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Evolving possibilities: postembryonic axial elongation in salamanders with biphasic (Eurcyea cirrigera, Eurycea longicauda, Eurycea quadridigitata) and paedomorphic life cycles (Eurycea nana and Ambystoma mexicanum)

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Abstract

Typically the number of vertebrae an organism will have post-embryonically is determined during embryogenesis via the development of paired somites. Our research investigates the phenomenon of *post-embryonic* vertebral addition in salamander tails. We describe body and tail growth, and patterns of postsacral vertebral addition and elongation in context with caudal morphology for four plethodontids (*Eurycea*), and one ambystomatid. *Eurycea nana* and *A. mexicanum* have paedomorphic life cycles; *E. cirrigera*, *E. longicauda* and *E. quadridigitata* are biphasic. Specimens were collected, borrowed and/or purchased, and cleared and stained for bone and cartilage. Data collected include snout-vent length (SVL), tail length (TL), vertebral counts and centrum lengths. *Eurycea* species with biphasic life cycles had TLs that surpassed SVL following metamorphosis. Tails in paedomorphic species elongated but rarely exceeded body length. Larger TLs were associated with more vertebrae and longer vertebrae in all species. We observed that rates of postsacral vertebral addition varied little amongst species. Regional variation along the tail becomes prominent following metamorphosis in biphasic developers. In all species vertebrae in the posterior one-half of the tail taper towards the tip. We suggest a developmental link might exist between the ability to continually add vertebrae and regeneration in salamanders.

Introduction

The vertebrate body axis undergoes segmentation and specialization into distinct regions during embryogenesis. Typically the process of somitogenesis (segmentation) during the embryonic period establishes an organism's total number of vertebrae. During somitogenesis, paraxial mesoderm that flanks the neural tube and notochord separates into discrete segments of mesoderm bound by epithelium. These segments are called somites and will contribute cells to the dermis, muscle, cartilage and connective tissue of the axial skeleton of the developing embryo (Keynes and Stern, 1988; Brent and Tabin, 2002). Post-embryonically, *vertebrate* axial segmentation is most easily visualized as the resulting vertebral column.

Vertebrates exhibit little diversity in terms of options for functional axial units, yet they exhibit great diversity in the number and types of vertebrae that result from somitogenesis (e.g., thoracic vs. lumbar; Richardson et al., 1998; reviewed in Dubrülle and Pourquie,

2004). For instance, snakes have an exceptionally large number of trunk vertebrae, and this number has been found to vary with body size (termed pleomerism, Lindsey, 1975). Even though snakes grow their entire life, growth is attributed to elongating trunk vertebrae, rather than addition of vertebrae, with the tail seemingly a negligible part of the story (Arnold, 1988; Arnold and Bennett, 1988; Lindell, 1994; Shine, 2000). While somite formation is an embryonic developmental process that typically leads to a determinate number of axial segments, examples of species that alter their segment numbers post-embryonically are found amongst both invertebrates and vertebrates (e.g., salamanders – Vaglia et al. 1997; centipedes – Minelli, 2005). Specifically, vertebrate species known to add segments post-embryonically do so via terminal addition to the tailbud. (Vaglia et al., 1997; Babcock and Blais, 2001). Our research investigates ongoing addition of caudal vertebrae in salamanders, with current emphasis on species within the genus *Eurycea* (Plethodontidae; Hemidactyliini; Ryan and Bruce, 2000; Chippendale et al., 2004) as well as one *Ambystoma* species, *A. mexicanum* (Ambystomatidae).

As a taxon, salamanders (Amphibia: Urodela) exhibit almost no diversity in body plan, yet they do vary in life cycle. Some are direct developing and hatch from the egg as miniature adults. Others have biphasic life cycles where an aquatic larval period of variable length is followed by metamorphosis into the terrestrial juvenile/adult. During metamorphosis the tail goes from being laterally compressed and having an extensive dorsal fin to being more rounded, longer and lacking some or all of the fin (e.g. Bruce, 1982a, b). Yet other salamanders have paedomorphic life cycles where the sexually mature adults retain larval features, such as gills and a laterally compressed, finned tail. Species thus far shown to continually elongate via the addition of caudal segments are found across all life history patterns, represent different families, inhabit different environments and exhibit different tail morphologies (Noble; 1931; Jockusch, 1997; Vaglia et al., 1997; Babcock and Blais, 2001) (Table 1). Many of the species that continually elongate also undergo tail autotomy, or 'voluntary' dropping of the tail when threatened by predation. However, not all species that undergo tail autotomy continually elongate (e.g. Dickie, 1999).

To further explore post-embryonic axial elongation, we examined five salamander species. Three species in our study (*Eurycea cirrigera*, *E. longicauda* and *E. quadridigitata*) have biphasic life cycles, and two species (*E. nana* and *Ambystoma mexicanum*) are predominately paedomorphic (natural metamorphosis unknown or rare). In addition to the species representing different life cycles, they span a range of body sizes (total length), from the proportionately longer tailed *E. longicauda* to the petite *E. quadridigitata*.

Our goals were to 1. determine whether and to what extent *Eurycea* and *A. mexicanum* add vertebrae after hatching, 2. assemble centrum length profiles of tail vertebrae, and 3. build upon our understanding of axial elongation in salamanders, especially plethodontids. Centrum length profiles provide snapshots of caudal vertebral patterning over time and may reveal regional specializations that develop across an organism's life cycle (Bergmann et al., 2003, 2004). For instance, morphologic specializations such as decreasing centrum lengths posterior to the sacrum have been shown for salamanders that occupy aquatic habitats (Worthington and Wake, 1972). Of the five species in our study, *A. mexicanum* tails are also not known to autotomize, although amputated tails do regenerate (Wake and Dresner, 1967).

Distinct functional adaptation for aquatic locomotion and lack of autotomy might suggest that *A. mexicanum* tails do not elongate via the addition of caudal vertebrae.

Materials and Methods

Specimen collection

Samples used in this study were collected during the 2007 and 2008 field seasons; sample sizes are shown in the appropriate figures throughout the paper. Egg clutches, larval, juvenile, and adult *E. cirrigera* were collected from their natural habitats, under rocks and logs near streambeds in Putnam County and Hoosier National Forest, Indiana between late March and mid-July. Large larvae, juvenile and adult *E. longicauda* were collected from similar field sites but were most abundant from early April through late June. While *E. longicauda* and *E. cirrigera* larvae do resemble one another, we consistently found that *E. longicauda* larvae have darker pigment on their sides and lower jaw, as well as a more defined dorsal stripe, similar to what Franz (1967) described (Fig. 1). *E. quadridigitata* (Dwarf salamander) and *E. nana* (San Marcos salamander, Hays County Texas; http:// amphibiaweb.org) specimens were obtained courtesy of David Cannatella and Travis LaDuc from the Texas Natural History Collections at the University of Texas (Appendix A). Various sizes of *A. mexicanum* were purchased from the Ambystoma Genetic Stock Center, University of Kentucky.

Specimen preparation and measurements

Some field-collected individuals were anesthetized on site in a 0.5 – 1.0 % solution of MS-222 (Tricaine), staged, measured for snout-vent length (SVL) and tail length (TL) and released when alert and mobile. Other individuals were taken to the lab, over-anesthetized in a 3 - 5% solution of MS-222, preserved, processed, and cleared and stained for bone and cartilage (procedure modified from Wassersug, 1976). During the clearing and staining process, trypsin was omitted as a macerating agent. Prior to preservation, specimens were staged, and snout-vent length (SVL) and tail length (TL) recorded. Following clearing and staining, caudosacral and caudal vertebrae were identified and counted, and centrum lengths of individual vertebrae were measured from the base to the tip of the tail. Figure 2 provides a basic orientation to the postsacral vertebrae and their landmarks. Criteria established by Vaglia et al. (1997) were used to distinguish between caudosacral and caudal vertebrae. From a lateral view, the haemal arch on the last caudosacral vertebrae is smaller and directed more ventrally than the haemal arch on the first caudal vertebra. It also exhibits a greater haemal spine angle than those on subsequent caudal vertebrae (Babcock and Blais, 2001). Images, vertebral counts and centrum measurements were taken and caudal morphology described using a Nikon SMZ1000 with Spot Insight Imaging software.

Statistical analyses

Statistics were performed using Sigma Plot/Stat version 11, Systat software and SPSS. For comparisons amongst species, snout-vent length (SVL) and tail length (TL) data were ln transformed. We ran simple linear regressions to estimate slope and y-intercept values for relationships between (1) ln SVL and ln TL and (2) TL and number of postsacral vertebrae. Two-sample t-tests were used to compare slopes and y-intercepts between all possible

pairings of salamander species, as well as between the two genera (*Eurcyea* vs. *Ambystoma*). A total of 11 t-tests were run for ln SVL vs. TL and TL vs. number of postsacral vertebrae. We recognize that running multiple t-tests increases the risk of committing a Type I error. To account for this, we adjusted the value of alpha using the Bonferroni approach. The desired alpha of 0.05 was divided by 11 (number of t-tests), resulting in a Bonferroni-adjusted alpha of 0.004. Thus, values were significant at $p < 0.004$, and error measurement was to the nearest 0.01mm.

Results

Body axis elongation

Tail growth is positively allometric with respect to body growth in all species studied. The tails of young *E. cirrigera* larvae are as much as 43% shorter than the bodies. In pre-metamorphic individuals with SVLs ranging from 19 – 25 mm, SVL and TL are nearly equal. Post-metamorphically, tails exceed SVL by as much as 41% (Fig. 3). Similar to *E. cirrigera*, SVL and TL were nearly equal in *E. longicauda* larvae just prior to metamorphosis (SVL = 21 – 28 mm). Following metamorphosis, tails reached lengths up to 68% longer than the bodies. A smaller sample size (n = 8) makes it difficult to define the trend for *E. quadridigitata*, but it appears that SVL and TL are nearly equal in individuals having 15 – 17 mm SVLs, with adults displaying tails up to 44% longer than the body.

In analyses of SVL to TL, two-sample t-tests revealed that slopes and y-intercepts did not differ significantly among the multiple species of *Eurycea*. Given the lack of differences, data for all *Eurycea* species were combined and compared to *Ambystoma*. The combined slope for all *Eurycea* differed significantly from *A. mexicanum*, which supports a difference in growth rates between the two genera (Fig. 4; Table 2). Y-intercept values did not differ between the genera. The relation of body to tail growth in paedomorphic *E. nana* most resembles other *Eurycea*. However, *E. nana* does resemble *A. mexicanum* in having tails that hover around a 1:1 size relation with the body at maximum known adult body sizes. Data on the range of SVLs and SVL:TL ratios observed amongst the metamorphosing and adult individuals for all species used in this study can be found in Appendix B.

Vertebral counts

Longer *Eurycea* tails contained more postsacral vertebrae. We use the term *postsacral* to indicate all vertebrae located posterior to the sacrum (= caudosacral plus caudal). These results support previous studies that have documented continual addition of postsacral vertebrae in a number of plethodontid species (Table 1). Most individuals across all species in our study exhibited three caudosacral vertebrae, though occasional individuals had either two or four.

In analyses of postsacral vertebrae to tail length, two-sample t-tests revealed that slopes and y-intercepts did not differ significantly among the multiple species of *Eurycea*. Thus, as for TL to SVL comparisons, data for all *Eurycea* species were combined and compared to *Ambystoma*. With Bonferroni adjustment, the combined slope for all *Eurycea* did not differ significantly from *A. mexicanum* ($0.01 < p < 0.05$). In contrast, the y-intercepts did differ

significantly (p < 0.001), indicating that elongating *A. mexicanum* tails begin with fewer vertebrae than found in the *Eurcyea* species (Fig. 5; Table 2). Presuming that the rate of tail elongation and vertebral addition are consistent through life, as suggested by Figure 5, it is unlikely that the number of postsacral vertebrae in *A. mexicanum* will ever 'catch up' to the numbers found in *Eurycea*.

Centrum Lengths

The general trend for tail vertebrae is that they elongate across life stages and through time. Of all species, *E. cirrigera* and *E. longicauda* have the most similar profiles. In *E. cirrigera*, the vertebral centra of larvae taper very gradually from the base (CS1) to the caudal tip of the tail (Fig. 6). Between the larval and metamorph/juvenile stages, all postsacral vertebrae lengthen. Regardless of the number of postsacral vertebrae present across a range of adult snout-vent and tail-lengths, for all three *Eurycea* species exhibiting biphasic development, tapering in the posterior 1/3 of the tail is most pronounced. Post-metamorphic vertebrae reveal more periodic rises and falls in centra lengths than earlier stages, but in general we are unable to discern specialized increases or decreases in lengths of individual vertebrae. In adult *E. cirrigera* the most posterior caudosacral vertebrae (typically CS2 and CS3) are consistently the shortest vertebra in the anterior tail, followed by a series of caudal vertebrae that are slightly longer and vary little in length up through the anterior one-third, or mid-tail, depending on tail size (Fig. 6). This might be one example of regional variation that develops in the tail over time.

Relative to metamorphosing and young juvenile *Eurycea* with biphasic life cycles, tails of the paedomorphic species, *E. nana* and *A. mexicanum*, exhibit greater growth of vertebral centra in the anterior one-third to one-half of the tail (Fig. 7A). Elongation of postsacral vertebrae over time in the paedomorphic species is shown in figure 7C. When the centrum length profiles are analyzed using tails of only older adults, rather than younger, biphasic *Eurycea*, a different pattern emerges. Now vertebral centra in the anterior tail of biphasic Eurycea are longer than those found in *A. mexicanum* and *E. nana* (Fig. 7B). Such a shift in the centrum length profile would be expected considering that our *E. nana* sample includes individuals that have reached the maximum known body/tail sizes for that species, and those sizes are up to 50% shorter than those found amongst the biphasic developers. It is clear that amongst the biphasic developers, both ongoing addition of vertebrae *and* elongation are at work throughout much of the lifespan.

In summary, postsacral vertebrae located in the anterior one-third of the tail of the paedomorphic species were longer than those found in younger stages of biphasic *Eurycea*, and shorter than those found in older adults (Figs. 7A, B). Furthermore, all three biphasic *Eurycea* species developed more caudal vertebrae than either of the paedomorphic species. We attribute this to differences in tail length, and to differences in the initial numbers of vertebrae present in the tail, rather than to rate of addition.

Morphological evidence of vertebral addition

Our exploration of caudal vertebral development has revealed that caudal vertebral centra elongate, and that caudal vertebrae are added over time in the salamander species

investigated. Clearing and staining provided morphological evidence for terminal (tail tip) addition of vertebrae (Fig. 8). In *A. mexicanum*, the development of terminal vertebrae is preceded by a distinct cartilaginous rod. The vertebrae then appear as miniature rectangular ossifications surrounding the rod. As the vertebrae continue to develop they become slightly spool-shaped (Fig. 8H, I). The mode of development of terminal vertebrae in post-metamorphic *Eurycea* is more obscure in the absence of histological detail. While it seems likely that a precursor cartilaginous rod would be present prior to development of caudal vertebrae, clearing and staining does not reveal a rod such as that seen in *Ambystoma*. However, similar to *A. mexicanum*, the terminal caudal vertebrae in biphasic *Eurycea* are small and rectangular in shape. Unlike *A. mexicanum*, these caudal-most vertebrae show hints of ossified neural and haemal arches (Fig. 8A–E). The caudal ossification pattern in *E. nana* differs slightly from that of biphasic *Eurcyea*. Initially newly developing vertebrae in *E. nana* look like miniature ossified letter x's. This shape then fades and is replaced by ossification in the anterior and posterior articular portions of the vertebral centrum, or perichordal tube (Fig. 8F, G). *E. quadridigitata* is unique in having the most completely formed and ossified miniature caudal vertebrae to the furthest extent of the tail tip. Where in other species, 3–5 vertebrae are seen forming via slight ossification of the vertebral centra (vertebral body), but lack arches, in *quadridigitata* we often see both arches and vertebral bodies forming in close unison (Fig. 8D).

While more detailed studies on the histological and cellular detail of tail tip are in progress, a brief mention of outstanding vertebral morphology is worthy of note in the context of comparing two different genera as well as biphasic vs. paedomorphic species. All *Eurycea* species share nearly identical caudal vertebral morphology. In *Eurycea*, tail vertebrae develop gradually, but become more complex earlier in development than in *A. mexicanum*. Caudal vertebral morphology in paedomorphic *E. nana* retains some larval characteristics throughout much of development. For example, ossification of the vertebral centra and neural and haemal arches occurs late in development. Also, pre- and post zygapophyses processes remain visibly cartilaginous until late in development. Furthermore, they are less tightly articulated in the posterior one-third of the tail in the adult individuals examined when compared to biphasic *Eurycea*.

A. mexicanum caudal vertebrae morphology is distinct from *Eurycea* in exhibiting little differentiation and elaboration of vertebral arches and processes. The paedomorphic *A. mexicanum* exhibits an even more dramatic larval caudal morphology into late adulthood than found in *E. nana*. For instance, pre- and post-zygapophyses processes, and the posterior tips of haemal arches remain cartilaginous throughout much of the tail. In addition, vertebral centra ossify late, and features such as transverse processes and neural and haemal spines do not develop beyond the caudosacral region until late in adulthood, if at all. In large adults it is not uncommon to find only spool-shaped vertebral centra with rudimentary neural arch formation, and no evidence of haemal arches (Fig. 8H). The extent to which the spinal cord appears to be 'exposed' in the tail is astounding. And as mentioned earlier, the tail tip is characterized by a long cartilaginous rod.

Discussion

Continual elongation, or indeterminate growth, of the vertebrate body axis is widespread in groups such as fish, amphibians, lizards and snakes. Here we explore continual elongation of the posterior body axis (tail), especially its unique ability to add terminal caudal vertebrae. Little documentation for the ongoing addition of caudal vertebrae is available, outside of key studies that have noted or explored this phenomenon in amphibians (Milner, 2008; Table 1). Our work (1) builds upon our knowledge of which salamander species exhibit caudal vertebral addition, (2) describes allometric changes in the tail and trunk across biphasic and paedomorphic life cycles, (3) describes patterns of vertebral addition before, during and after metamorphosis, and (4) discusses vertebral addition and elongation in context with tail morphology across life stages.

The salamander species in our study elongate post-embryonically and continue to elongate as adults. In all cases tail length (TL) scales with positive allometry to snout-vent length (SVL), and longer tails are associated with more vertebrae. Moreover, longer tails achieve their length both by adding vertebrae to the tail tip, and by lengthening pre-existing vertebrae. In biphasic *Eurycea* most vertebrae are added after metamorphosis, and potential regional variation (i.e. the shortened second and third caudosacral vertebrae) becomes prominent following metamorphosis. The rate at which paedomorphic tails add postsacral vertebrae does not differ significantly from that of the biphasic species, though they hatch with a small number of vertebrae. In all species, vertebrae taper between the tail base and tip, but the position of onset of tapering varies across life stages and among adults of different species.

Body axis elongation

The role of somites and somitogenesis in body axis elongation during embryogenesis is well known, and the molecular mechanisms receive ongoing attention and exploration. Typically the number of embryonic somites determines the number of postembronic vertebrae, with the exception of those groups/species that add or subtract after hatching (e.g. Handrigan and Wassersug, 2007). Much of the work on the evolution of body size references somitogenesis as a key mechanism for generating shifts in body size and form. Lability in somite formation, whether in the amount of cells available for developing somites, or the rate at which somites form, could account for relatively small changes in body size, as well extreme changes in form, such as gigantism.

Because body size has been correlated with number of vertebrae in groups such as fish (pleomerism, Lindsay, 1975), and vertebral number reflects an *embryonic* process, the developmental importance of elongation *post-embryonically* has received little attention. Furthermore, most work has focused on developmental plasticity of the trunk region (salamanders – Jockusch, 1997; snakes – e.g. Lindell, 1994, Head and Polly, 2007). In snakes the tail structure is virtually inconsequential when it comes to shifts in body form or axial elongation. A series of studies looking at axial growth and development in snakes have demonstrated that longer bodies (i.e. trunk) are most often positively correlated with vertebral number (Lindell, 1994, 1996; Shine, 2000). Notable deviations from this pattern are found in some snake species that inhabit very specific ecological niches or specialized

environments, such as those species that burrow. Such bodies have evolved with the number of vertebrae that will function most optimally in their respective environments. Relative to snakes, salamanders exhibit little variation in trunk vertebral number; although, in some genera variation is more pronounced and varies with population and species (Wake, 1966; Jockusch, 1997). Our research emphasizes that post-embryonic elongation, specifically the development of caudal structures, is a developmental process worthy of consideration in investigations of the evolution of body size.

Do salamanders add vertebrae throughout the life cycle?

Studies of Iguanian lizards have shown that numbers of caudal vertebrae vary with species, but no direct evidence has been provided for continual elongation during the lifespan of individuals (Bergmann et al. 2003, 2004). Polly et al. (2001) report for snakes that total vertebral number does not change after hatching. In snake species where body and tail proportions are highly correlated with habitat, segments are usually gained or lost simultaneously in the trunk and tail over evolutionary time, though it has been noted that changes in the tail region seem to occur with greater frequency and magnitude (Head and Polly, 2007). As found in snakes, the number of trunk vertebrae in salamanders remains fixed after hatching, and the number of tail vertebrae varies across species. However, the ability of some salamander species to add terminal caudal vertebrae throughout life is a unique feature not documented in snakes. From his observations of two individual salamanders of the same species, Noble (1931) appears to be the first to surmise: "The increase in number of vertebrae may continue during life. In a small but fully formed *Batrachoseps attenuatus* 2.3 cm from snout to vent, I find there are 22 dorsal vertebrae and 28 caudals, a number which approximates that found in some species of plethodon. In an adult 4.75 cm head and body length, there are 22 dorsals and 61 caudals. Hence the number of tail vertebrae more than doubles during the active terrestrial life of this species" (p. 231, Noble, 1931, 1st addition, Biology of the Amphibia).

Our work supports Noble's observation and the few other studies that have demonstrated that tail vertebrae are added post-embryonically in some salamander species (Table 1). As a result, tremendous variation is observed in numbers of caudal vertebrae – across salamander species, within species and even within individuals over the course of a life cycle. Furthermore, addition of tail vertebrae is not specific to species with biphasic life cycles. As Table 1 shows, direct-developing and paedomorphic species also add tail vertebrae. It would be interesting to determine whether trends exist between life cycle and timing of post-embryonic vertebral addition. Dickie (1999) discussed how the majority of vertebrae in direct-developing *Aneides* are added at small sizes (within the first few years of growth). On the other hand, direct- developing *Batrachoseps* adds at a constant rate through life, resulting in approximately one new vertebra per two millimeters of tail length (Dickie, 1999). Many of the biphasic species add a large number of new vertebrae either soon after hatching, or following metamorphosis (Vaglia et al, 1999; Babcock and Blais, 2001; current study). The padeomorphic pattern remains elusive, but our initial data suggest that *A. mexicanum* adds vertebrae at a steady rate throughout life. It also is important to recognize that the initial post-embryonic number of postsacral vertebrae for *mexicanum* is lower than for the other species investigated.

Segmental and regional growth

Research with lizards, especially chamaeleons (Chamaeleonidae) and anoles (Iguanidae), has shown that regional growth of caudal vertebrae reflects an organism's ecological and functional needs, ranging from habitat and primary mode of locomotion to specializations such as sexual dimorphism and tail autotomy (Bergmann and Russell, 2001; Bergmann et al., 2003; Bergmann et al. 2004). While our study did not set out to correlate ecological and functional variables with regional growth of caudal vertebrae, we documented some patterns. One finding is that individual caudal vertebrae lengthen over time in all species examined. Specific regional patterning, such as the shortened second and third caudosacral vertebrae in adult *E. cirrigera* develop after metamorphosis (Fig. 6). Gorman (1998) described a different pattern for the salamander *Desmognathus monticola*, where centrum length increased between the sacrum and the third caudosacral vertebra before tapering towards tail.

A second finding is that caudal vertebrae in larval *E. cirrigera* and adult paedomorphs (*E. nana* and *A. mexicanum*) taper quite uniformly from the base to the tip of the tail. A similar pattern has been observed in the adult tails of several *Ambystoma* species with biphasic life cycles (Worthington and Wake, 1972; Babcock and Blais, 2001 – *A. jeffersonianum*), and in *Siren intermedia* (Gillis, 1997). In contrast, the onset of postsacral tapering in adult *Eurycea* with biphasic life cycles is shifted from the base towards the posterior one-half to one-third of the tail. This pattern resembles that shown for the biphasic *Hemidactylium scutatum* (Vaglia et al., 1997) and *Desmognathus ocoee* (Babcock and Blais, 2001.

Tapering centrum lengths are associated with propogating waves along the laterally undulating tail in aquatic environments (Worthington and Wake, 1972; Gillis, 1997). Thus, the presence of such a pattern is consistent with the functional needs of aquatic larvae, and paedomorphs that occupy an aquatic environment throughout their life. At this time it is difficult to speculate as to why *Ambystoma* species with biphasic life cycles would exhibit larval centrum length patterning as adults. Additional insight might be gained from a more thorough investigation across species within families, as well as amongst species with differing life cycles.

Morphology supports post-embryonic addition of caudal vertebrae

Along with SVL/TL ratios and caudal vertebral counts, morphology of post-metamorphic caudal segments provides further confirmation that the posterior tail is a region of ongoing development and growth. The pre- to post-metamorphic transition in caudal vertebral ontogeny has been described for a few salamander species (Worthington, 1971; Babcock and Blais, 2001). Some of the key events involved in the metamorphic transition include ossification of centra, neural and haemal arches, formation of neural and haemal keels and formation and elaboration of transverse processes. The timing of these events varies with species, and also along the length of the tail. We found in both *Eurcyea* and *Ambystoma* species that the posterior tail is a region of 'ongoing ontogeny.' At the most extreme, terminal vertebrae in adult individuals are visible as small, rectangular ossifications. More anteriorly positioned vertebrae are larger, more completely ossified and have neural and haemal arches and transverse processes at various states of development.

While the morphology of terminal caudal vertebrae provides good evidence that new, immature vertebrae are developed and continue to develop throughout the salamander life cycle, it also suggests that the pre- and post-embryonic processes of developing vertebrae are different. The posterior tails of adult *Eurcyea* do not reveal an obvious cartilaginous rod, although vertebrae still appear to ossify around a central tail structure. Histology and immuno-histochemical work on the posterior salamander tail are in process to help us decipher the cellular origins of new vertebrae that form in the absence of embryonic somites. We also hope to reveal how this process might differ amongst species.

The ability to add vertebrae forever…

Elongation is typically measured as the ratio of body length to body width or depth, or head length (Jockusch, 1997); thus, evolution of elongate body forms should not be confused with the ability to continually add caudal vertebrae. Organisms with elongate bodies are often associated with particular habitats and locomotor functional needs (e.g. lizards - Greer, 1987; Griffith, 1990; Caputo et al., 1995; fishes - Lindsey, 1975; Swain 1992a, b; snakes – Arnold and Bennett, 1988; Garland, 1988). The evolution of elongate body forms reflects changes that occur in the trunk and appendages, namely the addition of vertebrae, and reduction of digits and/or limbs (Lande, 1978; Greer, 1987; Cohn and Tickle, 1999; Caldwell, 2003; Woltering et al., 2009). Changes to the tail would not be expected to produce a radically different looking organism. However, because of the variety of functions tails exhibit (from fat storage to swimming to autotomy), and its ability to continually add vertebrae across a lifespan, the tail is perhaps the most evolutionarily labile portion of the body axis. Additional research is needed to decipher the mechanism(s) underlying ongoing production of vertebrae in species exhibiting different life cycles from direct developing to paedomorphic. Such mechanisms could involve processes that are retained from early development, 'remodeled' from early development and/or are altered completed.

Segmentation, or somitogenesis, in the developing embryo has been described as a 'clock,' – a process in which groups of molecular signals are expressed at regular time intervals as they contribute to formation of somites, and to subsequent differentiation of those somitic cells (Palmeirim et al., 1997; Dubrulle and Pourquié, 2004; Gomez et al., 2008; Vonk and Richardson, 2008). A change in the number of paired somites that forms during embryogenesis affects the resulting number of vertebrae, and often, though not always, the post-embryonic body size/length. There are of course exceptions, such as giant taxa that have fewer vertebrae than expected for their size. These exceptions remind us to consider the other developmental processes at work, such as growth (Head & Polly, 2007). In tails that add vertebrae post-embryonically, key developmental players, the somites, are not present; thus, populations of cells in the vicinity of the tail tip must retain some embryonic cell potential qualities. Tam and Tan (1992) demonstrated that when the tail bud of a 13.5-day mouse embryo is transplanted into an 8.5-day embryo, the cells participate in somitogenesis longer than would be expected for their developmental age. This suggests that the cells are able to alter their function as influenced by their environment. It is possible that cells in the tail tips of adult salamander continually produce embryonic signals that maintain the cells in a dedifferentiated state until needed to form the next vertebrae and associated tissues. We also hypothesize a developmental link between the ability to continually add

vertebrae and the ability to regenerate portions of lost tail or limbs in salamanders. Understanding how caudal vertebrae continue to develop in an adult organism has great significance for understanding regeneration and potential links between the two processes. We also can imagine constructing models that demonstrate how changes in caudal vertebral number, growth and timing of addition create possibilities for evolving different vertebrate forms as related to the posterior body.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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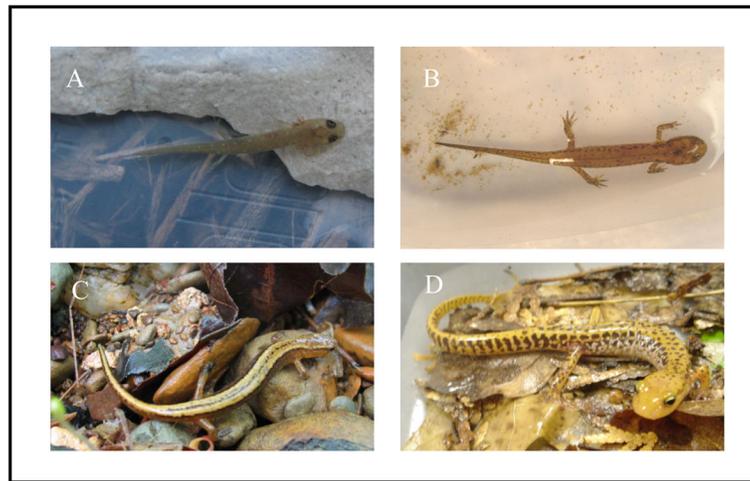
Fig. 1.
Larval and adult forms of two *Eurycea* species. **A, B.** Larvae, *E. cirrigera* (A); *E. longicauda* (B). – **C, D**. Adults, *E. cirrigera* (C), *E. longicauda* (D).
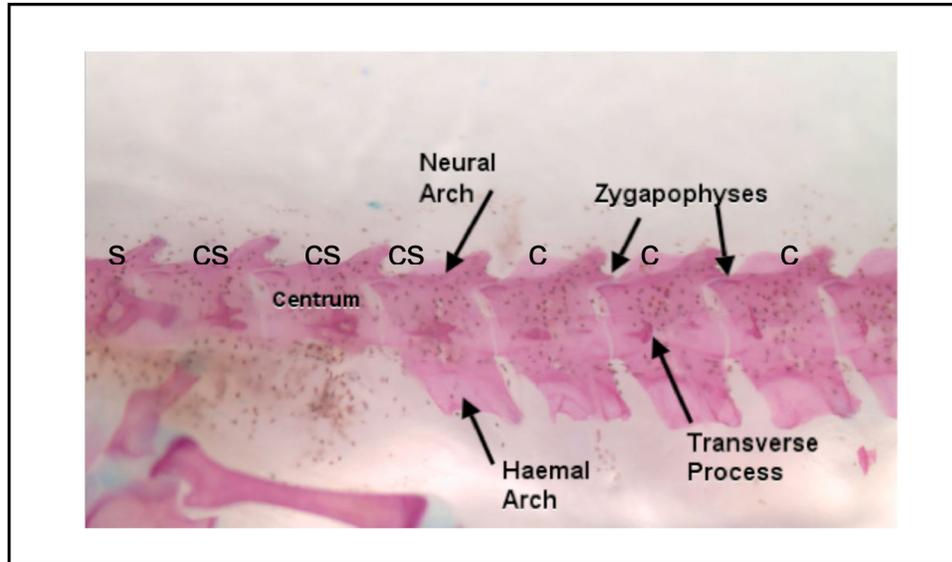
Fig. 2.
An example of cleared and stained caudal vertebrae in an adult *E. cirrigera*. Anterior (head) is to the left. Lateral view of the tail, including the sacrum (S), caudosacral (CS) and caudal (C) vertebrae. The various morphological landmarks indicated ossify and become more complex as individuals increase in age and size.
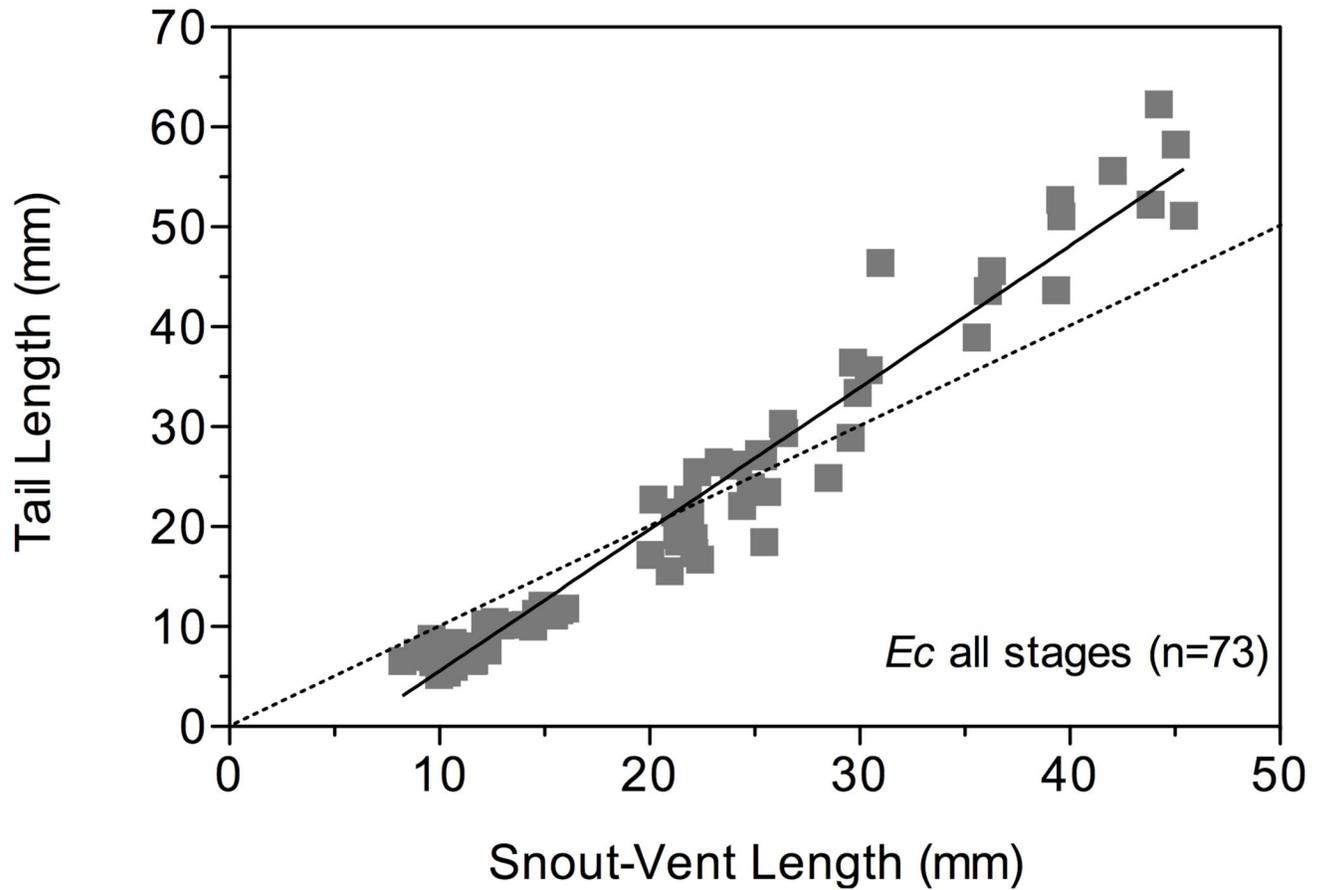
Fig. 3.
Growth relation of snout-vent length to tail length across all life stages of *Eurycea cirrigera* (*Ec*). Hatchlings (n=5), small larvae (n= 27), large larvae (n= 15), metamorphs and juveniles (n=14) and adults (n=12) are represented. The relationship across stages is linear (p < 0.001; $r^2 = 0.954$; slope = 1.345 to 1.493). Hatchlings are outliers in these data and account for the range in slope. Dotted line = line of isometry, representing a 1:1 ratio of tail length to snout-vent length.
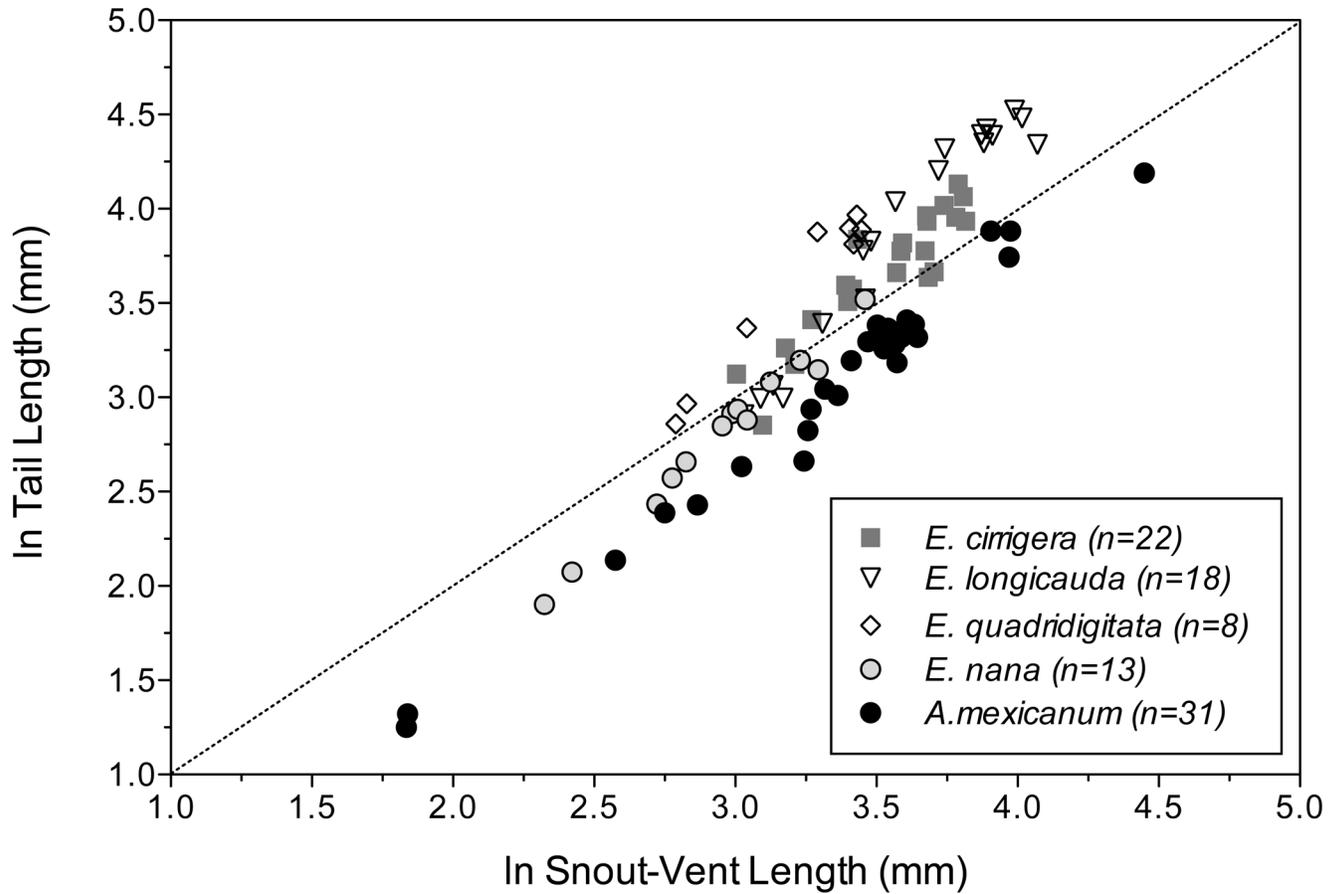
Fig. 4.
Least squares regressions of tail length (TL) to snout-vent length (SVL) for juvenile-adult *Eurcyea* and *Ambystoma*. Slopes and r^2 values are shown in Table 2. Dotted line = line of isometry, representing a 1:1 ratio of tail length to snout-vent length. Axes are logarithmically transformed.
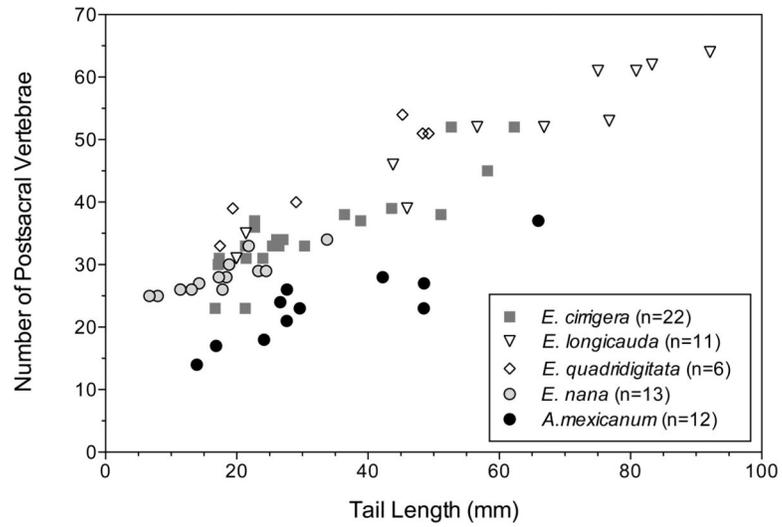
Fig. 5.
The number of caudal vertebrae increases as the tail elongates. Least squares regressions of number of postsacral vertebrae to tail length (TL) for juvenile – adult *Eurcyea* and *Ambystoma*. Slopes and r^2 values are shown in Table 2.
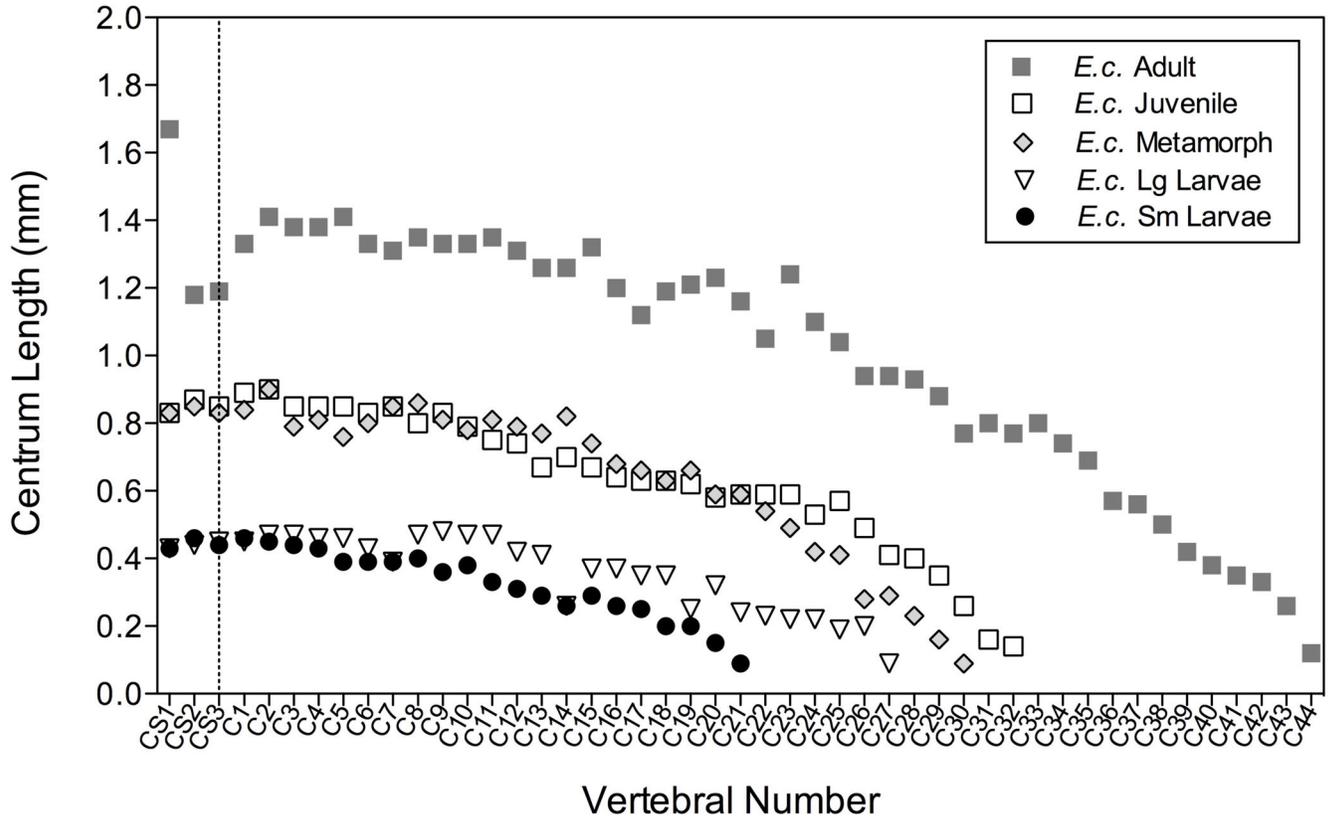
Fig. 6.
Centrum lengths (mm) of caudosacral (CS), and caudal (C) vertebrae in *Eurycea cirrigera* at different life stages. The centrum length profile of one individual is shown for each life stage. Snout-vent and tail lengths are as follows: adult (SVL – 39.55 mm; TL – 52.70), juvenile (SVL – 24.0 mm; TL – 26.08 mm), metamorph (SVL – 23.3 mm; TL – 26.43 mm), large larvae (SVL – 20.04 mm; TL – 17.16 mm), and small larvae (SVL – 14.46 mm; TL – 9.98 mm). The dotted line at CS3 indicates a large transition in centrum length between the posterior caudosacral vertebrae and most anterior caudal vertebrae. This pattern was present in all adults examined (data not shown).
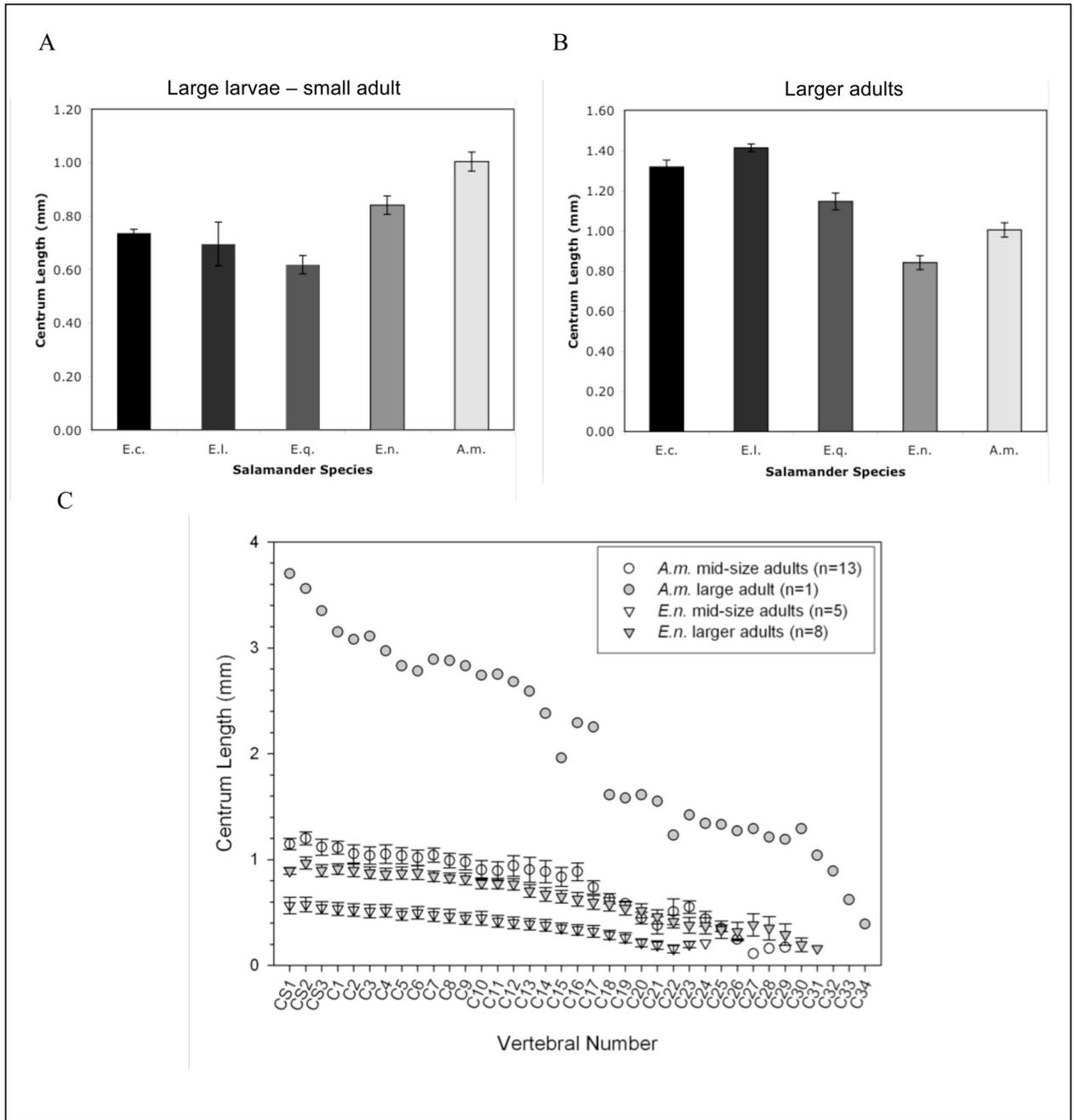
Fig. 7.
Vertebral centrum lengths vary across species and over time. **A & B.** – Average centrum length for vertebrae located in the region of caudal vertebrae one – twelve (C1 – C12). **(A)** Large larvae, transitioning, juvenile and very young adult stages of the biphasic species investigated. The approximate range of tail lengths are as follows: *E.c.* (17 – 27 mm); *E.l.* (20 – 22 mm); *E.q.* (17 – 29 mm); *E.n.* (17 - 34 mm); *A.m.* (24 – 49 mm). **(B)** Larger adults of the biphasic species. The approximate range of tail lengths are: *E.c.* (39 – 62 mm); *E.l.* (44 – 88 mm); *E.q.* (45 – 53 mm); *E.n.* (17 – 34 mm); *A.m.* (24 – 49 mm). Note that the range of tail lengths is identical in both graphs for the paedomorphic species. **C.** – Comparison of centrum length profiles of two different size classes in the paedomorphic species, *E. nana* and *A. mexicanum*. These profiles reveal how the vertebral centra

lengthen over time. The range of tail lengths are as follows: *E.n.* mid-size (7 – 14 mm; SVL:TL ratio – 1.18 – 1.52); *E.n.* larger (17 – 34 mm; SVL:TL ratio – .94 – 1.17); *A.m.* mid-size (24 – 49 mm; SVL:TL ratio – 1.47 – 1.02); *A.m.* large (66 mm; SVL:TL ratio – 1.30).
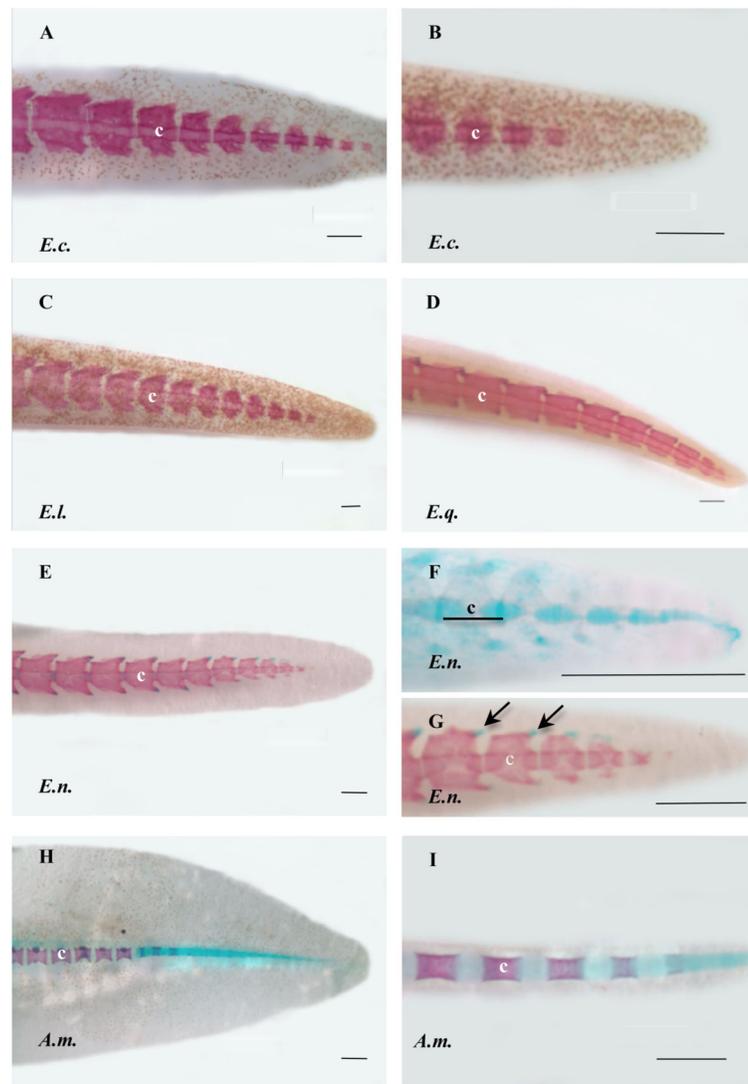
Fig. 8.

Lateral views of the morphology of caudal vertebrae in the posterior tails of adult salamanders. Anterior (head) is to the left in all images. Abbreviations in lower left hand corner of each image indicate species name, as used throughout the manuscript. Lowercase 'c' indicates the vertebral centrum, and an example is provided in each image for orientation. – **A, B.** In these lower and higher magnified views of an *E. cirrigera* tail, what is most notable is the lack of complex features found in the most posterior vertebrae of post-metamorphic, elongating tails. Evidence of such newly developing vertebrae is found in even the oldest adults of all species investigated. – **C.** *E. longicauda* caudal vertebrae exhibit similar morpohology to those of *E. cirrigera*; whereas, the vertebrae in *E. quadridigitata* exhibit some differences, such as neural and haemal arches that are oriented more horizontally (**D**). – **E–G.** While the morphology of *E. nana* caudal vertebrae most resemble those of *cirrigera* and *longicauda*, the cartilaginous posterior zygaphophyses are much more visible (arrows in **G**, the magnified version of **E**). The cartilaginous larval tail (**F**) also clearly shows how cartilage formation initiates in the anterior and posterior portions of what will become one vertebral centrum. Ossification will also occur first in these regions. – **H, I.** Caudal vertebral morphology in *A. mexicanum* differs widely from the *Eurcyea* species. Little to no elaboration of the vertebral bodies are apparent, even in the oldest adult salamander. Furthermore, a long cartilaginous rod is retained in the tail tip.

Table 1

Salamander groups/species known to exhibit ongoing addition of postsacral vertebrae

Family	Species	Life Cycle	Citations
Ambystomatidae	*Ambystoma gracile*	Biphasic	Lindsey, 1966
	A. jeffersonianum	Biphasic	Babcock & Blais, 2001
	A. mexicanum	Paedomorphic	Vaglia, current study
	A. opacum	Biphasic	Worthington, 1968, 1971
Plethodontidae	*Aneides ferreus*	Direct	Dickie, 1999
	A. flavipunctatus	Direct	Dickie, 1999
	A. lugubris	Direct	Wake, 1963; Wake et al., 1983
	Batrachoseps attenuatus	Direct	Noble, 1931; Jockusch, 1997; Dickie, 1999
	Desmognathus ocoee	Biphasic	Babcock & Blais, 2001
	Eurcyea bislineata	Biphasic	Wake & Lawson, 1973
	E. cirrigera	Biphasic	Vaglia, current study
	E. longicauda	Biphasic	Vaglia, current study
	E. nana	Paedomorphic	Vaglia, current study
	E. quadridigitata	Biphasic	Vaglia, current study
	Hemidactylium scutatum	Biphasic	Vaglia et al., 1997
	Oedipina	Direct	Wake & Lynch, 1976

Table 2

Results of linear regression analyses for interspecies comparison of ln snout-vent length vs. ln tail length, and tail length vs. number of postsacral vertebrae. Results of t-test pairwise comparisons using slope values are shown only for the pairing of all *Eurcyea* species vs. *A. mexicanum*.

Species (lg larvae -adult)	N	Slope	SE	Y-Int.	SE	R^2	F		Tcalc	Df	Tcrit
SVL vs. TL											
E. cirrigera	22	1.250	0.120	-0.735	0.424	0.844	108.3				
E. longicauda	18	1.656	0.097	-2.075	0.352	0.948	289.2				
E. quadridigitata	8	1.592	0.124	-1.523	0.400	0.965	163.7				
E. nana	13	1.381	0.049	-1.273	0.146	0.986	782.2				
A. mexicanum	31	1.179	0.031	-0.895	0.107	0.980	1411		Tcalc	Df	Tcrit
All *Euryea*	61	1.479	0.059	-1.466	0.201	0.914	624.4		4.501	88	1.987 p< 0 001
TL vs. Postsacral											
E. cirrigera	22	0.454	0.058	21.08	1.98	0.754	61.13				
E. longicauda	11	0.447	0.041	23.62	2.67	0.929	117.1				
E. quadridigitata	6	0.552	0.085	25.47	3.18	0.913	41.77				
E. nana	13	0.341	0.057	22.14	1.09	0.764	35.58				
A. mexicanum	12	0.353	0.058	11.50	2.120	0.785	36.52		Tcalc	Df	Tcrit
All *Eurycea*	52	0.489	0.026	20.99	1.042	0.878	358.8		2.14	60	2.000 p < 0.0 5