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RESEARCH ARTICLE

Facultative Transitions Have Trouble Committing, But Stable Life Cycles Predict Salamander Genome Size Evolution

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Abstract

Facultative traits can provide phenotypic lability in dynamic environments, but it is unclear how weaving between disparate habitats impacts non-facultative traits that are carried along the way. The life cycles of salamanders are associated with distinct ontogenies, aquatic-to-terrestrial (*biphasic*), completely-terrestrial (*direct development*), and completely-aquatic (*larval form paedomorphic*). Salamanders have some of the largest genomes among vertebrates, and the most extreme expansions have been attributed to paedomorphosis and life cycle simplification. Recent analyses of genome size evolution across amphibians have rejected this hypothesis for salamanders. Our analyses show that treatment of facultatively paedomorphic salamanders, which are alternatively biphasic, in part explains this discrepancy. Nearly all of the facultatively paedomorphic species analyzed have genome sizes that overlap with the optimum of biphasic species. We found that obligate paedomorphs, alone and when combined with direct developers, have significantly larger genome sizes than biphasics plus facultative paedomorphs. In general, salamander genome size variation fits life cycle models better than those for larval ecology, adult ecology, or aquatic habitat stability. Obligate transitions to a simple life cycle appear to have been an important route for lineages to evolve significant divergence in genome size from biphasic ancestors. Our analyses support the classic association between genome size variation and life cycle complexity in salamanders, which may ultimately reflect patterns of time limited development.

Keywords Adaptive zones · Complex life cycles · Direct development · Facultative paedomorphosis · Metamorphosis

Introduction

Transitions between major adaptive zones such as distinct habitats can impose new selective regimes that substantially alter patterns of evolution (Simpson 1944). Subsequent diversification can arise from specialization to sub-habitats, but the evolution of some traits may still be constrained by overarching limitations of the adaptive zone (Simpson 1944, 1953; Van Valen 1971; Uyeda et al. 2011; Dumont et al. 2012; Bonett and Blair 2017; Ledbetter and Bonett 2019). Such bounding of trait distributions may explain why some phenotypes are relatively static through time (Estes and Arnold 2007; reviewed in Futuyma 2010), but exhibit pulses of punctuated divergence associated with adaptive zone shifts (Simpson 1944; Uyeda et al. 2011; Arnold 2014). Some organisms have environmentally-induced polymorphisms that allow them to facultatively explore different adaptive zones without commitment (West-Eberhard 1989; Whiteman 1994; Janson et al. 2008; Denoël and Ficetola 2014). There has been considerable theory and research regarding the canalization of plastic traits (West-Eberhard 2005; Pfennig et al. 2010; Moczek et al. 2011). However, non-plastic (non-facultative) traits would also be carried through fluctuating selective regimes and may ultimately: (1) express values that are constrained by one regime, (2) rapidly shift trait values to fit the current regime, or (3) evolve trait values that are different than the other regimes (Fig. 1).

Life cyclwater bodies"), but it was necessary toe variation in salamanders coincides with discrete habitats. The majority of salamander families include *biphasic* (bi) species with an aquatic larval stage that metamorphoses into

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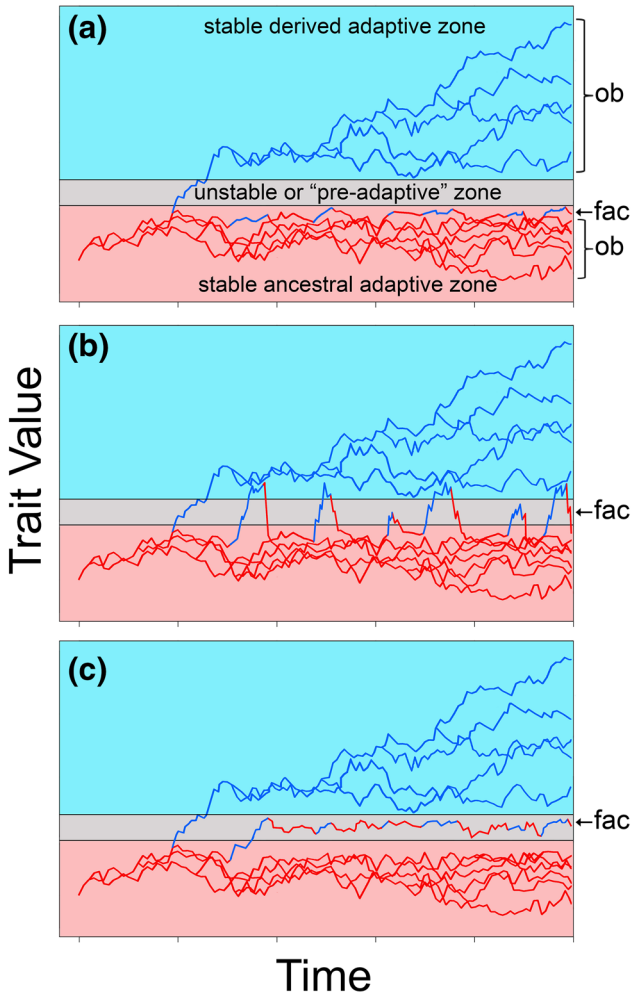

Fig. 1 Trait evolution and lineage divergence through time across adaptive zones. Lines represent a clade diversifying through time with respect to the evolution of a trait that is strongly influenced by two discrete adaptive zones (red or blue). Most lineages obligately (ob) persist in just one of the adaptive zones, but the facultative (fac) lineage may frequently transition between the two. Obligate lineages in the different adaptive zones (blue and red boxes) have different trait optima (mean) and rates of evolution (variance). Grey middle box is the unstable ("instable") or "pre-adaptive" zone (Simpson 1944). The non-facultative trait value for the facultative lineage may either **a** be constrained by one of the two adaptive zones despite frequent transitions, **b** rapidly shift to the alternative optimum upon shifting adaptive zones, or **c** may lead to the derivation of a novel adaptive zone by optimizing on different trait values, here shown as intermediate

a terrestrial adult. There have been at least two significant deviations from the biphasic pattern: *direct development* (dd), where transformation to a fully terrestrial salamander occurs *in ovum*, thus eliminating the free-living aquatic stage; and *larval form paedomorphosis*, where reproduction occurs while maintaining the aquatic larval morphology and ecology (Hanken 1992; Bonett et al. 2014). Several lineages of salamanders are *obligate paedomorphs* (pd) that have permanently transitioned to a primarily aquatic lifestyle. By comparison, *facultative paedomorphs* (fac) represent largely environmentally dependent aquatic transitions, but still maintain the potential to metamorphose into a terrestrial form (Denoël and Joly 2001; Denoël et al. 2005; Denoël and Ficetola 2014). Life history transitions in facultative paedomorphs are unstable, and can occur among generations (metamorphic ←⟶paedomorphic) and even within a single ontogeny (paedomorphic →metamorphic).

While genotype largely dictates phenotype, structural properties of the genome such as size are functionally significant traits that influence cell size (Cavalier-Smith 1991; Gregory 2002b; Sessions 2008; Beaulieu et al. 2008; Mueller 2015), developmental timing (Jockusch 1997; Gregory 2002b), developmental rate (Sessions and Larson 1987; Wake and Marks 1993; Gregory 2002b) and, in some clades, metabolic rate (Licht and Lowcock 1991; Vinogradov 1995, 1997; Gregory 2002a, 2005; Starostova et al. 2009). Salamanders exhibit some of the largest vertebrate genomes (Gregory 2019), and there has been a long history of speculation and tests of the underlying drivers of these patterns (Gregory 2002b; Sessions 2008). The most compelling correlations have been found between the genome sizes of plethodontid salamanders and developmental traits like hatching time (Jockusch 1997) and differentiation rate (Sessions and Larson 1987). Aestivation coupled with enormous genomes observed in some gigantic aquatic salamanders and lungfish fueled the hypothesis that genome expansion is an adaptation for low metabolic rate, mediated by large cell size (Cavalier-Smith 1991). However, metabolic rate is only correlated with salamander genome size at high temperatures (Licht and Lowcock 1991) and is unlikely to fully explain all of the evolutionary nuances (Uyeda et al. 2017).

Based on the "degree of paedomorphosis", Martin and Gordon (1995) correlated life cycle mode with genome size in salamanders. The "oldest" obligately paedomorphic families have the largest genomes, while families that include several facultatively paedomorphic species have on average a more modest genome size increase compared to "younger" families. They hypothesized that the large genome sizes of paedomorphic salamanders resulted from lost functionality of genes controlling post-metamorphic (terrestrial) adult cell types, which ultimately lead to their greater accumulation of "junk DNA". Regardless of the underlying driver(s), the association between genome size and life cycle has been well adopted (Wake and Marks 1993; Martin and Gordon 1995; Gregory 2002b). Synthesizing studies from a variety of species, Gregory (2002b) hypothesized that genome size is minimized in species with multistage life cycles that require time-dependent developmental processes. This in part could explain why larval form paedomorphs have larger genomes than biphasic salamanders.

Liedtke et al. (2018) recently showed a major increase in the rate of evolution and optimal genome size in the

stem leading to salamanders. They also found correlations between genome size and developmental rate in frogs, but rejected an association between life cycle complexity and genome size overall, including no support for a relationship between genome size and paedomorphosis in salamanders. The disparity between this study and prior assessments of salamander genome size evolution may lie in how facultative life cycle transitions are treated. Here we use phylogenetic comparative analyses to test whether genome size is associated with life cycle complexity in salamanders, specifically how facultative life cycles compare to obligate. We evaluate three alternative hypotheses concerning the effect of facultative life cycles on genome size: (1) genome size does not deviate from the ancestral biphasic adaptive zone, (2) genome size evolves to match the paedomorphic adaptive zone, or (3) genome size may evolve values that are different than the other adaptive zones. Recent analyses have also shown a relationship between salamander genome size and habitat stability, with the suggestion that large genomes limit colonization of ephemeral aquatic habitats (Lertzman-Lepofsky et al. 2019). Thus we also compared the fit of genome size to models of life cycle evolution as well as those that characterize larval and adult ecology. Finally, we test for correlations between genome size and timing of metamorphosis to evaluate the hypothesis that time-dependent developmental processes can limit genome size evolution. These analyses are important for understanding how cellular properties such as genome size relate to ontogenetic complexity, as well as how transient adaptive zone shifts influence macroevolution.

Methods

Genome Sizes, Life Cycles, and Ecologies

Mean genome sizes in *pg* (*C*-value) of 163 species of salamanders were used from the recent compilation by Liedtke et al. (2018), based on the Genome Size Database (Gregory 2019), which is derived from many sources (see supplementary materials, Table S1). Life cycle categories of each species were coded consistent with our recent compilation (Bonett and Blair 2017). The complete data set included 17 obligately paedomorphic, 74 direct developing, and 72 biphasic species. These species represent several independent transitions to obligate paedomorphosis from a biphasic life cycle, and three to four transitions between direct development and biphasy in the family Plethodontidae (Chippindale et al. 2004; Mueller et al. 2004; Bonett et al. 2014; Bonett and Blair 2017). Of the biphasic species, 22 are known to exhibit facultative paedomorphosis and were alternatively coded in some analyses (see below). Due to low sample size, the 3 viviparous or ovoviviparous species of *Salamandra* were removed from the dataset.

We also compared the fit of genome size to ecological models based on larval and adult ecology (Bonett and Blair 2017), and aquatic habitat stability (Lertzman-Lepofsky et al. 2019). Of the 89 species with free-living aquatic larvae (biphasics and paedomorphs), 48 primarily dwell in lentic habitats and 41 in lotic habitats. All extant direct developing salamanders and most biphasics have terrestrial adults, and all obligate paedomorphs have aquatic adults (when not aestivating). Some biphasic newts (Salamandridae) metamorphose, but never leave the water and were coded as aquatic (Table S1). Our data set includes 144 salamanders with terrestrial adults and 19 with aquatic adults. Lertzman-Lepofsky et al. (2019) categorized "habitat stability" based on the Habitat Classification Scheme from the International Union of the Conservation of Nature (IUCN). We utilized their categories (direct development, "permanent water bodies", and "ephemeral water bodies"), but it was necessary to reclassify some species (see Table S1).

Modeling Genome Size Evolution

All analyses are carried out in the R (v. 3.6.0) statistical computing language (2018). Phylogenetic Analyses of Variance (phyloANOVA) on linear models with Randomized Residuals in a Permutation Procedure (RRPP) implemented in the R package RRPP v. 0.5.2 (Adams and Collyer 2018; Collyer and Adams 2018) were used to test whether life cycle complexity or life cycle mode are associated with average genome size. Linear models in RRPP were fit using the *lm.rrpp* function, and *pairwise* was used to calculate 95% confidence intervals and test for significant differences in least squares distance among groups. This method has been shown to exhibit substantially higher power than methods that rely on phylogenetic simulation (Adams and Collyer 2018). These analyses were based on a pruned consensus tree (Appendix S1) of the posterior distribution of 1000 Bayesian time-calibrated phylogenies (Bonett and Blair 2017) calculated using TreeAnnotator from BEAST v. 2.4.0 (Bouckaert et al. 2014), and transformed into a variance–covariance matrix in R package ape v. 5.3 (Paradis et al. 2004) assuming Brownian Motion. We performed these analyses on the full 163-taxon data set, and also with the omission of *Necturus*, which have the largest genomes (see Results). We present analyses of Log_{10} transformed average genome size. Median and average genome sizes are almost identical and produce the same results.

The R package OUwie v. 1.5.7 (Beaulieu et al. 2012) was used to test whether the rate of evolution (σ^2) and optimum (θ) of genome size differ between life cycle modes, levels of life cycle complexity, or ecologies under Brownian Motion (BM) or Ornstein Uhlenbeck (OU) processes (Butler and

King 2004; O'Meara et al. 2006; Beaulieu et al. 2012). We specifically tested whether genome size is best fit to seven different categorical life cycle and ecological models primarily based on Bonett and Blair (2017) with additions. (1) *2 Life Cycles Model* (i.e. complexity model): simplified life cycles (obligate paedomorphic and direct development) are different than a complex life cycle (biphasics and facultative paedomorphs). (2) *3 Life Cycles Model*: obligately paedomorphic, biphasic (including facultatively paedomorphic), and direct developing species each have different patterns of genome size evolution. (3) *4 Life Cycles Model:* obligately paedomorphic, biphasic, facultatively paedomorphic, and direct developing species each have distinct patterns of genome size evolution. (4) *Facultative Model*: facultative paedomorphs plus obligate paedomorphs are compared to direct developers and biphasics. (5) *Adult Ecology*: species with terrestrial transforming adults (biphasics and direct developers) are compared to aquatic non-transforming adults (obligate paedomorphs) and a few biphasic newts that remain in the water. (6) *Larval Ecology*: comparison between primarily lentic (pond-dwelling) larvae, primarily lotic (stream-dwelling) larvae, and development *in ovum* (direct development). (7) *Habitat Stability*: comparison between species that breed in "permanent" aquatic habitats, "ephemeral" aquatic habitats, and direct development. We also removed the 22 facultatively paedomorphic species and re-estimated the parameter distributions under the *2 Life Cycles Model* to assess where the genome sizes of facultative paedomorphs fell with respect to optimal genome size distributions based on life cycle complexity.

Each of the models were fit to 1000 stochastic character maps across the 1000 post-burnin chronograms using the *make.simmap* function in phytools v 0.6–99 (Revell 2012). For each of the seven models (listed above) two allowed different θ and σ^2 (Thomas et al. 2006) or a single θ and different σ^2 for each group, and compared these alternatives to BM and OU models where these parameters were the same across the tree (BM_1 and OU_1 models, respectively). Differences in model fit were based on changes in Akaike Information Criterion (ΔAIC), AIC weights (w_i), and changes in Bayesian Information Criteria (ΔBIC), which more heavily penalize model complexity than AIC (Schwarz 1978). Lower AIC or BIC indicates a better fitting model. Models with AIC or BIC differences less than 2 ($\Delta AIC < 2$) were considered equally fit (Burnham and Anderson 2002). 95% confidence intervals (CI) were calculated for σ^2 and θ of the best-fit models. The selection parameter (α) did not improve OU models over their BM analog, and therefore we only included the results of the BM models.

We used Phylogenetic Independent Contrasts (Felsenstein 1985) in the *nlme* package (Pinheiro et al. 2019) to test for correlations between genome size and hatching time (for direct developers) and minimum larval period (for biphasics). For direct developers in particular, this allowed us to evaluate whether genome size is related to the duration of development, and therefore evaluate the time-dependent development hypothesis (Wake and Marks 1993; Gregory 2002b). Obligate paedomorphs never fully transform, so they cannot be quantified or directly compared to direct developers or biphasics in the same manner.

Results

Differences in Genome Size Evolution Among Life Cycle Modes

Phylogenetic ANOVAs using RRPP showed significant differences in genome size variation among life cycle modes ($F_{(2,160)} = 31.82$; $P < 0.001$; Fig. 2a; Table S2). Obligately paedomorphic salamanders have the largest average genome sizes (57.1 ± 14.1 pg) and were significantly different than

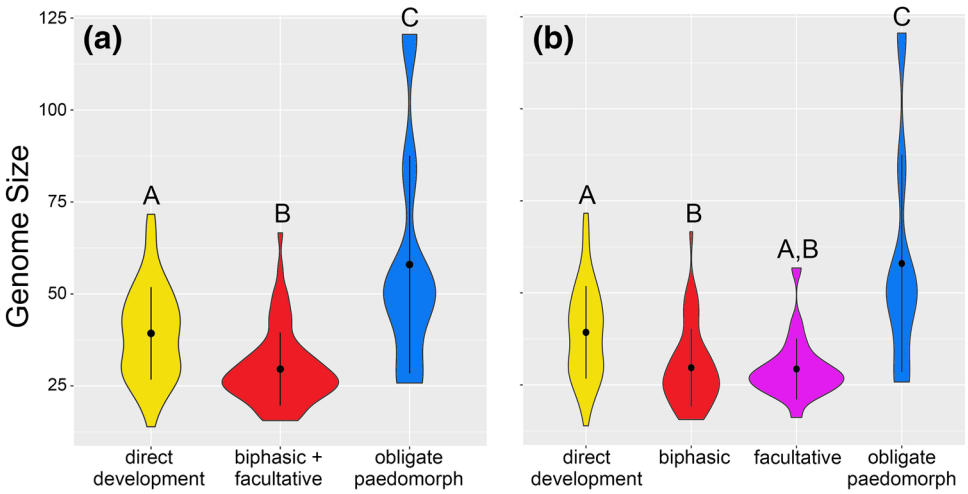

Fig. 2 Comparisons of salamander genome sizes among life cycle modes. Violin plots of average genome size in picograms for different life cycles. Facultative paedomorphs are either combined with biphasic species **a** or treated as a separate life cycle mode **b**. Capital letters above each distribution indicate significantly similar and different groups based on phylo-ANOVA using RRPP ($P < 0.05$; see the supplementary materials, Tables S2 and S3)

all other life cycle groups ($Z > 6.89$; $P < 0.001$ for both comparisons). The average genome size of direct developers (39.3 ± 2.9 pg) was more similar to biphasics (29.8 ± 3.0 pg), but was still significantly different ($Z = 1.99$; $P < 0.05$). Our samples of facultative paedomorphic salamanders had the same average genome sizes (29.3 ± 3.5 pg) as biphasic salamanders, and these two groups were not significantly different ($Z = -0.225$; $P = 0.512$; Fig. 2b; Table S3). Facultative paedomorphs were not significantly different than direct developers ($Z = 0.692$; $P = 0.243$; Fig. 2b).

Obligate paedomorphs of the genus *Necturus* include salamanders with the largest known genomes (up to 120 pg). We largely recovered the same RRPP results as presented above when we repeated these analyses without the two species of *Necturus* with the largest genomes (*N. punctatus* and *N. lewisi*; Tables S4 and S5) or without all three of the species of *Necturus* (Tables S6 and S7). The only disparity in the results is that (without *Necturus*) the difference in average genome size between direct developers and facultative paedomorphs is significant ($P < 0.05$; Tables S5 and S7).

When obligate paedomorphs and direct developers are grouped together (simple life cycle) they have a significantly larger genome than biphasics (complex life cycle. $F_{(1,161)} = 5.16$; $P < 0.025$; Table S8). This is consistent with the estimated differences in genome size optima presented below.

Modeling Optima and Rates of Genome Size Evolution

The overall best fit continuous trait models were Brownian Motion that allowed for different optima and rates ($BM_{\theta\sigma}{}^2$) of genomes size evolution between simple and complex life cycles (*2 Life Cycles Model*; $w_i = 0.3353$), between the three major stable life cycle modes (*3 Life Cycles Model*; $w_i = 0.2611$), and facultative paedomorphs as a distinct group (*4 Life Cycles Model*; $w_i = 0.1887$). Based on AIC, these three models were equally fit ($\Delta AIC < 2$; Table 1). However, when model complexity is more severely penalized via BIC then the *2 Life Cycles Model* is still the best overall fit model, and substantially better than both the *3* and *4 Cycles Models* ($\Delta AIC = 6.69$ and 13.54, respectively; Table 1). Compared to lineages with complex life cycles, those with simple life cycles (obligate paedomorphs and direct developers) were estimated to have higher optimum genome size (θ: complex $= 28.9 \pm 0.244$; simple $= 59.7 \pm 0.546$) and nearly two times higher rate of genome size evolution (σ^2: complex $= 0.00016 \pm 6.552e^{-7}$; simple $= 0.00029 \pm 7.921e^{-7}$). Parameter estimates for the *3 Life Cycles Model* show that both direct developers and obligate paedomorphs have optimal genome sizes that are two times higher than biphasics (θ: dd $= 60.9 \pm 0.681$; bi (+ fac) $= 31.6 \pm 0.177$; pd $= 81.2 \pm 1.124$), but rate of genome size evolution is only higher in direct developers (σ^2: dd $= 0.00031 \pm 8.391e^{-7}$; bi (+ fac) $= 0.00017 \pm 6.835e^{-7}$; pd $= 0.00012 \pm 1.501e^{-6}$; Table 1). The parameters for the *4 Life Cycles Model* are generally the same as the *3 Life Cycles Model*, but facultative paedomorphs (θ: fac $= 23.1 \pm 0.483$) have a lower genome size optimum than biphasics (θ: bi $= 29.7 \pm 0.289$).

Regardless of whether or not genome sizes of direct developers evolve differently than obligate paedomorphs (*3 Life Cycles Model*), or facultative paedomorphs evolve different than biphasics (*4 Life Cycles Model*), all three of the equally best fit models (via AIC) show that salamanders with simple life cycles have higher genome size optima than those with complex life cycles. Furthermore, considering both evaluation criteria the *2 Life Cycles Model* was a substantially better than a model that considers a single rate and optimum across the tree (BM_1; $\Delta AIC = 10.76$, $\Delta BIC = 4.59$). Models that combine facultative and obligate paedomorphs (*Facultative Model*) were always a relatively poor fit ($\Delta AIC = 5.84$, $\Delta BIC = 7.32$). The distribution of facultative paedomorphs (Average 29.3 pg) largely overlaps with the optimum for a complex (biphasic) life cycle (Fig. 3). The only facultatively paedomorphic species to fall within the optimal range for simple (or obligately paedomorphic) life cycle is *Dicamptodon ensatus*, which has a genome size 25% larger than any other facultative paedomorphs, and is part of a clade (Dicamptodontidae) that includes only obligately or facultatively paedomorphic species (discussed more below). Models based on *Larval Ecology* (lentic, lotic, or *in ovum*) or *Adult Ecology* (terrestrial adults vs. aquatic adults) were a worse fit that the *2 Life Cycles* model (ΔAIC and $\Delta BIC > 6$). The aquatic *Habitat Stability* model (direct development, permanent, ephemeral) was also a worse fit than the best life cycle complexity model ($\Delta AIC > 2$, $\Delta BIC > 7$).

We found that genome size is correlated with developmental time (hatching time) for direct developers (Adjusted $r^2 = 0.4335$; $P < 0.0003$; Fig. S1a). However, the minimum time to metamorphosis (embryonic development plus the larval period) for biphasics was not correlated (Adjusted $r^2 = 0.0060$; $P < 0.2402$; Fig. S1b).

Discussion

Variation in life cycle complexity has been implicated as a driver of salamander genome size evolution (Wake and Marks 1993; Martin and Gordon 1995; Gregory 2002b; Sessions 2008), but recent analyses across amphibians did not support this pattern (Liedtke et al. 2018). The analyses presented here demonstrate that life cycle simplification is associated with an increase in genome size in salamanders. This relationship is more clearly resolved when facultative paedomorphs are grouped with their biphasic relatives, rather than with obligate paedomorphs. Facultative

Table 1 Comparison of the fit of BM life cycle and ecological models to salamander genome sizes

Model	−lnL	*k*	AIC	ΔAIC	w_i	BIC	ΔBIC
2 Life cycles $BM_{\theta\sigma}^2$	157.64	4	− 307.27	0.00	0.3353	− 294.90	0.00
3 Life cycles $BM_{\theta\sigma}^2$	159.39	6	− 306.77	0.50	0.2611	− 288.22	6.69
4 Life cycles $BM_{\theta\sigma}^2$	161.06	8	− 306.12	1.15	0.1887	− 281.37	13.54
Habitat stability $BM_{\theta\sigma}^2$	158.56	6	− 305.13	2.14	0.1150	− 286.56	8.35
Facultative $BM_{\theta\sigma}^2$	156.72	6	− 301.43	5.84	0.0181	− 282.88	12.03
Larval ecology $BM_{\theta\sigma}^2$	156.55	6	− 301.11	6.16	0.0154	− 282.54	12.37
Adult ecology $BM_{\theta\sigma}^2$	154.40	4	− 300.80	6.47	0.0132	− 288.42	6.48
2 Life cycles BM_{σ}^2	153.35	3	− 300.70	6.57	0.0125	− 291.42	3.49
Larval ecology BM_{σ}^2	154.20	4	− 300.40	6.87	0.0108	− 288.02	6.88
Facultative BM_{σ}^2	153.98	4	− 299.97	7.30	0.0087	− 287.58	7.32
Habitat stability BM_{σ}^2	153.98	4	− 299.78	7.49	0.0079	− 287.58	7.32
3 Life cycles BM_{σ}^2	153.82	4	− 299.64	7.63	0.0074	− 287.26	7.64
4 Life cycles BM_{σ}^2	153.98	5	− 297.98	9.29	0.0032	− 282.49	12.41
BM_1	150.25	2	− 296.51	10.76	0.0015	− 290.31	4.59
Adult ecology BM_{σ}^2	150.94	3	− 295.89	11.38	0.0011	− 286.60	8.31

Seven alternative partitions were tested: *2 Life Cycles* (simple, complex), *3 Life Cycles* (paedomorphic, biphasic, direct development), *4 Life Cycles* (paedomorphic, biphasic, direct development, facultative paedomorphic), *Facultative* (facultative plus obligate paedomorphic, biphasic, direct development), *Adult Ecology* (terrestrial adults, aquatic adults), *Larval Ecology* (lotic, lentic, direct development), and aquatic *Habitat Stability* (permanent, ephemeral, direct development; Lertzman-Lepofsky et al. 2019; see Table S1). These models were compared to one another as well as to the *BM1* (Brownian Motion 1) with a single rate and optimum across the tree. Models were fit using OUwie (Beaulieu et al. 2012) with the potential for the optimum (θ) and/or rate of evolution (σ^2) to vary among groups within each model (indicated by subscript). ΔAIC, AIC Weights (*wi*), and ΔBIC were used to assess model fit. *k* is the number of parameters for each model. Parameter estimates and 95% confidence intervals for the overall best-fit models based on AIC (*2 Life Cycles* $BM_{\theta\sigma}^2$, *3 Life Cycles* $BM_{\theta\sigma}^2$, and *4 Life Cycles* $BM_{\theta\sigma}^2$) and BIC (*2 Life Cycles* $BM_{\theta\sigma}^2$) are listed below. The models were fit to the posterior distribution of 1000 chronograms pruned from Bonett and Blair (2017)

2 Life Cycles $BM_{\theta\sigma}^2$

θ: complex = 28.9 ± 0.244; simple = 59.7 ± 0.546

σ^2: complex = 0.00016 ± 6.552e^{-7}; simple = 0.00029 ± 7.921e^{-7}

3 Life Cycles $BM_{\theta\sigma}^2$

θ: dd = 60.9 ± 0.681; bi (+fac) = 31.6 ± 0.177; pd = 81.2 ± 1.124

σ^2: dd = 0.00031 ± 8.391e^{-7}; bi (+fac) = 0.00017 ± 6.835e^{-7}; pd = 0.00012 ± 1.501e^{-6}

4 Life Cycles $BM_{\theta\sigma}2$

θ: dd = 55.4 ± 0.599; bi = 29.7 ± 0.289; fac = 23.1 ± 0.483; pd = 70.8 ± 1.081

σ^2: dd = 0.00032 ± 8.855e^{-7}; bi = 0.00016 ± 1.408e^{-6}; fac = 0.00017 ± 2.174e^{-6}; pd = 0.00013 ± 1.695e^{-6}

paedomorphosis is an environmentally-induced alternative to the biphasic strategy, and is a potentially ephemeral phenotype. Therefore, in this case, a non-facultative trait such as genome size has shown limited divergence from species that express a biphasic life cycle. We further demonstrate that life cycle complexity explains genome size variation better than models of larval ecology, adult ecology, or aquatic habitat stability.

Persistent Utilization of an Adaptive Zone

Simpson (1944) described adaptive zones as environmental features or lifestyles that play a major role in influencing patterns of trait evolution. Strength of selection (Losos et al. 1997), evolvability (Wagner and Altenberg 1996), gene flow (Slatkin 1987) and population size (Lande 1980; Slatkin 1987) can influence the rate of trait divergence after a transition. However, impacts of the adaptive zone on macroevolution of a lineage may also be contingent upon: (1) the persistence of key ecological parameters of the adaptive zone (e.g. availability of a given habitat, food type, etc.), and (2) continuous utilization by members of the lineage across generations (Simpson 1944). Subsequent phenotypic alterations may retain lineages in a given adaptive zone, while phenotypic lability (through plasticity or rapid evolution) may permit frequent transitions between adaptive zones. Facultative transitions represent a special case where persistence in an adaptive

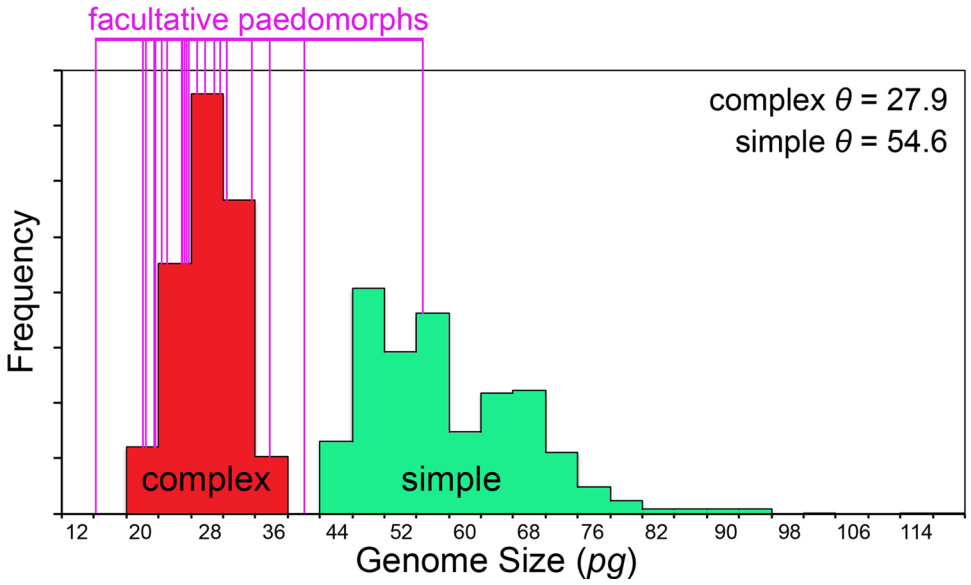

Fig. 3 Genome sizes of facultative paedomorphs compared to optima based on life cycle complexity. Optimal genome sizes for complex (red) and simple (green) life cycles estimated without facultative paedomorphs in OUwie. The genome sizes of 22 facultatively paedomorphic species (purple) are plotted with respect to these distributions. Optimal genome size distributions were estimated from across 1000 stochastic character maps under the *2 Life Cycles* $BM_{\theta\sigma^2}$ model

zone is inherently unstable and lability is directly tied to environmental consistency.

The biphasic life cycle is the likely ancestral condition for amphibians (Duellman and Trueb 1994) and salamanders (Bonett and Blair 2017). Several lineages of obligately paedomorphic salamanders appear to have transitioned to the aquatic adaptive zone in the Cretaceous (Bonett et al. 2013; Bonett and Blair 2017) and subsequently lost their ability to metamorphose into a terrestrial form (Bonett 2016). This has substantially reduced the likelihood for reversal, and has largely restricted these lineages to the aquatic adaptive zone. Reversals from larval form paedomorphosis to metamorphosis are only likely in a few derived clades of obligate paedomorphs (Bonett et al. 2014, 2018). Direct development is prominent and potentially ancestral for the family Plethodontidae (Bonett et al. 2014; Bonett and Blair 2017). Despite the likelihood of some major reversals from direct development to a biphasic life cycle (Chippindale et al. 2004; Mueller et al. 2004; Bonett et al. 2014), the invariance of direct development across several major clades of plethodontids speaks to its persistence through time. Therefore, obligate paedomorphosis and direct development represent relatively stable departures from biphasic adaptive zone. In contrast, salamanders with facultative life cycles occupy the geographic and temporal interface between biphasic and paedomorphic adaptive zones and can spontaneously shift between them. Most facultatively paedomorphic species occur in temperate regions that endured dynamic climatic cycles during the Pleistocene (Denoël et al. 2005). Therefore, the conditions faced by most modern facultative paedomorphs have likely been ephemeral over geologic time scales.

We found that obligate paedomorphs and direct developers, collectively or individually, have significantly higher genome size optima compared to biphasic and facultatively paedomorphic lineages (Table 1). The genome sizes of lineages that have the ability to express facultative paedomorphosis have not significantly diverged from their biphasic relatives (both groups with a mean of ~ 29 pg; Figs. 2, 3). If anything, facultative paedomorphs have reduced genome sizes compared to biphasics, but there is only limited support for this model (under AIC not BIC; Table 1). This suggests that facultative or very recent adaptive zone shifts in salamanders have not substantially impacted genome size evolution at the measured scale (picograms). In contrast, lineages with an ancient and extended history of paedomorphosis, as well as many clades of direct developers have dramatically expanded the sizes of their genomes.

Life Cycle Complexity and Genome Size Evolution

Genome size is highly variable across eukaryotes and has been attributed to mutation rate (Lynch and Conery 2003), metabolic rate (Vinogradov 1995, 1997), temperature (Grime 1982; Thompson 1990), developmental timing (Jockusch 1997), developmental rate (Sessions and Larson 1987; Gregory 2002b), and life cycle complexity (Martin and Gordon 1995; Gregory 2002b). Some of the largest genomes are found in obligately paedomorphic and direct developing salamanders (Sessions 2008, Fig. 2), both of which share reductions in life cycle complexity compared to biphasic species. This has been the basis for suggestions that loss of life cycle stage is associated with genome expansion (Wake and Marks 1993; Martin and Gordon 1995; Gregory 2002b). Despite recent analyses that reject this relationship for amphibians (Liedtke et al. 2018), we find that salamanders with simplified life cycles (direct developers and obligate paedomorphs), collectively or individually, are

associated with an approximately two fold increase in optimal genome size (Fig. 2; Table 1). Possible explanations for how and why genome expansion is associated with life cycle simplification are found in three prior hypotheses: (1) "junk DNA" (Martin and Gordon 1995), (2) "frugal metabolic strategy" (Szarski 1983; Licht and Lowcock 1991), and (3) "time-limited developmental rate" (Wake and Marks 1993; Gregory 2002b). It is important to note that these are not necessarily exclusive because each hypothesis addresses a somewhat different issue. The first is a mechanism for how genomes expand, the second addresses potential benefits, and the third potential limitations (for biphasic species at least).

The "junk DNA" hypothesis by Martin and Gordon (1995) was based on a family-level analysis that showed a positive correlation between genome size and "clade age", with "old" obligate paedomorphic families having the largest genome sizes. They suggested that the lack of tissue transformation (metamorphosis) in obligate paedomorphs would have left genomic regions that originally coded for underlying developmental genetic mechanisms vulnerable to decay. They predicted that this resulted in the accumulation of "junk" in these genomic regions. They did not address direct developing salamanders, but presumably the same argument could be made for and changes in genome content associated with the elimination of traits for a free-living larval stage.

This somewhat aligns with more recent explanations for genome size expansion in general (Lynch and Conery 2003), and runaway gigantic salamander genomes (Dodsworth et al. 2016; Mohlhenrich and Mueller 2016). Analyses of genome complexity across a wide range of organisms suggested that genome size is related to mutation rate and genetic drift (Slatkin 1987; Wagner and Altenberg 1996). This is in part because non-coding regions can still accrue deleterious mutations and are potentially genomic hazards (Lynch and Conery 2003). Recently, Mohlhenrich and Mueller (2016) showed that salamanders have lower mutation rates compared to frogs and suggested that this may reduce the overall hazard of carrying large insertions mutations, which are primarily gained through transposable element expansions (Sun et al. 2012; Sun and Mueller 2014). There is considerable evidence for stage-specific gene expression in amphibians (Das et al. 2006; Row et al. 2016; Wollenberg Valero et al. 2017; Sanchez et al. 2018). Relaxed selection in genomic regions that express stage-associated genes in paedomorphic and direct developing salamanders could further reduce genomic hazards by providing more real estate where transposable elements, subsequent mutations, and large-scale deletions could occur at reduced cost (Martin and Gordon 1995).

Both positive and negative trade-offs of genome size in salamanders have been proposed (Goin et al. 1968; Gregory 2002b; Sessions 2008; Lertzman-Lepofsky et al. 2019). In general, genome size is correlated with cell size (Gregory 2001; Sessions 2008; Beaulieu et al. 2008; Mueller 2015), and in some vertebrate clades these variables are inversely related to metabolic rate (Licht and Lowcock 1991; Vinogradov 1995, 1997; Gregory 2002a, 2005; Starostova et al. 2009). Salamanders and lungfish with gigantic genomes were thought to benefit from reduced metabolic rate due to larger cell size (Cavalier-Smith 1991). However, tests of this hypothesis only showed a correlation between genome size and metabolic rate when salamanders were kept at high temperatures (Licht and Lowcock 1991). Given that major shifts in metabolic rate within salamanders are not necessarily concurrent with shifts in genome size, any benefits of a large genome may be dependent on other factors such as the presence/absence of lungs (Uyeda et al. 2017), aestivation (Cavalier-Smith 1991; Gregory 2002b), migration, or the temperature during critical periods of activity. Amphibians with large genomes may be excluded from utilizing ephemeral aquatic habitats for breeding (Goin et al. 1968; Lertzman-Lepofsky et al. 2019). We did not include metabolic categories in our analyses, but we found that life cycle complexity is a better fit to genome size than larval habitat, adult habitat, or aquatic habitat stability (Table 1). For the latter this is in part due to the fact that there are some obligate paedomorphs such as amphiumids and sirenids that have large genome sizes, but can persist in ephemoral aquatic habitats by burrowing and even aestivating during dry conditions (Etheridge 1990; Smith and Secor 2017).

Since genome size predicts cell size, this attribute can govern rates of differentiation and cell migration during development (Wake and Marks 1993; Gregory 2002b). In salamanders this has been demonstrated through experimental and comparative analyses that showed correlations between genome size and hatching time (Jockusch 1997), and differentiation rate during regeneration (Sessions and Larson 1987). Amphibian metamorphosis involves extensive, and sometimes rapid, remodeling of multiple tissue systems (Shi 2000). This was the basis for speculation that the loss of discrete metamorphosis in larval form paedomorphs relaxed selection on cell size, permitting larger cells and larger genomes (Wake and Marks 1993; Gregory 2002b).

Superficially it seems inexplicable that salamanders with the largest genomes are at opposite ends of the spectrum with respect to the timing of metamorphosis. Direct developers complete transformation into a terrestrial form before hatching, while obligate paedomorphs never fully transform. However, it may not be the overall developmental time that matters, but rather the need to pass through some developmental stages rapidly, which could constrain genome size. Based on a review of genome sizes of diverse organisms, Gregory (2002b) suggested that time-limited development mattered more than overall developmental

time. Most biphasic salamanders may be subject to multiple time-limited developmental events, including rapid embryonic development to achieve early hatching as well as rapid metamorphosis coinciding with aquatic to terrestrial transitions (Fig. 4). In contrast, obligate paedomorphs progress through varying amounts of morphogenesis after hatching (Hanken 1992; Bonett 2018), but these changes tend to be more subtle and may not be time limited, as individuals can remain in the aquatic habitat.

This consequence could extend to direct developing (see below) or biphasic salamanders that transform very slowly. For example, *Dicamptodon ensatus* have a relatively large genome (~56 pg), are difficult to induce to metamorphose, and can take a long time to transform (Wagner 2014). Therefore, constraints on genome size evolution may ultimately be better described by the speed at which transformation needs to progress (Fig. 4), rather than discrete life cycles characterizations.

Congruent Genome Size Evolution of Salamanders and Frogs

The categorical relationship between genome size and direct development across amphibians appears paradoxical. On average direct developing salamanders show an increase in genome size, while direct developing frogs do not (Gregory 2002b; Liedtke et al. 2018). This may be explained by the overall differences in the duration of transformation between direct developing frogs and salamanders, and even among direct developing salamanders. First, frogs that directly develop *in ovum* can form in as little as two weeks (e.g. Ovaska and Estrada 2003), whereas direct developing salamanders can take from two to several months to transform (Jockusch 1997). Second, there is evidence of a positive correlation between the time to metamorphose and genome size in frogs (Goin et al. 1968; Liedtke et al. 2018; Womack et al. 2019), and the effects of genome size on skeletal development are time and size dependent (Womack et al. 2019). In other words, these variables are related in frogs and genome size does have developmental consequences, especially when morphogenesis is rapid. Likewise, Jockusch (1997) showed a positive correlation between genome size and hatching time for a subset of largely direct developing plethodontid salamanders when raised at common temperatures. We also found genome size to be positively correlated with metamorphic timing for direct developers (Fig. S1a). These species are more-or-less continuously differentiating, usually under the protection of a guarding parent (Wells 2010). This means that their time to metamorphosis may more accurately reflect the duration of morphogenesis, with fast direct developing salamanders having comparatively smaller genomes (Fig. 4a). It is the slow direct developers that exhibit larger genome sizes (e.g. *Hydromantes italicus* and *Bolitoglossa subpalmata*; Fig. 4d). We found no correlation between genome size and minimum time to metamorphose across a broad sampling of biphasic salamanders (Fig. S1b). Unlike direct developers, total morphogenesis (embryonic development plus metamorphosis) in biphasic salamanders is interrupted by a larval period that can vary greatly in length, from weeks to years. This intervening stage is largely dedicated to growth and may obscure time-dependent morphogenic patterns in salamanders. However, many biphasic and facultatively paedomorphic salamanders undergo rapid transformation (Fig. 4b, c). More temperature controlled morphogenic rate data are needed across species to further evaluate the specific limitations of genome size on salamander development.

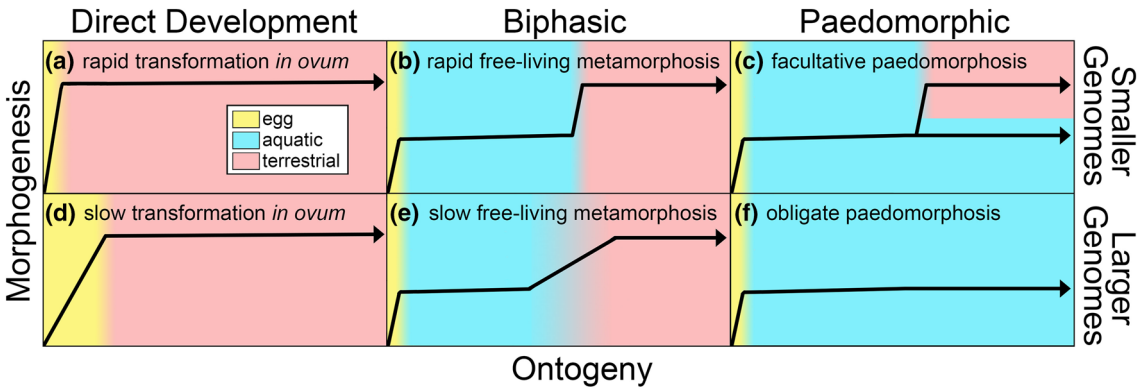

Fig. 4 Alternative patterns of salamander morphogenesis as they relate to genome size. Genome size is expected to be limited when *a* direct developing lineages rapidly transform *in ovum*, **b** biphasic lineages rapidly metamorphose between free-living stages, and **c** facultatively paedomorphic species frequently require rapid metamorphosis. Opportunities for genome expansion may occur when the rates of morphogenesis are slow or truncated such as **d** slow direct development *in ovum*, **e** slow free-living metamorphosis, and **f** obligate paedomorphosis

Conclusions

Some polymorphic species can facultatively transition between major adaptive zones, but the impact of such lifestyle swings on non-facultative traits is uncertain. Even though some salamanders can facultatively exhibit paedomorphosis, we found that their genome size is more similar to biphasic relatives than obligate paedomorphs. Obligate paedomorphs and direct developers collectively have significantly larger genomes and higher rates of genome size evolution, compared to biphasics and facultative paedomorphs. This supports the classic hypothesis that multi-stage complex life cycles limit genome size due to time-limited developmental windows such as rapid metamorphosis (Wake and Marks 1993; Gregory 2002b). Life cycle simplification with extended durations of morphogenesis (or eliminating morphogenesis) could relax these constraints and permit genomic expansion. This may have been an important factor that led to gigantic genomes in salamanders. This problem requires more fine-scale genomic resolution to map changes in genome size/content (Sun et al. 2012), and cellular/developmental comparisons to evaluate whether rates of morphogenesis influence genome size evolution (Mueller and Jockusch 2018).

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Compliance with Ethical Standards

Conflict of interest The authors declare that we have no conflict of interest with regards to this research.

Research Involving Human and Animal Rights This study did not directly involve live animals or experiments.

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