

Terrestriality constrains salamander limb diversification: Implications for the evolution of pentadactyly

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Abstract

Patterns of phenotypic evolution can abruptly shift as species move between adaptive zones. Extant salamanders display three distinct life cycle strategies that range from aquatic to terrestrial (biphasic), to fully aquatic (paedomorphic) and to fully terrestrial (direct development). Life cycle variation is associated with changes in body form such as loss of digits, limb reduction or body elongation. However, the relationships among these traits and life cycle strategy remain unresolved. Here, we use a Bayesian modelling approach to test whether life cycle transitions by salamanders have influenced rates, optima and integration of primary locomotory structures (limbs and trunk). We show that paedomorphic salamanders have elevated rates of limb evolution with optima shifted towards smaller size and fewer digits compared to all other salamanders. Rate of hindlimb digit evolution is shown to decrease in a gradient as life cycles become more terrestrial. Paedomorphs have a higher correlation between hindlimb digit loss and increases in vertebral number, as well as reduced correlations between limb lengths. Our results support the idea that terrestrial planigrade locomotion constrains limb evolution and, when lifted, leads to higher rates of trait diversification and shifts in optima and integration. The basic tetrapod body form of most salamanders and the independent losses of terrestrial life stages provide an important framework for understanding the evolutionary and developmental mechanisms behind major shifts in ecological zones as seen among early tetrapods during their transition from water to land.

KEY WORDS

body form, Caudata, constraint, digit loss, elongation, integration, paedomorphosis, phenotypic evolution, tetrapod evolution

1 | INTRODUCTION

Phenotypic optima can shift in response to selection, but evolutionary trajectories can be limited by underlying constraints (Arnold, 1992; Futuyma, 2010). Integration between traits can result from shared genetic, functional or developmental mechanisms that produce correlations between traits (Pigliucci, 2003). Patterns of trait evolution and integration often vary as lineages move between

adaptive zones, which can impact the strength of trait correlations, variances or optima (Revell & Collar, 2009). Organisms with complex life cycles, such as many insects and amphibians, transition through multiple environments across ontogeny (Moran, 1994; Sherratt, Vidal-García, Anstis, & Keogh, 2017; Wilbur, 1980). For some traits, this can produce developmental constraints that force a phenotypic trade-off between life stages. However, ecological and developmental constraints across ontogeny can be lifted through metamorphosis

(Moran, 1994; Wilbur, 1980) or by losing life cycle stages (Bonett & Blair, 2017; Bonett, Phillips, Ledbetter, Martin, & Lehman, 2018). Shifts in life cycle complexity should also impact the relationships among traits, but few studies have addressed this question in a phylogenetic context.

The tetrapod limb is a classic model of evolutionary and developmental biology (Alberch & Gale, 1983, 1985; Alberch, Gould, Oster, & Wake, 1979; Coates, 1994; Coates & Clack, 1990; Shubin, Daeschler, & Coates, 2004; Shubin, Daeschler, & Jenkins, 2006; Shubin, Tabin, & Carroll, 1997; Shubin & Wake, 1995). Early tetrapods such as *Ichthyostega* and *Acanthostega* had limbs with seven or more digits that were reduced to five after a likely shift to a fully terrestrial lifestyle (Coates & Clack, 1990). Pentadactyl limbs may represent the optimal configuration for both stability and flexibility necessary during plantigrade locomotion (Clack, 2012). Subsequently, this state has become developmentally canalized and derived increases beyond pentadactyl limbs are rare (Hayashi et al., 2015; Saxena, Towers, & Cooper, 2016), even though numerous tetrapods exhibit loss of digits (Alberch & Gale, 1985; Carroll, 1988). Modern vertebrate analogs have been used to shed light on the functional morphology and evolution of early tetrapods (Ashley-Ross, 1994; Ijspeert, Crespi, Ryczko, & Cabelguen, 2007; King, Shubin, Coates, & Hale, 2011; Pierce, Hutchinson, & Clack, 2013). Among them, most salamanders and many modern "lizard-like" squamates have pentadactyl hindlimbs and locomotor patterns similar to the earliest fully terrestrial crown-group tetrapods, offering a comparative context to test the ecological associations of early limb evolution.

A complicating factor in understanding the evolution of the tetrapod limb is the frequent associations of digit loss and limb reduction with body elongation (Bergmann, 2015; Brändle, Huelsenbeck, & Wiens, 2008; Gans, 1975; Lande, 1978; Wiens & Hoverman, 2008; Wiens & Slingluff, 2001). In squamates alone, this pattern has manifested over 20 times (Wiens, Brändle, & Reeder, 2006) and is associated with multiple locomotory strategies (e.g. burrowing and grass swimming) (Wiens & Slingluff, 2001; Wiens et al., 2006). Salamanders show similar patterns of body form evolution (Wiens & Hoverman, 2008), but are set apart from squamates by the presence of distinct life cycle stages and significant ontogenetic variation among lineages. Strategies range from fully aquatic (paedomorphic) or fully terrestrial (direct developing), to a complex life cycle with an aquatic larval form that metamorphoses into a terrestrial adult (biphasic, also referred to as "metamorphic"). Traits that remain static across life stages (e.g. vertebral and digit numbers) may need to be optimized for performance across disparate environments. Lineages that shift to life cycles with different ecological regimes or fewer stages may be freed from such constraints and exhibit greater rates of phenotypic diversification (Bonett & Blair, 2017) as well as changes in patterns of trait correlations (Tomašević Kolarov, Cvijanović, Denoël, & Ivanović, 2017). Alternatively, the increased demands of terrestrial locomotion may constrain limb and body form phenotypes and create shifts in trait correlations.

Here, we use salamanders as a model to test for the effect of shifting selective regime on patterns of tetrapod limb evolution. Specifically, we use comparisons of evolutionary rate and optima

(phylogenetic mean) to test for altered patterns of constraint and limb reduction. Constraint can be tested through comparisons of evolutionary rates among lineages, where groups with relatively low rates of phenotypic evolution are considered to be subject to higher levels of constraint (Beaulieu, Jhuang, Boettiger, & O'Meara, 2012; Oufiero & Gartner, 2014). If terrestriality or life cycle complexity imposes a constraint on limb evolution, then this should manifest in higher rates of evolution in salamanders with an aquatic adult stage or a simple life cycle. We also test for shifts in integration of limbs and body form, and serial homology between forelimbs and hindlimbs in salamanders by comparing relative evolutionary correlations. Regimes with relatively higher evolutionary correlations among traits were considered more integrated. This study provides an example of how shifts in life cycle and ecology can impact multivariate trait space and demonstrates the utility of clades with multiple life cycle transitions to test for evolutionary constraint.

2 | MATERIALS AND METHODS

2.1 | Morphometric and phylogenetic data

Morphometric data used in all analyses were derived from the data sets of Wiens and Hoverman (2008) and Bonett and Blair (2017). The morphological data set contained 199 taxa for which both limb lengths and digit numbers were available and 156 taxa with data on number of trunk vertebrae. This included 20 taxa that deviate from ancestral digit numbers of four toes on forelimbs and five toes on hindlimbs. The traits used in our analyses included forelimb length (FLL), hindlimb length (HLL), number of forelimb digits (FLD), number of hindlimb digits (HLD), number of trunk vertebrae (NoV; between the atlas and the sacrum), head length (HL), body width (BW), trunk of the body (SVL-HL) and trunk elongation (SVL-HL/BW). Limb lengths were divided by body width to control for body size variation. Continuous traits were Log_{10} transformed.

The phylogenies used were a posterior distribution of 1,000 Bayesian trees taken from Bonett and Blair (2017). Tree analyses were based on three mitochondrial genes and four nuclear protein-coding genes. Trait models were tested across 1,000 simmaps simulated over our 1,000 Bayesian trees with unconstrained ancestral nodes (Figure 1). We coded life cycle states following Bonett and Blair (2017): taxa with aquatic larval and terrestrial adult stages were biphasic (bi), taxa that obligately retain larval morphologies and fully aquatic ecologies into adulthood were paedomorphic (pd), and taxa that completely transform in ovum and hatch as fully terrestrial juveniles lacking aquatic larval traits were coded as direct developers (dd). All analyses were conducted in the R v3.5.0 statistical software (R Core Team, 2018).

2.2 | Phenotypic constraint and optima of limb form

We tested rates and optima of limb evolution across three selective regime hypotheses as outlined in Bonett and Blair (2017). The first model, Three Life Cycles, splits all three salamander life cycle strategies (bi vs pd vs dd) into distinct regimes. The Two Life Cycles model

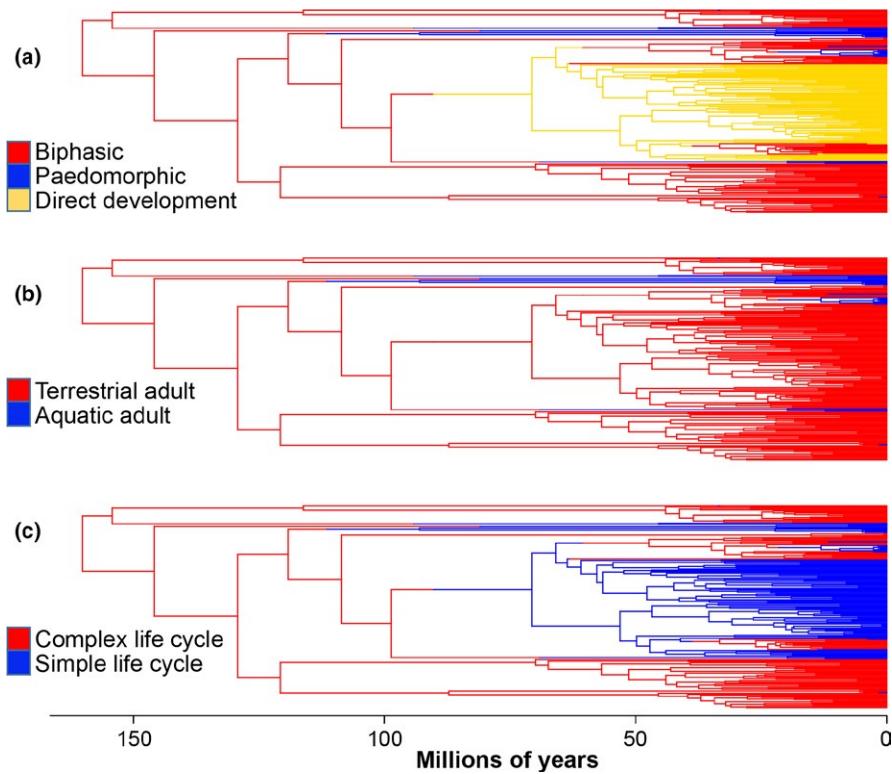


FIGURE 1 Character maps of alternative regime hypotheses. (a) Three Life Cycles regime, (b) Adult Ecology regime (terrestrial adults bi + dd vs aquatic adults pd) and (c) Two Life Cycles regime (complex bi vs simple life cycles pd + dd). The phylogeny is a consensus of the post-burn-in Bayesian cladograms used in comparative analyses from Bonett and Blair (2017)

compares salamanders with simple (pd and dd) vs complex (bi) life cycles. The Adult Ecology model groups species with terrestrial (bi and dd) vs aquatic (pd) adults.

We used the package *OUwie* v1.50 (Beaulieu et al., 2012) to estimate rates of evolution and optima of limb morphologies in order to assess whether shifting between regimes influences phenotypic constraint or evolution. Trait optima (θ) allow us to test for differences in phylogenetic mean among selective regimes. Rates of phenotypic evolution (σ^2) can be used to assess whether regimes have differing levels of constraint. Low rates of evolution may indicate high levels of constraint, whereas shifts to relatively higher rates of evolution suggest release of constraint (Oufiero & Gartner, 2014). The traits modelled were FLL, HLL (both divided by body width), FLD and HLD. We tested the fit of one regime Brownian Motion (BM) and Ornstein–Uhlenbeck (OU) models as well as BM and OU models that allowed trait optima and rate of evolution to vary across regimes ($BM\sigma^2$, $BM\theta\sigma^2$, $OU\theta$ and $OU\theta\sigma^2$).

All evolutionary models were tested across our three selective regime hypotheses, totalling 14 model comparisons per trait (see Supporting Information Tables S1–S4). OU models additionally consider the pull of a trait towards an optimum known as strength of selection (α) (Butler & King, 2004; Cressler, Butler, & King, 2015; Hansen, 1997). However, we did not compare models that vary α across regimes as it often does not improve model selection when α is low (Beaulieu et al., 2012; Kaliontzopoulou & Adams, 2016). Models were compared using delta Akaike information criterion (ΔAIC) and AIC weights (w_i) (Burnham & Anderson, 2002). To validate model fit, we simulated 100 data sets using σ^2 , θ and α parameter estimates from the best-fitting model for each trait using the “*multiOU*” function implemented in the package *phytools* v0.6-44 (Revell, 2012).

The simulated data sets were then fitted to the respective model in *OUwie* v1.50, and the difference in mean parameter estimates was assessed qualitatively.

2.3 | Coevolution between limbs and body form

To test for differences in patterns of integration among selective regimes, we implemented the package *ratematrix* v1.0 (Caetano & Harmon, 2017, 2018), which uses Metropolis–Hastings Markov Chain Monte Carlo (MCMC) to estimate the posterior distribution of evolutionary covariance and correlations fitted to a pool of phylogenetic trees with mapped character states. Regimes that have relatively higher evolutionary correlation between traits were considered to have stronger patterns of integration. We estimated posteriors from five pairwise trait interactions: trunk elongation to both forelimb and hindlimb length, numbers of vertebrae to both numbers of forelimb and hindlimb digits, and forelimb to hindlimb length. We conducted bivariate analyses instead of a model including all traits based on the recommendation by Adams and Collyer (2018), where type 1 error may be inflated when more than two traits are added to a model. Ten short (1 million generations) MCMC chains were run with uninformative priors, and the four chains with the highest likelihood were continued to 4 million generations. The first 50% of iterations were discarded as burn-in, leaving 2 million generations for analyses. We used summary statistics described in Caetano and Harmon (2017, 2018) to infer percent overlap between posterior distributions of trait covariance among regimes. If life cycle regimes had overlap of less than 5% between their covariance posterior distributions, then we considered those life cycles to have differing levels of phenotypic integration between traits. We further described this direction by calculating evolutionary

TABLE 1 Average rate (σ^2) and optima (θ) parameter estimates \pm standard deviation from our best-fitting evolutionary models across 1000 stochastically mapped chronograms. Adult Ecology was the best-fitting model for forelimb digits (FLD) and length (FLL). Three Life Cycles was the best-fitting model for hindlimb digits (HLD) and length (HLL). The three life cycles compared were biphasic (bi), paedomorphic (pd) and direct development (dd)

Trait	bi+dd θ	pd θ	bi+dd σ^2	pd σ^2
Adult ecology				
FLD	$4.00 \pm 4.00e^{-3}$	3.62 ± 0.09	$2.06e^{-9} \pm 2.50e^{-24}$	2.39 ± 0.74
FLL	2.05 ± 0.023	0.89 ± 0.23	$8.20e^{-3} \pm 6.00e^{-4}$	$0.026 \pm 3.30e^{-3}$
Trait	bi θ	pd θ	dd θ	bi σ^2
Three life cycles				
HLD	4.81 ± 0.14	1.09 ± 1.22	5.35 ± 0.20	$1.20e^{-3} \pm 1.30e^{-4}$
HLL	2.33 ± 0.033	0.65 ± 0.29	2.47 ± 0.024	$0.011 \pm 1.10e^{-3}$
				pd σ^2
				dd σ^2

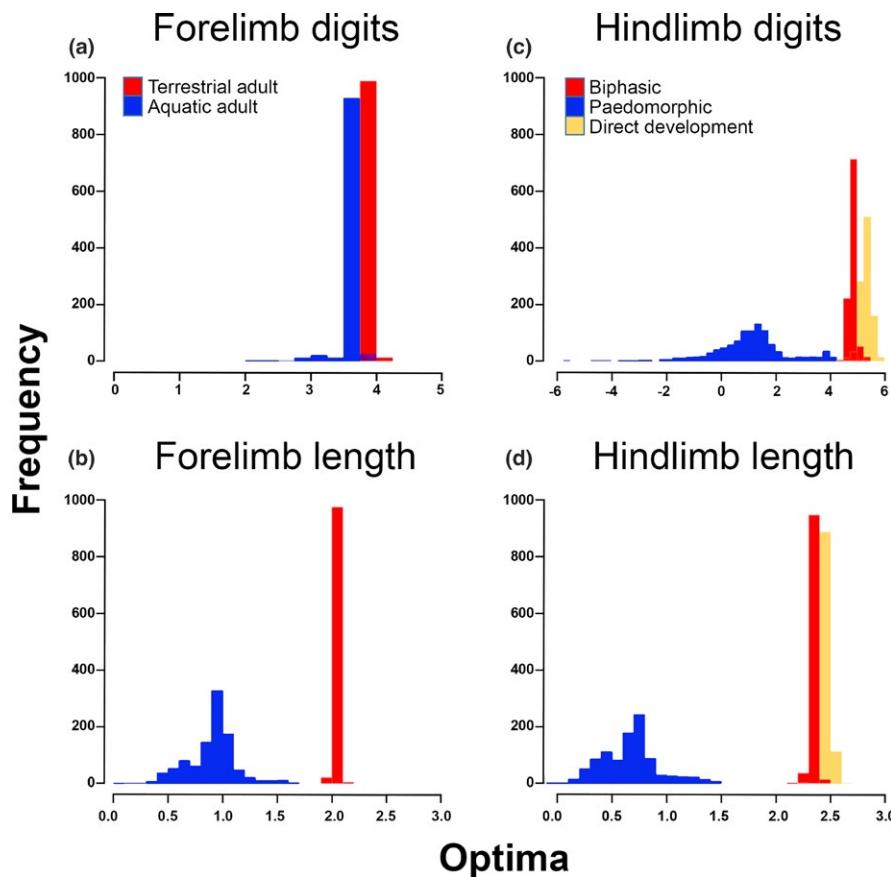


FIGURE 2 Histograms of optima (θ) parameter estimates from the best-fitting evolutionary models across 1,000 stochastically mapped chronograms. Forelimb digits (a) and forelimb length (b) were best fit by the Adult Ecology model that separates salamanders with an aquatic adult (blue) and terrestrial adult (red). Hindlimb digits (c) and hindlimb length (d) were best fit by the Three Life Cycles model that separates all three salamander life cycle strategies: biphasic (red), paedomorphic (blue) and direct development (gold)

correlation between traits using the “extractCorrelation” function. Life cycles with higher absolute correlation have relatively higher integration between traits and vice versa.

3 | RESULTS

3.1 | Paedomorphosis influences rates and optima of limb evolution

In order to assess the role of life cycle variation and adult ecology on rates and optima of limb evolution, we used BM and OU models

that allow us to investigate whether salamander life cycles have different trait optima and rates of evolution. We found OU models that allow rates and optima to vary among groups are favoured across all our morphological traits. Furthermore, for each trait, the Two Life Cycles model (simple pd + dd vs complex bi) was never the best fit, suggesting that life cycle complexity is not the best explanation of limb evolution patterns (Supporting Information Tables S1–S4). Adult Ecology models (terrestrial bi + dd vs aquatic pd) were the best fit for both FLL and FLD (Supporting Information Tables S1 and S2; $\Delta AIC_c \geq 2.48$ and 93.64 , $w_i = 0.75$ and 1.00 , respectively). There was marginal support for the Three Life Cycles model (bi vs

pd vs dd) explaining FLL ($\Delta\text{AICc} = 2.48$, $w_i = 0.22$). In both cases, it is clear that the evolution of an aquatic adult decreases optima and increases rate. FLD optima were lower for salamanders with aquatic compared to terrestrial adults, and the rate was over one billion times higher (Table 1, Figure 2a). This large disparity in rate between regimes is likely explained by complete fixation of FLD number at four in all species with terrestrial adults compared with FLD variation in aquatic salamanders. The same pattern is seen in FLL where optima for salamanders with terrestrial adults are higher than those with aquatic adults, and the rate of evolution is three times higher (Table 1, Figure 2b).

The best-fitting hindlimb models separated salamanders by all three life cycles (Supporting Information Tables S3 and S4; HLD $\Delta\text{AICc} \geq 4.85$, $w_i = 0.92$; HLL $\Delta\text{AICc} \geq 0.64$, $w_i = 0.44$). However, there was also some support for the Adult Ecology (bi + dd vs pd) OU $\theta\sigma^2$ ($\Delta\text{AICc} = 0.64$, $w_i = 0.32$) and OU θ ($\Delta\text{AICc} = 2.00$, $w_i = 0.16$) models when explaining HLL. Paedomorphs had the lowest HLD optimum followed by biphasics, and direct developers had the highest HLD optimum (Table 1, Figure 2c). Paedomorphs had a rate twenty times higher than biphasics, which in turn had a rate six times higher than direct developers (Table 1). Paedomorphs also had the lowest HLL optimum and direct developers the highest (Table 1, Figure 2d). HLL rates are 2.5 times higher in paedomorphs than biphasics and 1.7 times higher than direct developers. The Adult Ecology model (bi+dd vs pd) still showed that aquatic adults (pd) had lower optima

and higher rates of evolution. Overall higher rates of phenotypic diversification in aquatic salamander limb traits suggest that terrestrial stages impose constraint, and lower optima suggest limb reduction. Reverse OU simulations yielded similar parameter estimates between groups except for HLD simulations, where θ were significantly underestimated when α was lower than 0.017. However, when manually set to a greater α parameter, results matched θ estimations from raw data (Supporting Information Table S5).

3.2 | Paedomorphosis changes covariance between traits

We used Bayesian MCMC modelling to test for shifts of limb and body form evolutionary correlation between salamander life cycles. We found significant differences in posterior correlation between all three life cycle strategies in FLD (% overlap <0.001). In HLD, there was overlap between salamanders with terrestrial adults (% overlap bi-dd = 0.40). For both the HLL and FLL to trunk elongation simulations, there was no significant difference between rate matrix posterior estimations except for correlation structure between paedomorphs and direct developers in FLL (HLL % overlap: bi-pd = 0.06, bi-dd = 0.74, pd-dd = 0.053; FLL % overlap: bi-pd = 0.07, bi-dd = 0.46, pd-dd = 0.025). While limb length-to-body elongation rate matrices show more than 5% overlap between posterior parameter estimates, patterns trend towards higher negative correlation

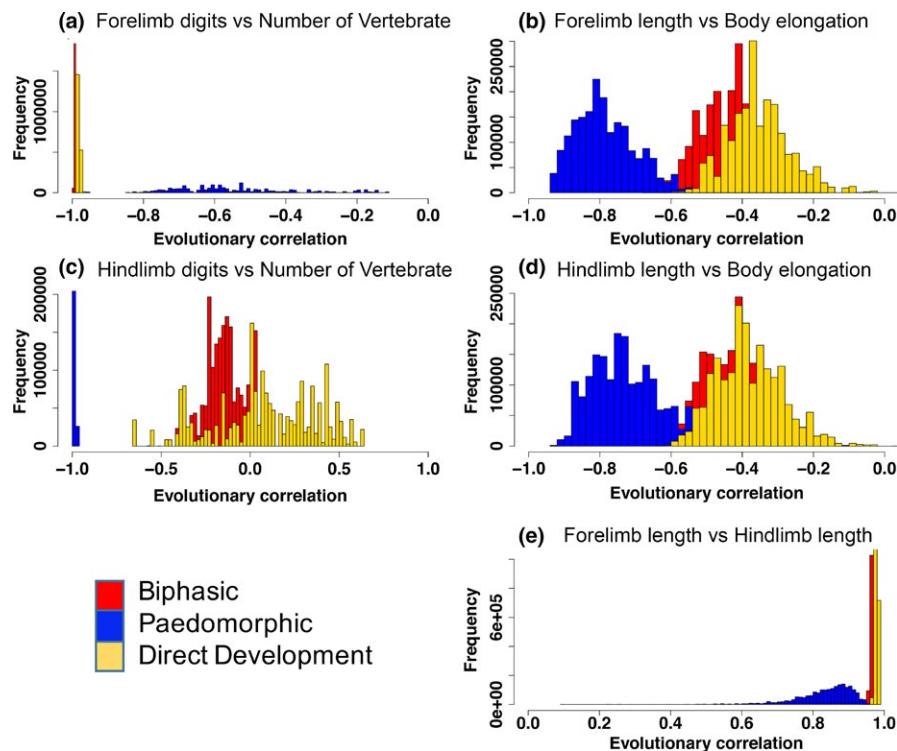


FIGURE 3 Posterior distribution of rate matrix parameter estimates of evolutionary correlation. Histograms represent a summary of the posterior distribution of correlation between traits among species (deemed evolutionary correlation). We compared forelimb digits vs numbers of vertebrae (a), hindlimb digits vs numbers of vertebrae (b), forelimb length vs body elongation (c), hindlimb length vs body elongation (SVL-HL/BW) (d) and forelimb length vs hindlimb length (e). Three rate matrices were fitted to the phylogeny: paedomorphic (blue), biphasic (red) and direct development (gold)

in paedomorphic lineages. FLL-to-HLL comparisons show significant differences in correlation between aquatic and terrestrial adult ecologies (% overlap: bi-pd <0.001, bi-dd = 0.12, pd-dd <0.001). Paedomorphs exhibit higher negative evolutionary correlation between HLD and NoV, but lower correlation between FLD and NoV and FLL to HLL (Figure 3). Higher negative evolutionary correlation between HLD and NoV in paedomorphs suggests that these traits show patterns of functional integration.

4 | DISCUSSION

Shifts in adaptive zones can impact patterns of morphological evolution (Dial, Shubin, & Brainerd, 2015; Landis & Schraiber, 2017; Simpson, 1944; Thompson, 1998; Uyeda, Harmon, & Blank, 2016). Locomotion in aquatic vs terrestrial habitats presents disparate challenges that can result in distinct selective regimes (Citadini, Brandt, Williams, & Gomes, 2018; Gillis & Blob, 2001). Here, we show that reversals from terrestrial to aquatic environments result in higher rates of limb form evolution and lower trait optima. Reversals were also associated with increased integration between numbers of vertebrae and hindlimb digits as well as a reduction of integration between hind and forelimb length. Our results support the hypothesis that shifting from terrestrial to aquatic environment lifts existing constraints on limb form as evidenced by elevated rates of morphological evolution. This shift has altered patterns of correlation, and therefore integration, between limbs and body form.

4.1 | Terrestriality constrains limb evolution

Terrestrial and aquatic environments create vastly different mediums for locomotion (Gillis & Blob, 2001), and transitions between them can alter rates and optima of locomotory trait evolution (Bonett & Blair, 2017; Bonett et al., 2018; Citadini et al., 2018). Locomotion on land requires more force to overcome gravity in many taxa (Clarac, Libersat, Pflüger, & Rathmayer, 1987; Deban & Schilling, 2009; Gillis & Blob, 2001), which could induce constraint on associated morphology. Our data suggest strong patterns of constraint on digit number in terrestrial environments, likely associated with the functional demands of plantigrade locomotion. When re-invading aquatic habitats, this constraint is lifted and replaced with novel selection pressures leading to higher variance in limb evolution and often digital reduction in salamanders.

Evidence of digit number constraint due to plantigrade locomotion can be observed in early tetrapods during the transition from aquatic to terrestrial environments. The earliest tetrapods, *Acanthostega* and *Ichthyostega*, had eight and seven digits, respectively. Later, *Tulerpeton* was reduced to six digits (Coates & Clack, 1990). Skeletal evidence suggests that *Acanthostega* was likely adapted to tail-propelled swimming, while *Ichthyostega* may have been the first known tetrapod that could facultatively shuffle on land coinciding with fewer digits (Ahlberg, Clack, & Blom, 2005; Pierce, Clack, & Hutchinson, 2012). Almost all crown-group tetrapods have been reduced to five or fewer

digits following transitions to full terrestriality. Subsequent evolution beyond pentadactylly is rare, likely due to genetic and developmental constraints (discussed in Shubin et al., 1997).

Terrestrial salamander life cycles exhibit lower rates of limb evolution compared to paedomorphs. In hindlimb digits, this pattern manifests on a scale where evolutionary optima are increasingly truncated and rates of trait of evolution are elevated as salamanders transition from more terrestrial to aquatic life cycles. Locomotion on land may be achieved by a smaller range of phenotypes resulting in reduced limb variation and fixation of digit number. This is apparent when we compare numbers of digits in salamanders with terrestrial vs aquatic adults. Forelimb digits are always fixed at four in salamanders with terrestrial adults, and while hindlimb digits have been reduced seven times (See Lamb and Beamer (2012) for evidence of additional reductions), in each case it was only by one digit. In contrast, paedomorphic salamanders display a larger range of limb and digit phenotypes. Within terrestrial habitats, aspects of plethodontid salamander foot morphology are constrained in climbing compared to terrestrial species (Adams, Korneisel, Young, & Nistri, 2017). This further suggests that rates of evolution can be more stringently optimized as species move to locomotory regimes with higher performance demands.

Many other tetrapods exhibit higher variance after reversals to aquatic environments. Aquatic clawed frogs of the genus *Xenopus* have a prehallux which is likely a rudimentary sixth digit (Hayashi et al., 2015), while larval forms of most frogs have delayed limb development to metamorphosis (Gans, 1975). Many fully aquatic reptile and mammal lineages such as ichthyosaurs, mosasaurs, plesiosaurs, cetaceans and sirenians have repeatedly lost or gained digits and phalangeal elements or lost entire limbs (Cooper, Berta, Dawson, & Island, 2007; Fedak & Hall, 2004; Motani, 1999; Richardson & Chipman, 2003; Savage, 1976; Wu, Li, Zhou, & Dong, 2003). Fossil amphibians such as microsaurs and lysorophians have repeatedly evolved limb-reduced forms that have been attributed to aquatic locomotion (Carroll & Gaskill, 1978; Olson, 1971; reviewed in Schoch, 2009). Repeated changes in morphological variance following transitions to aquatic environments support a hypothesis that terrestriality constrains limb morphology. However, we do note that other transitions away from plantigrade locomotion result in limb or digit reduction, as it has also been associated with burrowing and grass swimming (Brandley et al., 2008; Polly, 2007; Wiens & Slingluff, 2001; Wiens et al., 2006). In fact, limb reduction likely evolves in a multimodal distribution within diverse environments (Gans, 1975). Results discussed here explain one adaptive pressure on limb morphology that may be shared across many tetrapod lineages. We hypothesize that patterns of limb constraint in salamanders are analogous to those facing early tetrapods during the transition from aquatic to terrestrial environments.

4.2 | Ecologically dependent correlation of digit and vertebral number

Among extant taxa, salamanders and “lizards” are the lineages that exemplify a generalized tetrapod body form with a short trunk and

four limbs of more or less equal length. Limb and digit reduction have long been recognized to coevolve with body elongation in tetrapods (Gans, 1975; Lande, 1978). Strong correlations between body elongation and limb reduction (including digit loss) have been recovered in several comparative analyses (Brandley et al., 2008; Wiens & Slingluff, 2001). However, previous studies have not directly supported a correlation between these variables for salamanders (Wiens & Hoverman, 2008). Rather, digit loss has been associated with paedomorphosis and correlated with major decreases in absolute body size (Wiens & Hoverman, 2008). Superficially, this finding seems counterintuitive given that paedomorphosis and aquatic ecologies are associated with large body size in amphibians (Laurin, Canoville, & Quilhac, 2009; Laurin, Girondot, & Loth, 2004; Wiens & Hoverman, 2008). However, digit loss has been most extreme in relatively small lineages from clades with otherwise large or gigantic paedomorphic species (e.g. *Amphiumidae* and *Sirenidae*; Wiens & Hoverman, 2008; Bonett, Trujano-Alvarez, Williams, & Timpe, 2013). We found that digit loss in salamanders has an ecologically dependent relationship with body elongation. Specifically, hindlimb digit number has a significantly stronger negative relationship with numbers of trunk vertebrae in aquatic (paedomorphic) lineages, compared to lineages with terrestrial adult stages (biphasics and direct developers). We also found a stronger correlation between hindlimb length and body elongation in paedomorphs compared to biphasics and direct developers, but the difference between these groups was not significant (Figure 3).

Lateral undulation assisted by a pelvic driven, limb (or fin)-based gait was potentially the ancestral mode of locomotion in the sarcopterygian lineage that gave rise to tetrapods (King et al., 2011). This locomotory strategy continues to be utilized by many salamanders and limbed squamates. When propulsion is dominated by lateral undulation, limbs are often adpressed to the sides of the body. The strong correlation between limb and digit reduction in squamates has been functionally explained as selection for more efficient undulation on, or through, a diversity of terrestrial habitats (Brandley et al., 2008; Gans, 1975; Siler & Brown, 2011; Wiens et al., 2006). In contrast, limbless lateral undulation by salamanders is used during anguilliform swimming or only brief, fast bursts when out of water. On land, all biphasic and direct developing salamanders still primarily walk using all four limbs. Even the most elongate (worm-like) terrestrial salamanders (*Batrachoseps*, *Oedipina* and *Plethodon*) primarily walk when on land, rather than laterally undulate with their limbs adpressed.

Wiens and Hoverman (2008) speculated that the loss of salamander digits may result from a reduced need for limbs in an aquatic environment, but point out that being aquatic and paedomorphic is "not sufficient" to explain patterns of digit loss in salamanders. Digit reduction and loss are indeed most extreme in permanently aquatic lineages with elongate bodies (amphiumids, sirenids and some proteids), but not in those with shorter body forms (other proteids, cryptobranchids and some plethodontids). Amphiumids, cryptobranchids, proteids and sirenids have all likely been aquatic since the Cretaceous (Bonett et al., 2013; Demar, 2013; Holman, 2006).

This suggests that length of time that a salamander lineage has been in the aquatic adaptive zone does not necessarily equate to a more elongate body and a smaller limb, but rather that there are alternative adaptive peaks for salamanders in diverse aquatic habitats. One peak involves body elongation and limb reduction for anguilliform swimming and burrowing as seen in amphiumids and sirenids. Another adaptive peak (or peaks) consists of dorsal–ventral flattening of the body and maintaining limbs for bottom walking in lotic environments (cryptobranchids and *Necturus*), or even shortening the trunk and lengthening the limbs as in some aquifer-dwelling paedomorphs (*Eurycea rathbuni* and *Eurycea wallacei*; Wiens, Chippindale, & Hillis, 2003; Bonett & Blair, 2017). In summary, patterns of hindlimb digit number and vertebral column elongation are more highly correlated in obligately paedomorphic species, which have colonized different aquatic adaptive zones. In contrast, many salamanders with a terrestrial ecology have maintained consistent hindlimb digit numbers, which, in elongate terrestrial salamanders, demonstrates a decoupling of digit from vertebral numbers.

Vertebrate embryos share their greatest similarity at the phylogenetic stage (Seidel, 1960; Slack, Holland, & Graham, 1993), which is marked by pronounced inductive signalling between embryonic regions (Galis & Metz, 2001; Galis, Van Alphen, & Metz, 2001). In amniotes, both the limb buds and somites are developing during this time and may be strongly integrated by shared inductive signals. Amphibian limb development is more modular (Galis, Wagner, & Jockusch, 2003; Galis et al., 2001) and therefore somewhat independent from somitogenesis and vertebral column development. In salamanders, the appearance of limb buds varies during embryonic and larval development (Collazo & Marks, 1994). This independence should allow for evolutionary flexibility to produce diverse combinations of appendicular and axial traits.

4.3 | Parcellation of serial homologous structures in aquatic environments

Serial homology is generated by duplicating existing structures, and their associated developmental programs, in a new location (Hall, 1995). The shared origin of serially homologous structures is thought to facilitate initially strong correlation (integration) due to common developmental pathways (Olson & Miller, 1958; Young & Hallgrímsson, 2005). Forelimbs and hindlimbs are evolutionarily linked by shared genetic, developmental and functional constraints generally hypothesized to originate from serial homology (Hallgrímsson, Willmore, & Hall, 2002; Hallgrímsson et al., 2009; Margulies, Kardia, & Innis, 2001; Petit, Sears, & Ahituv, 2017; Shou, Scott, Reed, Hitzemann, & Stadler, 2005; Shubin et al., 1997). However, we acknowledge that serial homology in this system has been challenged (see Diogo & Ziermann, 2015).

We show a significant decrease in correlation between limb lengths in aquatic compared to terrestrial salamanders. Our previously discussed results show patterns of limb reduction and elevated rates of morphological evolution in aquatic salamanders (Supporting Information Tables S1–S4) suggesting loss of constraint. These

results corroborate loss of constraint that leads to lower levels of integration between limb lengths. In spite of shared homology, limbs seem to be more free to vary independently of each other in aquatic environments. Fully terrestrial direct developing salamanders often have the most similarity between fore- and hindlimb lengths. Salamanders of the family Sirenidae show the greatest disassociation as they have entirely lost hindlimbs but retain functional forelimbs (Azizi & Horton, 2004), which emphasizes the independence that can be reached in paedomorphic lineages. However, recently diverged lineages such as terrestrial and semi-aquatic salamanders of the genus *Desmognathus* show the next largest disparity in length between the hind- and forelimb, while some more recently derived paedomorphic lineages of the genus *Eurycea* have relatively similar limb lengths. This suggests again that aquatic life history is not sufficient to predict patterns of integration between forelimbs and hindlimbs and it is likely that multiple optima are present within our defined regimes.

Recently, Tomašević Kolarov et al. (2017) investigated limb correlation in a polymorphic newt species that has both paedomorphic and biphasic ("metamorphic") individuals. They found that both morphs have similar patterns of hindlimb to forelimb correlation, but paedomorphs had higher partial correlations between some limb measurements such as tibia to radius length. These newts are facultatively paedomorphic and live in a range of disparate habitats where polymorphism has been attributed to variations in environmental conditions (Denoël & Ficetola, 2014). Differences in correlation between morphs may result from developmental plasticity rather than broad-scale evolutionary patterns. Paedomorphic newts in more stable aquatic environments may have reduced developmental error rates due to less perturbation in habitats with low environmental variation. More research is needed to test whether environmental variation has an impact on limb integration across salamander taxa.

Deviations from quadrupedal movement in vertebrates have been shown to create functional disassociation between limbs due to adaptations for specialization. Examples can be seen in marsupials (Kelly & Sears, 2011), primates (Young, Wagner, & Hallgrímsson, 2010) and flying tetrapods (Bell, Andres, & Goswami, 2011). In each case, parcellation of shared developmental mechanisms between limbs has to occur, fuelled by independent selection (Young & Hallgrímsson, 2005). Here, we show that life cycle shifts can reduce correlation between limbs, and that this is not necessarily associated with limb specialization. In fact, combined with other analysis we hypothesized that correlation between limbs may be reduced due to lack of selection for, or selection against, retaining limbs in the most elongate paedomorphic salamanders. Changes in selective regime can have drastically different effects on each limb in some lineages (e. g. Sirenidae). This hypothesis may be further supported by increased variance in limb length and digit number in paedomorphs as this is thought to be a sign of drift due to lower levels of integration (Young, 2017). As fully aquatic salamander lineages shift from quadrupedal to undulatory movement, locomotion selects for disassociations between forelimb and hindlimb and between limbs and vertebral column.

5 | CONCLUSION

Shifting to aquatic environments produces higher rates of limb evolution and limb reduction in salamanders. It also produces significant shifts in patterns of integration between digits and vertebral number, and forelimbs and hindlimbs. These results suggest that terrestriality imposes strong constraint on limb evolution that can be lifted upon reversion to aquatic habitats. This study has implications for how we understand the evolution of terrestriality and pentadactyly in early tetrapods as well as the many subsequent reversions to aquatic habitats, and provides an example of ecological and life cycle variation producing changes in patterns of integration among species.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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