biphasic species, as well as species that retain nearly all larval traits into adulthood (e.g., Proteidae, Sirenidae, some *Eurycea*). Notably, the direct-developing plethodontids, the most species-rich clade of salamanders, occupy the smallest area of the morphospace and have the lowest disparity.

Developmental canalization is hypothesized to reduce morphological variation in species with multi-stage life cycles (biphasic)[25]. Our results show that species exhibiting complete metamorphosis (including both biphasic and direct-developing forms), are less disparate that those that undergo differential metamorphosis (i.e., paedomorphic). Thus it is the process of complete metamorphosis (whether occurring at larval stage or *in ovum*), and not merely the presence of a multi-stage life cycle, that may canalize cranial shape variation in salamanders.

Changes in developmental strategy have impacted the dynamics of body form and limb evolution in salamanders over the last ~160My[25,33]. As shown in previous studies, a biphasic life cycle was recovered as the ancestral condition for Caudata[41,42], with direct-development evolving once, paedomorphy evolving several times independently[25,29,31], and biphasic (and sometimes then paedomorphic) strategies evolving and re-evolving multiple times among direct-developing plethodontids. Changes in developmental strategy are consistently associated with shifts in rate of cranial evolution, with paedomorphic and biphasic species showing faster rates than terrestrial direct-developing species. Furthermore, a reversal from a terrestrial biphasic to an aquatic paedomorphic life cycle is also accompanied by an increase in the rate of cranial evolution, as found in Ledbetter[33]. These results suggest that the rapid morphological evolution in paedomorphic taxa, which tend to live in inhospitable environments with poor access to food resources and mates (such as caves or organic muck habitats) [31,43], may facilitate the persistence of these species in such challenging conditions[44,45]. In contrast, complete terrestriality in direct-developing plethodontids may impose strong constraints on their cranial shape evolution, possibly associated with ballistic

tongue projection. This constraint is demonstrated by a low disparity and rate of evolution in this group, as previously also documented for limb morphology[33]. However, in contrast to previous studies, we did not find that direct-developing species, even if they remain in the same environment during their entire life, have a higher rate of evolution than biphasic forms[25], suggesting that cranial shape and body form are differentially impacted by developmental strategies. Moreoever, our results on cranial shape do not support those obtained at the intraspecific level in *Triturus* newts where metamorphosis did not induce reduced variability in external head shape, which may suggest distinct processes at the micro- and macroevolutionary scales.

Our analysis of patterns of evolutionary integration and modularity in species with different life cycles recovered a more modular pattern than documented in previous studies of the caudate skull[46,47]. As expected, paedomorphic species have a more integrated cranium than biphasic and direct-developing ones (the latter of which undergo prehatching metamorphosis)[48]. That direct-developing species have a (slightly) more modular cranium than biphasic forms is surprising and suggests that the impact of metamorphosis on cranial organization and evolution is retained even when the larval stage is entirely *in ovum*. Analyses of phenotypic integration and modularity within *Salamandra salamandra*[49] support the same pattern as observed at the evolutionary level, suggesting that the recruitment of all the bones of the suspensorium (pterygoid, jaw joint, quadrate and squamosal) into one strongly integrated, quasi-autonomous module may have facilitated its evolvability, resulting in a high rate of evolution and disparity for bones of the suspensorium across Caudata. This pattern may relate to changes in feeding mode during metamorphosis, which requires a radical change in morphology and ecology[24,50,51] and induces skull remodeling involving primarily the feeding apparatus. Unexpectedly, direct-developing species exhibit similar patterns to biphasic species, despite undergoing metamorphosis without an actively feeding larval stage. Our results thus suggest that metamorphosis in any form strongly impacts the pattern of shape evolution of cranial bones.

We further tested the hypothesis that metamorphosis strongly impacts the pattern of cranial shape evolution by assessing the per-landmark Procrustes variance and mean evolutionary rates for each cranial element in biphasic, direct-developing, and paedomorphic species. Our results show that, in biphasic species, nearly all cranial bones are constrained (with low disparity relative to their respective rate of evolution) except for those that are remodeled during metamorphosis (high disparity relative to rate for the vomer[52] and the pterygoid). The pattern is similar in direct-developing species except that nearly all cranial bones follow a Brownian motion model of evolution, where the disparity of each is increasing in line with its respective evolutionary rate. However, the overall pattern is different in paedomorphic species where most of the bones show high heterogeneity of disparity relative to rate, even within bones (see orbitosphenoid in Fig. 6). This difference may reflect variation due to differential levels of metamorphosis in paedomorphic taxa[29,50], as is evidenced by the variable absence of some bones (maxilla, prefrontal, nasal, and orbitosphenoid).

Conclusions

Metamorphosis is one of the most fascinating, spectacular, and surprisingly common developmental processes in the animal kingdom[8,23], often requiring an abrupt change in morphology and ecology during the lifetime of an individual as it transforms from a larva into an adult. Despite the potential for metamorphosis to impose compounding constraints on morphological evolution, our analyses suggest that completely metamorphic species, with or without an actively feeding, free-living larval stage, exhibit high evolutionary autonomy of cranial elements. This autonomy likely promotes the diversification of metamorphic, or ancestrally metamorphic, species by allowing rapid evolution of structures that engage in divergent functions and thus experience dynamic selection pressures both during ontogeny and through evolutionary shifts in life cycle complexity. The origin and evolution of different life-history stages, their maintenance through time, and their impact on species diversification still remain poorly understood and merit further attention. Future research, using longitudinal series with specimens at different developmental stages at the micro- and macroevolutionary levels is necessary to better understand how and why metamorphosis is an important driver of cranial diversity.

Methods

Data sampling. 152 specimens belonging to 148 species and representing all the families of Caudata (Supplementary Data 7) were sampled for this study. This sample was selected in order to represent the diversity of developmental life cycles within each family as fully as possible.

Developmental Life Cycle Traits and microhabitats. Developmental life cycle and microhabitats data were collected for each species from the existing literature (see Supplementary Figs. 1-3 and Data Table 8). The following definitions are used for each life cycle. Species are defined as paedomorphic (pd) when they retain aquatic larval traits when reproductively active. Species are defined as direct-developers (dd) when they fully transform in the egg and hatch directly as terrestrial miniature versions of the adults. Species are defined as biphasic (bi) when they exhibit a multiphasic life cycle (most of them exhibit a two-part life cycle), with an aquatic larval stage followed by metamorphosis into a more terrestrial adult[25,41,53]. Because direct-developers undergo metamorphosis in the ovum, we consider both biphasic and direct-developing species as metamorphic. It is important to note that this classification is an oversimplification of life cycle categories in some species. Some species in our dataset are facultative biphasic (f-bi), where some populations can either be metamorphs or paedomorphs, and this is often associated with changing habitats (see Supplementary Data 8). Other species are defined as puereparate viviparous (vipu) when they have embryos developing inside their body until the end of the gestation, and they give birth to fully developed terrestrial juveniles (or *stricto sensu* pueriparity in this study)[54]. Some species are facultatively viviparous, and these are mainly larviparous in our dataset (f-vila, Supplementary Data 8), delivering small aquatic larva in the water. Because neither of the two facultative viviparous species (*Salamandra algira* and *Salamandra salamandra*) in our dataset are pueriparous[54], we treat them as biphasic species in further statistical analysis (phylogenetic MANOVAs, modularity, and integration analyses), as they still have an active larva and encounter a full metamorphosis with a change of morphology and ecology. The strictly puereparate viviparous species were not included in several statistical analyses (such as the phylogenetic MANOVAs, modularity and integration analyses) as they are only represented by three species in our dataset (*Lyciasalamandra atifi*, *Lyciasalamandra luschani* and *Salamandra atra*). Another important point to raise is the complexity of the paedomorphic category, which includes several species that are encountering differential

metamorphosis[29,50], with variation of the composition of larval traits retained into adulthood. Using the literature[29,50], we defined four different categories of paedomorphy depending on traits that are modified during metamorphosis (Supplementary Data 8). Variability is also present among biphasic species with facultative biphasic species, where some populations can be paedomorphic in these species (f-bi). Other species that are coded as biphasic in all our analyses are in fact multiphasic such as *Notophtalmus*[55] (bi-tri), which have an aquatic larva, terrestrial juvenile, and aquatic adult, or *Ichthyosaura alpestris* and *Lissotriton vulgaris* that are seasonally changing between an aquatic and a terrestrial life as adults[56].
While coding of life cycles is necessarily oversimplified here for the purposes of robust statistical analyses, we encourage readers to consider the appropriateness of this coding scheme and possibilities of capturing more nuanced categories in future work. Nevertheless, at present, finer levels of classification cannot be used in most of our analyses as we need more than 20 species (ideally 30 species) per group for the modularity and integration analyses, and at least 5 species per group to test for shape differences depending on life cycle, disparity, and rate of evolution. Microhabitats (semi-fossorial, aquatic, semi-aquatic, terrestrial, arboreal, aquatic species living in cave and terrestrial species living in cave) were defined as finer scale than the broad habitats associated with life cycle (terrestrial and aquatic).

3-D scanning and processing. One hundred and seven scans were generated for this study (107 species), and 45 were collected from different online repositories (Supplementary Data Table 7). The following CT-scanners were used to scan specimens at high resolution: a Phoenix VTx L240-180 CT scanner (General Electric, Boston, MA, U.S.A.) at the X-ray tomography facility at the Museum National d'Histoire Naturelle (AST-RX platform, UMS 2700), a Phoenix nanotom X-ray|s at the Museum für Naturkunde; a Phoenix VTome|x M240 at the University of Florida's Nanoscale Research facility and made available on MorphoSource (morphosource.org); a Nikon Metrology HMX ST 225 CT scanner at the CT facility of the Natural History Museum. Specimens collected from Digimorph (digimorph.org) were scanned using an ACTIS scanner at the High-Resolution X-ray Computed Tomography Facility at the University of Texas at Austin. Avizo Lite 9 (FEI Visualization Sciences Group, Burlington, MA, USA) was used to segment and export the skull reconstructions of each specimen as PLY files. All the PLY files were imported into Geomagic Wrap (3D Systems, Rock Hill, South Carolina, USA) in order to clean, repair and decimate the meshes prior to the landmarking procedure.

Quantification of skull shape using 3D geometric morphometrics. The extreme variability in cranial region presence and morphology between metamorphic and non-metamorphic species has so far hindered robust comparisons of cranial shape using traditional morphometric approaches (Fig. 1 and Supplementary Data 9). To comprehensively capture cranial morphology across Caudata, we used a high-density surface geometric morphometric approach. Eighty-seven landmarks, 496 curve sliding semilandmarks, and 356 surface sliding semilandmarks were used to delineate 14 cranial regions (Fig. 1, Supplementary Data 9). A 3D sliding-semilandmark procedure[57-59] was used to precisely quantify the shape of each skull bone. This method allows the comparison of different shapes by transforming sliding semi-landmarks on curves and surfaces into spatially homologous landmarks[60]. All the landmarks and curve semilandmarks were manually collected by the same person (A-CF; Fig.1 and Supplementary Data 9) using the software package IDAV Landmark[61] (http://graphics.idav.ucdavis.edu/research/EvoMorph) and following the protocols described in several previous studies[62-67]. The curve semilandmarks generated from IDAV landmark were subsampled using the algorithm of Botton-Divet et al.[68]. Next, all the surface sliding semilandmarks were obtained using a semi-automated approach in the 'Morpho' R package v2.5.1[69]. First, a template was created with the same configuration of landmarks and curve

semilandmarks plus surface semilandmarks. To do so, we created an hemispheric template mesh using a 360 x 360 uniform-vertex sphere created in Meshlab (http://www.meshlab.net/) and modified in Blender (Stitching Blender Foundation, Amsterdam, Netherlands) on which we manually placed all the landmarks and curve and surface sliding semilandmarks. Secondly, this template is used to place surface semilandmarks semi automatically onto each specimen by fitting its coordinates (landmarks and curve semilandmarks) to those of each specimen. Surface semilandmarks were placed using the *placePatch*[69] function which determined their position through a thin-plate spline method. Each bone was patched separately following protocols as described elsewhere[59,65]. For a more accurate patching, different 'inflate' values across partitions and specimen were used because of the wide range of size and shape differences in our sample. Finally, all the sliding semilandmarks were slid to minimize bending energy criteria using the functions *RelaxLM* and *slider3d*[69].
All salamander species have nine cranial regions corresponding to 10 bones that are invariably present (otic region including both prootic and opistotic bones, occipital, premaxilla, frontal, parietal, parasphenoid, squamosal, quadrate, and vomer), but some cranial bones are variably present across the order (pterygoid, maxilla, prefrontal, orbitosphenoid, and nasal; Extended Data Figs. 1-3 and Supplementary Data 10). In order to represent the whole shape of the skull and to be able to compare cranial shape across the entire dataset, we decided to represent these absent regions by one landmark (the position was chosen as best-representing the location of the missing region). This was achieved by replicating this one landmark, forming an array with the same dimensions as the surface point dataset from specimens with that bone present[66,70]. Thus, an absent region is represented in this dataset as an infinitesimal surface, corresponding to exactly the same dimensions as those of a present region. This approach allows us to include all specimens and bones in the analyses. Because data were only recorded on the right side of each specimen, and to avoid alignment artifacts during the Procrustes superimposition[71], the landmarks and semilandmarks were mirrored using the *mirrorfill* function in the 'paleomorph' R package v.0.1.4. Finally, a global Procrustes alignment was performed using the *gpagen* function in the 'geomorph' R package v.3.1.2, and missing regions had non-zero (but negligible) size[66]. A mean shape was calculated for each species using the Procrustes coordinates and used in all further analyses.
Phylogenetic Tree. Comparative analyses were performed on the maximum clade credibility (MCC) tree estimated from a posterior sample of 1000 trees published by Jetz and Pyron[72]. The MCC was calculated using the TreeAnnotator program in BEAST[73] using the CAT (common ancestor tree) algorithm to avoid issues with the estimation of negative branch lengths[74]. This MCC tree was pruned to the species present in our dataset for further comparative analyses. Because some species were not present in the phylogeny, we substituted them with species that are closely related. Thus, *Thorius tlaxiacus*, *Thorius pinicola* and *Tylotriton himalayanus* from our dataset were substituted in the phylogeny by *Thorius arboreus*, *Thorius macdougalli* and *Tylotriton yangi*, respectively, following previous studies[75,76]. Finally, we scaled the MCC tree using the branch specific average rates obtained from the posterior samples of the Bayesian analyses (see Estimation of branch specific evolutionary rates and rate shifts section). This tree was used in downstream phylogenetic comparative analyses (phylogenetic MANOVAs, phylogenetic modularity, and correlation between disparity and rate per landmark) and has the advantage of taking into account the heterogeneity in rates that have been estimated by the Bayesian approach.

Data analyses

Cranial morphological variation

Impact of size on cranial shape. In order to assess the impact of size on shape, we used the centroid size as proxy of body size for each species. We performed a phylogenetic regression using the function *procD.pgls* from the 'geomorph' R package v.3.1.2[77,78] using the Procrustes coordinates, the log10 of the centroid size and the scaled MCC tree. Phylogenetic regressions were performed on four different Procrustes superimposed datasets: 1) the full dataset containing all the species, 2) the biphasic dataset, 3) the direct-developing dataset and 4) the paedomorphic dataset. Depending on the impact of the size on shape, further analyses were performed on the Procrustes coordinates after accounting for centroid size and phylogeny.

Visualization and test for shape differences. Shape differences were visualized using a principal component analysis as well as a phylogenetic principal component analysis[79] on which the phylogeny was mapped using the function *phylomorphospace* from the 'phytools' R package v.0.6-99[79]. To assess if cranial shape differs depending on life cycle, we performed phylogenetic analysis of variance (MANOVA) using the function *procD.pgls* from the 'geomorph' R package v.3.1.2[77].

Disparity differences. To assess and compare morphological disparities for each life cycle strategy (biphasic, paedomorphic, and direct-development) we used the function *morphol.disparity* in 'geomorph' v.3.1.2. Disparity is calculated as the Procrustes variance divided by the number of landmarks per bone for each life cycle using residuals of a linear model fit[80], and pairwise comparisons to identify differences among groups were also performed.

Evolutionary rates. Calculation of evolutionary rates for whole cranial shape as well as for each cranial element and comparisons across different life cycle strategies were performed based on a Brownian motion (BM) model of evolution using the function *compare.multi.evol.rates* in the 'geomorph' R package v.3.1.2.

Transitions of life cycle and rates of cranial evolution.

Estimation of branch specific evolutionary rates and rate shifts. Rates of evolution in the salamander skull were analyzed using the variable rates model implemented in BayesTraitsV3 (http://www.evolution.rdg.ac.uk/). A reversible-jump Markov Chain Monte Carlo (MCMC) algorithm was used to detect shifts in rates of continuous trait evolution (modelled by a BM process[81]). We used as input traits the phylogenetic principal components accounting for 95% of the overall variation in shape for the whole cranium (the first 42 phylogenetic PCs). Four independent chains were run for 200,000,000 iterations, sampling every 10,000 iterations and the first 25,000,000 iterations were discarded as burn-in. Trace plots were examined to ensure that the chains were stationary after burn-in. Effective sample size of the posterior samples (ESS>100) was assessed using the *effectiveSize* function and convergence of the chains was assessed using Gelman and Rubin's convergence diagnostic[82] (function *gelman.diag*); both functions are implemented in the R package 'coda' v.0.19-3 (Supplementary Fig. 6 and Tables 6-7). The results of the analyses were plotted on the tree using the function *mytreebybranch* (https://github.com/anjgoswami/salamanders/blob/master/mytreerateplotter.R) and summarized by the branch-specific average rate and the posterior probability of rate shifts, both estimated from the posterior samples using the *rjpp* and the *plotShift* functions in the 'btrtools' R package v.0.0.0.9000 (https://github.com/hferg/btrtools/tree/master/R).

Ancestral state estimation of life cycle in Caudata. Ancestral state estimations were conducted in order to compare the position of shifts in rates of morphological evolution to the acquisitions of the different life cycles in Caudata. We used a Markov model[83] for estimating the past transitions between life cycles at internal nodes in the phylogeny. Model fit was performed assuming that the transition rates between character states are either all equal

(ER), different for each state but symmetric (SYM) or asymmetric (ARD) using the algorithm implemented in the *rerootingMethod* function in phytools v.0.6-99[79]. The best model was selected using the Akaike information criterion[84] (AIC, Supplementary Tables 4-5).

Cranial modularity and integration

Cranial modularity integration. In order to test if the developmental strategy (complete metamorphosis in biphasic and direct-developing species versus differential metamorphosis in paedomorphic species) or if the change in ecology during development (change of diet and environment in biphasic versus no change of diet and environment in direct-developing, and paedomorphic species) impact the evolutionary integration in salamanders, we assessed the pattern and magnitude of phenotypic modularity and integration for each dataset depending on developmental strategies (biphasic, direct-developing, and paedomorphic). Cranial modularity was estimated using two methods developed for testing the degree of morphological integration with high dimensional data. The first method is a maximum-likelihood approach which calculates Akaike information criterion[84] (AIC) values to assess the best supported model of modularity based on trait correlations. This is conducted using the function *EMMLi* from the 'EMMLi' R package v.0.0.3[85]. The second method used is covariance ratio (CR) analysis which assesses the covariances within and among hypothesized modules and compares this ratio to a null hypothesis of random assignment of shape variables to partitions[86]. CR was estimated using the *modularity.test* function from the 'geomorph' R package v.3.1.2[86,87]. Different hypotheses of evolutionary modularity were tested (Supplementary Data 11) on the residuals of the Procrustes coordinates data after accounting for centroid size and the scaled phylogeny with the branch specific average rates obtained from the posterior samples of the Bayesian analyses. Integration analyses are susceptible to sample size differences, and dataset differ between each type of life cycle (20 paedomorphic, 53 direct-developing and 72 biphasic species). Hence, we also assessed the robustness of our results based on 100 random subsamples of 20 species for the biphasic and direct-developing species that were obtained using the *sample* function in 'base' R package v3.6.1. We compared the average results from these 100 runs to the results from the original analysis.

Correlation between rates and disparity per module depending on life cycle complexity. Disparity and rates were quantified for each module depending on life cycle strategy. Disparity was calculated for each module as the Procrustes variance divided by the number of landmarks per module using *morphol.disparity* in the R package 'geomorph'. Evolutionary rates were computed for each element based on a BM model of evolution using the function *compare.evol.rates* in the 'geomorph' R package v.3.1.2. The correlation between rate of morphological evolution and disparity was assessed using a non-parametric test of Spearman's rank correlation[88] using the *cor.test* function of the 'stats' R package v.3.7.0 to assess and explore the relationship between rate of morphological evolution and disparity.

Per-landmark rate and variance. To assess the correlation between per-landmark Procrustes variance and mean evolutionary rates per bone depending on life cycle, disparity and rates were quantified for each landmark depending on the life cycle strategy (biphasic, direct-developing, and paedomorphic datasets). Disparity was calculated for each landmark and semilandmark as the Procrustes variance. The evolutionary rate per landmark and semilandmark was calculated using a modified version of the *compare.evol.rates* function in 'geomorph' R package v.3.1.2 (https://github.com/anjgoswami/salamanders/blob/master/per_lm_rate_and_disparity.R)[17,65]. Next, to explore the relationships between disparity on morphological rate per landmark and semilandmark within each bone, a regression was performed using the *lm* function from the

'stats' R package v.3.7.0. In order to compare the correlation between within-landmark rate and variance calculated for the different datasets (biphasic, direct-developing and paedomorphic datasets) to the expectation of a Brownian motion model of evolution, we simulated morphological evolution under BM using the *sim.char* function in the 'geiger' R package v.2.0.6.2[89]. Mean variances were estimated after running 100 simulations for each landmark and semilandmark. Finally, a regression of evolutionary rate under Brownian motion on the simulated variance was performed and plotted with its 95% confidence interval.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Scan data that support the findings of this study have been deposited in the Phenome10K repository (http://phenome10k.org/) or are already available on MorphoSource and DigiMorph and URLs and DOIs are available in Supplementary Data 7. The Procrustes coordinates, centroid size, life cycle and microhabitat definitions are available in Supplementary Data 12. The table of module hypotheses used in modularity analyses is available in Supplementary Data 13. The MCC tree, scaled MCC tree and output of Bayesian analyses are available at https://github.com/anjgoswami/salamanders. All other data analysed in this study are included in Supplementary Information.

Code availability

The R and Bayestrait codes used in this paper are available at https://github.com/anjgoswami/salamanders.

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Author Contributions
A-CF and AG conceived and designed the study; A-CF, CB, MB, DCB, JB, ELS acquired and processed the CT data; A-CF acquired the geometric morphometric data; A-CF, CB, JC and RNF conducted analyses; A-CF wrote the initial draft of the manuscript, and CB, MB, JC, RNF, DCB, JWS, JB, ELS and AG all contributed to interpretation of the data and to the editing of subsequent drafts of the manuscript.

Competing interests
The authors declare no competing interests.

Figure legends:

Figure 1: Landmarks used to quantify cranial shape variation in Caudata. Top, anatomical landmark placed on scan of *Salamandra salamandra* and corresponding to the definitions in Supplementary Data 9. Red spheres represent anatomical landmarks. Bottom, sliding landmarks that describe the 14 bones of the cranium used in all shape analyses. A representative species for each family is also provided in Extended Data Figs. 1-3.

Figure 2: Phylomorphospace illustrating the first two principal components of cranial shape across Caudata. Symbols indicate family-level clade and colours represent life cycle strategies. Skull shapes at the positive and negative extremes of each axis are depicted with warped surfaces. Abbreviations are as follows: bi indicates biphasic species, or species that are considered as biphasic; dd indicates direct-developing species; pd indicates strictly paedomorphic species; pd1 indicates paedomorphic species with external gills, gill slits, tail fin, no eyelids, no maxillary bones, no septomaxilla and no prefrontal; pd2 indicates paedomorphic species with external gills, gill slits, tail fin, no eyelids, no septomaxilla, no prefrontal and with maxillary bones developing before adulthood; pd3 indicates paedomorphic species without external gills but with gill slits, tail fin, no eyelids, no septomaxilla and with maxillary and prefrontal bones developing before adulthood; pd4 indicates paedomorphic species with external gills, gill slits, tail fin, no eyelids, no septomaxilla and with maxillary and prefrontal bones developing before adulthood; vi indicates strictly puereparate viviparous species. See Extended Data Fig. 4 for results on fine-grained classifications.

Figure 3: Evolution of life cycle in Caudata. Ancestral state estimation using a re-rooting method using the symmetric rate model (best model following results of the AIC, Supplementary Table 4). Colours indicate life cycle strategies. See Extended Data Fig. 7 for results on fine-grained classifications.

Figure 4: Evolutionary rates and rate shifts for cranial shape in Caudata. Colour gradients on branches indicate the rate of shape evolution with warmer colours corresponding to a higher rate and cooler colours to a lower one. Grey triangles indicate the stem branch of clades with support for whole-clade shifts in evolutionary rate. Posterior probabilities (PP) of rate shifts are indicated by the relative size of the triangles (see Extended Data Fig. 10). Rates and shift

were estimated using BayesTraitsV3 using a variable-rates Brownian motion model. See Extended Data Fig. 9 for results on fine-grained classifications.

Figure 5: Modularity and integration in cranial shape for different caudatan life cycle strategies. Schematics of each life cycle strategy are provided in a) biphasic, b) direct-developing, and c) paedomorphic. Changes in environment are represented by colours with light blue for an aquatic environment and yellow for a terrestrial environment. Grey background indicates life cycles involving full metamorphosis, with a) or without b) a free-living, actively feeding larval stage. d), e), and f) Network graphs indicating integration within and between regions and respective covariance ratios for the indicated model. Networks illustrate the grouping of the 19 cranial regions using phylogeny-informed EMMLi analysis. Regions were grouped as modules (and colored accordingly) when the between-region correlation (thickness of the line in the network graph) was within 0.2 of the lowest internal correlation (indicated by circle size in the network graph). Procrustes variance *versus* rate per module and the linear regression of observed rate-variance relationship are displayed in g), h) and i).

Figure 6: Per-landmark evolutionary rate against Procrustes variance for biphasic (top), direct-developing (centre), and paedomorphic species (bottom). Red lines and grey areas represent the linear regression of observed within-landmark rate and variance relationship and its 95% confidence interval, respectively. Blue lines and blue areas indicate the expected relationship between within-landmark rate and variance given a Brownian motion model of trait evolution and its 95% confidence interval, respectively.