PERSPECTIVE

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Paedomorphic salamanders are larval in form and patterns of limb emergence inform life cycle evolution

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Abstract

Amphibians undergo a variety of post-embryonic transitions (PETr) that are partly governed by thyroid hormone (TH). Transformation into a terrestrial form follows an aquatic larval stage (*biphasic*) or precedes hatching (*direct development*). Some salamanders maintain larval characteristics and an aquatic lifestyle into adulthood (*paedomorphosis*), which obscures the conclusion of their larval period. Paedomorphic axolotls exhibit elevated TH during early development that is concomitant with transcriptional reprogramming and limb emergence. A recent perspective suggested this cryptic TH-based PETr is uncoupled from metamorphosis in paedomorphs and concludes the larval period. This led to their question: *"Are paedomorphs actual larvae?"*. To clarify, paedomorphs are only considered larval in form, even though they possess some actual larval characteristics. However, we strongly agree that events during larval development inform amphibian life cycle evolution. We build upon their perspective by considering the evolution of limb emergence and metamorphosis. Limbless hatchling larval salamanders are generally associated with ponds, while limbed larvae are common to streams and preceded the evolution of direct development. Permian amphibians had limbed larvae, so their PETr was likely uncoupled from metamorphosis, equivalent to most extant biphasic and paedomorphic salamanders. Coupling of these events was likely derived in frogs and direct developing salamanders.

KEYWORDS

Direct development, paedomorphosis, limb emergence, Caudata, life cycle evolution, high thyroid period, larva, developmental coupling, developmental consolidation

1 | INTRODUCTION

The term "larva" is applied across the animal kingdom. It often refers to an early life cycle stage that is followed by a dramatic anatomical metamorphosis and concomitant ecological transition.[1,2] Most amphibians have a *biphasic* life cycle with aquatic larvae that metamorphose into more terrestrial adults.[3,4] Characteristics of the amphibian aquatic larval stage include: a larval-shaped tongue skeleton and gill arches (hyobranchial apparatus), an incompletely developed skull, aquatic sensory systems, larval skin, absence of eyelids, presence of a tailfin and sometimes external gills.[5] Metamorphosis of lissamphibians generally involves the simultaneous transformation to more terrestrial characteristics and is recognized as the conclusion of the larval period for biphasic species.[2,5]

Many lineages have deviated from the biphasic life cycle through shifts in the timing of metamorphosis.[1,3-11] At one extreme, *direct developers* finish transformation

before hatching, permitting a completely terrestrial life cycle.[12] To the opposite extreme, is the phenomenon of *larval form paedomorphosis*, often understated as simply *paedomorphosis*.[13-16] This is when aquatic larval traits and ecology are maintained into adulthood. Larval form paedomorphosis occurs in nine of the ten salamander families,[3-5,17] and is expressed as an alternative life cycle mode to metamorphosis (biphasic) in some species.[18,19] The primary characteristic used to classify larval form paedomorphs is their larval-shaped hyobranchial apparatus, which is adapted for aquatic living. The tongue elements of this apparatus are arranged for gape-and-suction feeding underwater, and the internal gill arches support aquatic respiration as well as feeding.[5,20,21] The most highly paedomorphic salamanders also maintain additional larval characteristics including a tailfin, external gills, larval skin, lateral line, larval-skull morphology, and a lack of eyelids. Paedomorphs can also be *differentially metamorphic* where some tissues transform while others remain in the larval form.[16,22] For example, salamanders of the paedomorphic families Amphiumidae and Cryptobranchidae maintain many ancestrally larval characteristics, but resorb their external gills.

The development and activation of the hypothalamic-pituitary-thyroid (HPT) axis appears to mirror amphibian life cycle variation.[11,17,23-29] Alterations to a systemic regulator like thyroid hormone (TH) can simultaneously shift the developmental timing of many traits.[11,23-27] A reduction in TH release or TH sensitivity can result in the juvenilization of a broad array of otherwise TH transformable structures. A recent study showed that the tailfins of paedomorphic axolotls (*Ambystoma mexicanum*) changed their transcriptional responses to TH during the larval period.[30] That is, they found several genes were differentially regulated by TH in the tailfin of different larval "classes" despite the tailfin being maintained. This finding complemented decades old evidence for a High Thyroid Period (HTP) during the early part of the larval phase of axolotls that is coincident with a pulse of neuron proliferation and the emergence of limbs.[30,31] In a follow up perspective, the HTP and transcriptional reprogramming of the tailfin in larval axolotls were interpreted as a morphologically cryptic post-embryonic transition (PETr).[32] Under this model, larval form paedomorphs could be derived by uncoupling the TH-based PETr (including limb emergence) from metamorphosis. In contrast, the ontogenetically close timing of these developmental events was interpreted as "coupled" in all frogs, and direct developing and biphasic salamanders.[32] Finally, they posed the question, if the PETr marks the end of the larval period, "*are paedomorphs actual larvae*?".[32]

We strongly agree that attention to events during the larval period, and patterns of ontogeny in general, are important for understanding life cycle evolution. We build upon their perspective by reconstructing the evolution of limb emergence and metamorphosis with respect to hatching. We further examine the evolution of the coincidence ("coupling"[32]) of these events during development. Given the diversity in timing of limb development and metamorphosis in salamanders, we plot both events with respect to putative levels of TH. Lastly, we discuss what defines a salamander larva and how this relates to larval form paedomorphosis, which is not necessarily straightforward.

2 | EVOLUTION OF LIMB EMERGENCE

If limb emergence and metamorphosis represent TH induced transformations,[32] then how have the timing of these events evolved? Here we reconstruct the evolution of the timing of limb emergence relative to hatching in salamanders. The analyses reveal phylogenetically structured variation in salamander limb emergence, and provide insight into the evolution of life cycles, especially direct development.

In salamanders, the timing of limb emergence with respect to hatching varies extensively (Figure 1). Limbless hatchlings are relatively uncommon. This pattern primarily occurs in pond breeding ambystomatids and hynobiids.[34] Some salamandrids, plethodontids, and proteids are also limbless at hatching. In salamanders, forelimbs either emerge first or simultaneous with the hindlimbs.[34,35] When not emerging simultaneously, hindlimb development lags behind forelimb development to varying degrees. A greater lag between forelimb and hindlimb development has also been noted for pond-dwelling larvae compared to stream-dwelling larvae and direct developers.[34] Hatchlings with forelimbs already carrying at least two digits are common among salamandrids, spelerpine plethodontids, some proteids, sirenids and cryptobranchids. Dicamptodontids, a few salamandrids, most biphasic plethodontids, amphiumids, and rhyacotritonids develop both pairs of limbs before hatching. In general, stream-dwelling larvae hatch with at least front limbs or all four limbs.[34] Limbed hatchlings may be necessary in a stream setting for attachment, bottom walking, and climbing, whereas pond-dwelling larvae mostly locomote by undulating their broad tail.[34-36]

All direct developers are even further developed at hatching, as they have all four well-developed limbs and are already transformed (metamorphosed) into a terrestrial form. The largest and most diverse family of
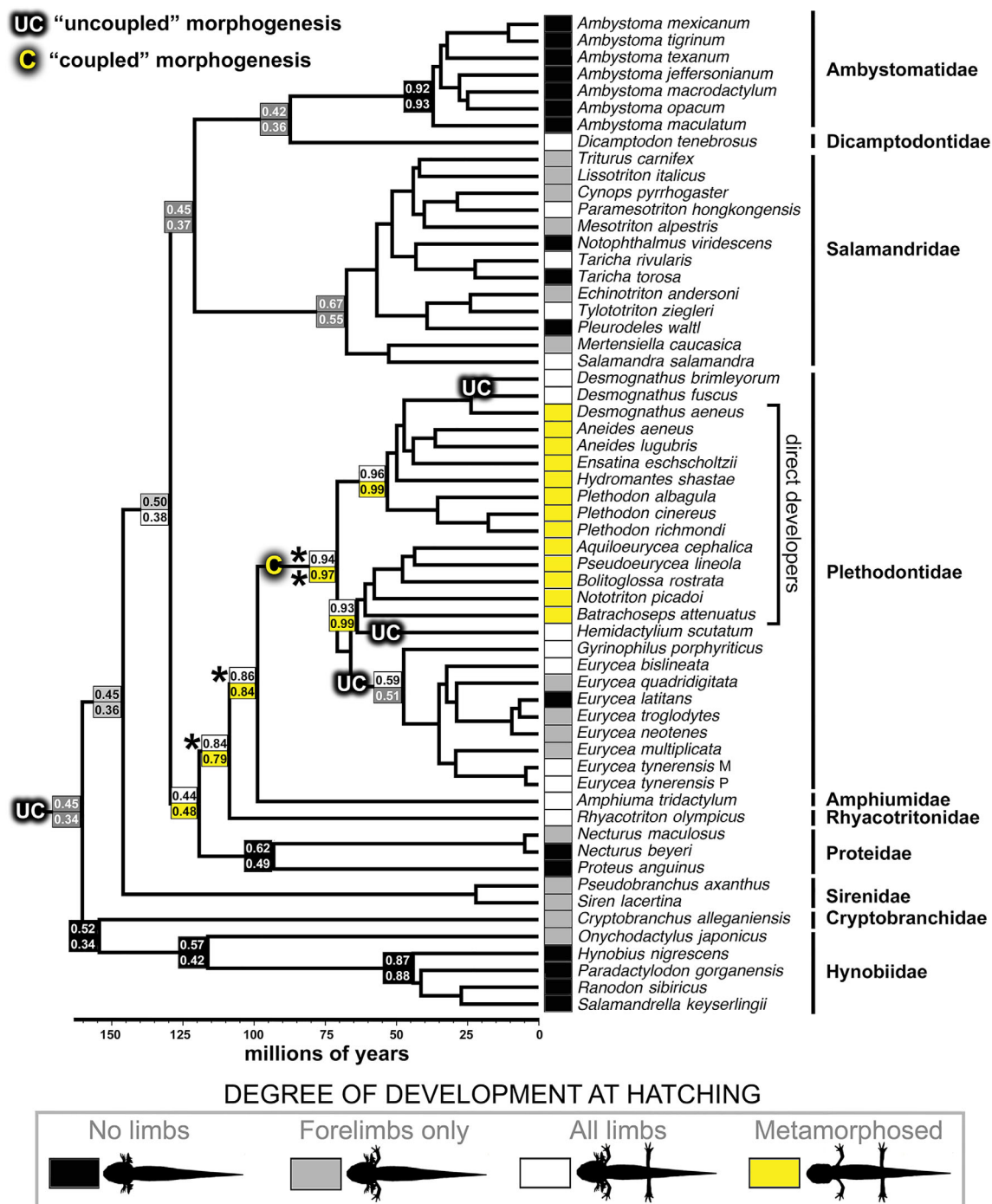
FIGURE 1 Phylogeny of salamanders with ancestral reconstructions of the degree of development at hatching. We considered four degrees of development at hatching: (1) No limbs (black); (2) Forelimbs only (gray); (3) All limbs (white); and (4) Metamorphosed (yellow, direct development). The phylogeny is a consensus tree pruned from Bonett and Blair.[4] Multistate Bayesian ancestral state reconstruction was performed in BayesTraits 3.0.5.[33] Numbers subtending major nodes are the highest proportional probability for the ancestral state reflected by the color of the box. Numbers and boxes above branches are from a reconstruction considering only the first three developmental degrees at hatching (No limbs, Forelimbs only, and All limbs), below the branch includes all four degrees at hatching (i.e., the addition of metamorphosis before hatching in the direct developers). Asterisks indicate when there is evidence that the degree of development at hatching with the highest proportional probability is significantly better than all alternatives. This was tested for major nodes leading to the evolution of direct development in plethodontid salamanders (See Appendix S1 for details). Ancestral transitions between "uncoupled" (UC) and "coupled" (C) morphogenesis of limbs and metamorphosis[32] are also shown. Limbs and metamorphosis were likely uncoupled in ancestral salamanders, became coupled during the evolution of direct development in plethodontids, and uncoupled (decoupled) again in three lineages of biphasic plethodontids

salamanders (Plethodontidae) includes hundreds of direct developing species.[16] The ancestral life cycle mode has been reconstructed as direct development, with at least three major reversals to biphasy.[4,15] This makes the larvae of biphasic plethodontids unusual compared to other larval salamanders.[37] The fact that hatchlings of most biphasic plethodontids already have four limbs (*Desmognathus*, *Hemidactylium*, *Gyrinophilus*, and many *Eurycea*) is additional evidence that they are derived from a direct developing ancestor that would have also had four well developed limbs at hatching. Furthermore, there was likely a shift to prehatching limb emergence in the most recent biphasic ancestors of direct developing plethodontids. This includes the common ancestors of rhyacotritonids, amphiumids and plethodontids, and of amphiumids and plethodontids. This suggests that there was likely a deep history of early limb development that preceded the evolution of direct development in plethodontids (Figure 1). It should also be acknowledged that the timing and morphology of the embryo at hatching can be somewhat altered by conditions such as temperature and moisture.[38-40] A more comprehensive set of larval stages scored from species raised under the same conditions would be ideal for further assessing the evolution of timing for early developmental events.

3 | EVOLUTION OF THE PETR AND METAMORPHOSIS IN AMPHIBIANS

Evidence from paedomorphic axolotls shows that emergence of both sets of limbs is more-or-less coincident with a broad increase in TH,[31] and TH inducible reprogramming of the tailfin.[30] These events were considered to be a major PETr that is uncoupled from metamorphosis, which does not naturally happen in the paedomorphic axolotl.[32] In contrast, limb emergence and metamorphosis were interpreted as coupled due to their close ontogenetic proximity in all frogs, and direct developing and biphasic salamanders.[32] Assuming that the independent appearance of limbs and metamorphosis represent at least two primarily TH induced developmental transitions when uncoupled, we can track these events during the evolution of amphibians.

First, let us consider the evolution of consolidation of developmental events by examining the ontogenies of ancient amphibians. During the early Permian many temnospondyls such as *Sclerocephalus* had more graded development,[41,42] indicating that their morphological transition from larva to adult was not induced by a single spike of a governing morphogen. Branchiosaurs, a group of largely gilled amphibians from the same period,

included the earliest known examples of metamorphosis. Metamorphosed branchiosaurs had consolidated development of the skull, mandible and vertebral column compared to *Sclerocephalus*. These changes, plus "late phase" skull formation and hyobranchial remodeling, were further consolidated during the evolution of metamorphosis of modern biphasic salamanders. Therefore, paleontological evidence suggests that metamorphosis evolved to be more dramatic in crown group amphibians,[42] which fits with models of increasing hormonal control of skeletal development in salamanders and frogs.[43] However, the larvae of branchiosaurs[42] and stem salamanders[44] had relatively well-developed limbs prior to metamorphosis, indicating limb emergence was ancestrally decoupled from metamorphosis.

Most modern biphasic salamanders actually develop their limbs well before metamorphosis, maintaining the ancestral uncoupling of these events (Figures 1 and 2). Uncoupling in biphasics and paedomorphs means the early TH spike that regulates limb development should remain insufficient to induce full transformation.[32] Paedomorphs may only have one early spike to produce limbs, but none later to instigate metamorphosis, unless they facultatively metamorphose.[25,32] The timing of the early spike could be before or after hatching. This has produced hatchlings that range from limbless to having all four limbs (Figure 2). In paedomorphic sirenid salamanders, a potentially early TH increase while still in the egg only produces front limbs.[47] Amphiumids and cryptobranchids may have a second TH increase that induces only a partial metamorphosis. Developmental coupling, or ontogenetic coincidence, of limb emergence with metamorphosis in frogs and direct developing plethodontid salamanders would be derived. An interesting facet of plethodontid salamanders is that there appears to have been up to three reversals to a biphasic life cycle.[4,15,37] If direct development in plethodontids is driven by a continuous TH surge that pushes an embryo through limb development and a full transformation prior to hatching, then this could be reversed ("decoupled") by evolving an insufficiency to induce metamorphosis before hatching (Figure 1).

Assessments of the ontogeny of TH release and TH sensitivity have been limited to relatively few salamander species.[11,24,25,30,48-57] Even though TH can induce metamorphosis of biphasic amphibians, it is indeed necessary, but insufficient, to complete metamorphosis of *Xenopus*.[54] The effects of the hypothalamic-pituitary-interrenal (HPI) axis, especially in conjunction with the HPT axis, are clearly important for anuran metamorphosis.[28,58,59] Evaluation of the molecular endocrine mechanisms of salamanders lags far behind frogs.[25] Technological advances, from hormone assays to

DEGREE OF DEVELOPMENT AT HATCHING
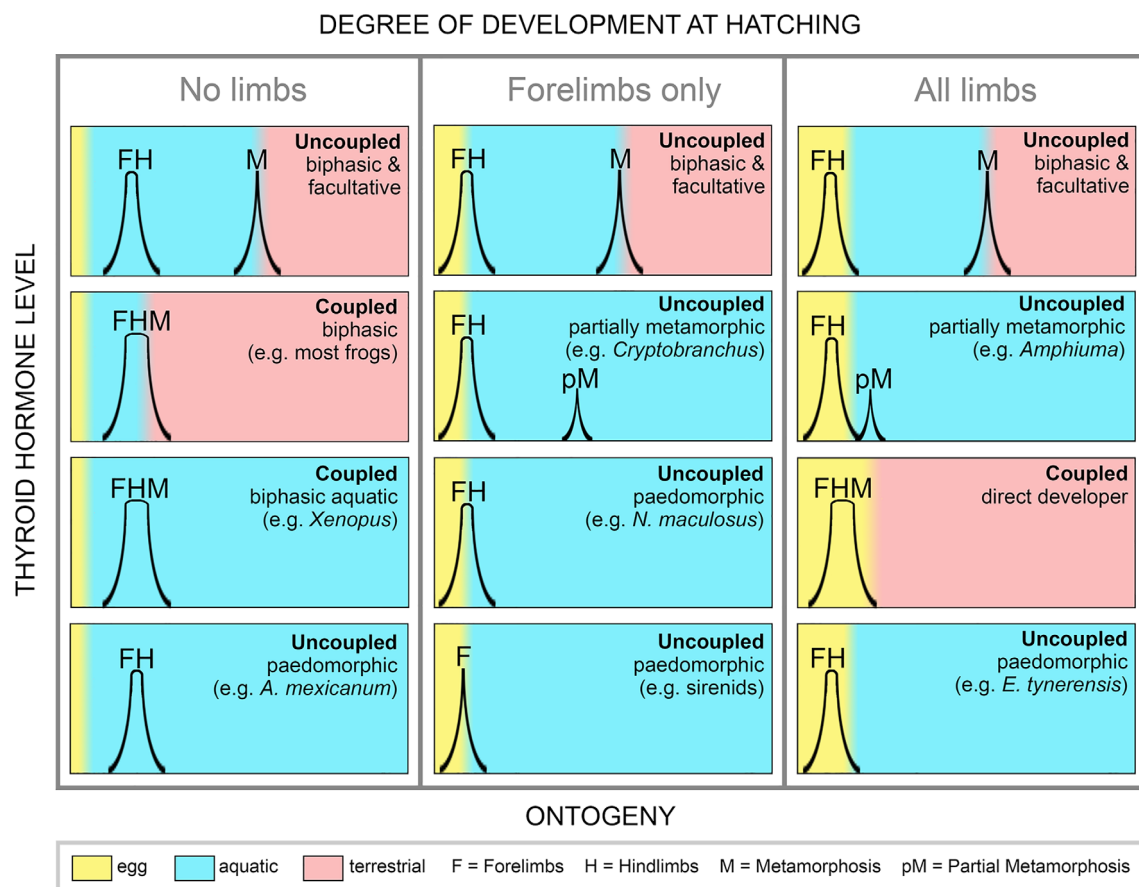
FIGURE 2 Plots depicting the diverse patterns of timing of salamander limb development and metamorphosis with respect to their habitat. Background colors show habitat transitions across ontogeny: egg (yellow); aquatic (blue); terrestrial (red). Letters indicate the timing of forelimb (F) and hindlimb (H) emergence, metamorphosis (M) and partial metamorphosis (pM). Peaks represent hypothetical high thyroid periods (HTP)[31,32] that correspond with limb emergence and metamorphosis. Some proximal morphogenic events, such as the loss of external gills in *Amphiuma*, may be controlled by the same morphogenic surge. We acknowledge that other morphogens may be the primary drivers of these events. "Coupled" and "uncoupled" refer to the occurrence of these morphogenic events (limb emergence and metamorphosis)[32] as simultaneous or not. It is important to distinguish "uncoupling" of developmental events (e.g., limb emergence, metamorphosis, etc.) as described by Tribondeau,[32] vs the "uncoupling" of developmental stages, which is a consequence of metamorphosis[45,46]

transcriptomics, should allow for more depth and accuracy in our assessment of the mechanisms of salamander morphogenesis. Evaluation of potential morphogens across the diverse developmental trajectories of salamanders are needed to understand the underpinnings of ontogenetic evolution.

4 | WHAT IS A LARVAL SALAMANDER?

Beyond a metaphor, to our knowledge, no one considers larval form paedomorphs to be actual larvae. This is because a larva is broadly recognized as a subadult stage.[1,3,5] Rather, larval form paedomorphic salamanders retain some combination of the many larval

characteristics mentioned above, particularly the larval shaped hyobranchial apparatus.[5,21] This structure is present before the PETr and is maintained in both larvae and adults. Therefore, adults maintain larval form characteristics, even if an early PETr is considered the end of the larval period. It has yet to be determined whether the reprogramming discovered in the axolotl tailfin applies to other tissues and species. Even still, some aspects of larval form paedomorphosis involve the absence of traits.[5] For example, all larvae and larval form paedomorphs lack eyelids. This characteristic is truly paedomorphic, as its perpetual absence means that it is not subject to a cryptic PETr. The same could be said for bones such as the maxilla, which are present in all metamorphic adults, but absent in all larvae and most larval form paedomorphs.

If the PETr, signified by limb emergence, is recognized as the end of the larval period,[32] this presents major issues for how salamander life cycles are described. One problem is that most biphasic species develop one or both pairs of limbs before hatching (Figure 1). If limb emergence marks the end of the actual larval period then most biphasic and paedomorphic salamanders would need to be recategorized as direct developers, or at least considered to lack a free-living 'larval' stage.

Another problem is most biphasic salamanders persist as a limbed larval form for an extended duration, sometimes for years before metamorphosis.[15,37,60] If this portion of the life cycle is not part of the larval stage, then what should it be called? This relates to a standing issue. There are already inconsistent criteria used to define the end of the larval period for paedomorphs of different families. For example, the post-hatching stage of paedomorphic ambystomatids and plethodontids is called a larva until it reaches reproductive maturity. In contrast, the hatchlings of large paedomorphic species like sirenids, proteids, and cryptobranchids are considered larval until they begin to resemble adults in overall form, which is well before adulthood.[61] During that intervening period (between "larva" and adult), they are considered juveniles, but not larvae. For sirenids this happens after the trunk further elongates. For proteids it is after the full formation of limbs, loss of yolk, and a shift in color pattern. Cryptobranchids are the most complicated because they fully develop limbs and reabsorb yolk shortly after hatching, but do not lose their external gills until one or two years later.

In practice, the simplest solution is to continue recognizing a biphasic salamander's aquatic larval stage as the period from hatching until a substantial metamorphosis, which includes the transformation of the hyobranchial apparatus. Diverse larval substages can still be recognized during this period.[62] Most paedomorphs should probably be considered larval until maturation, despite potentially early PETrs that induce a variety of morphogenic patterns. The exceptions are amphiumids and cryptobranchids which undergo partial metamorphosis (Figure 2),[16,22] so the end of their larval period would be marked by a loss of external gills. If this solution is unpalatable then one alternative is to refer to individuals after their PETr as *larval form juveniles* until they metamorphose or mature.

In the end, even if a clearly homologous PETr emerges across amphibians, should we call it the end of the larval phase or something else? Since events that mark the PETr, such as limb emergence, often occur within the egg then technically they are primarily embryonic. So, perhaps the PETr should mark the end of the embryonic stage, as the name suggest, rather than the end of the larval stage. Regardless of categories, from an analytical perspective, the most important consideration is to align homologous developmental events when comparing ontogenies. The fact that the developmental patterns of salamanders do not fit into discrete categories is what makes them so interesting!

5 | SUMMARY

Attention to events during larval development can be informative for understanding patterns of life cycle evolution and speculating on developmental mechanisms. The TH-induced PETr that includes the development of limbs was likely uncoupled from metamorphosis in ancestral amphibians, paedomorphs, and most biphasic salamanders. Coupling, or coincidence, of these events is derived in frogs and direct developing salamanders. Limbless larval salamanders, produced from hatching before a PETr, often dwell in lentic habitats. Whereas hatchlings in lotic habitats tend to bare limbs, a pattern that appears to precede the evolution of direct development. More developmental data are needed to understand whether variation in the timing of limb development corresponds to predicted variation in TH release and sensitivity. We suggest that the free-living aquatic larval stage carries its long-standing definition of the period between hatching and metamorphosis of key terrestrial features in biphasic species. This would be consistent with the several thousand studies published that refer to aquatic gilled juvenile salamanders as larvae. If the term larva is restricted to the earliest limbless period then many more salamanders would have to be considered direct developers, or lacking a larval stage. The current definition of the larval period in paedomorphs is inconsistent. Post-hatching, they could be referred to as larvae until they reach maturation, or as larval form juveniles during the intervening period between a PETr (or partial metamorphosis) and maturation. It is important to evaluate the degree of conservation of morphogenic drivers and developmental changes during the PETr, an event that has been understudied compared to metamorphosis of amphibians. The nuances of salamander ontogenies make them difficult to categorize, but interesting to study.

AUTHOR CONTRIBUTIONS

Ronald M Bonett: Conceptualization (equal); data curation (lead); formal analysis (lead); visualization (lead); writing – original draft (lead); writing – review and editing (equal). **Nicholus M Ledbetter:** Conceptualization (equal); writing – review and editing (equal).

CONFLICT OF INTEREST

The authors declare no conflicts of interests with regards to this perspective.

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SUPPORTING INFORMATION

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