Metamorphosis imposes variable constraints on genome expansion

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Abstract

Genome size varies ~ 100,000-fold across eukaryotes. Genome size is heavily shaped by transposable element accumulation, the dynamics of which are increasingly well understood. However, given that traits like cell size and rate of development co-vary strongly with genome size, organism-level trait evolution likely shapes genome size diversity as well. Metamorphosis — a radical transformation of morphology — has been hypothesized to impact genome size because it can be a vulnerable part of the life cycle. Thus, selection may act to limit metamorphic duration, indirectly constraining the rate of development as well as genome and cell sizes. Salamanders have large and variable genomes — 3 to 40 times that of humans — and species exhibit a range of metamorphic and non-metamorphic life histories. Using salamanders, we test the hypothesis that different types of metamorphic repatterning during the life cycle impose different constraints on genome expansion. We show that metamorphosis during which animals are unable to feed imposes the most severe constraint against genome expansion. Other types of metamorphosis that differ in energetic provisioning impose less severe constraints. More generally, our work demonstrates the utility of phylogenetic comparative methods in testing the role of constraint in shaping phenotypic evolution.

Introduction

Across the tree of life, few characters exhibit the tremendous scale of variation of genome size, encompassing a ~100,000-fold range across eukaryotes (Gregory 2021). Decades of research have revealed the consistent covariation of two organismal features with genome size: a negative correlation with cell division rate, and a positive correlation with cell size (Gregory 2001, 2005). Genome size has also been associated with a variety of organismal or ecological factors including: developmental rate or complexity (Gregory 2002b), temperature (Hessen, et al. 2013), metabolic rate (Waltari and Edwards 2002; Roddy, et al. 2019), invasiveness (Pandit, et al. 2014), or speciation and extinction rates (Vinogradov 2004; Jeffery, et al. 2016), but these associations vary across studies.

Comparative biologists often think of adaptive explanations for character associations, which would suggest that genomes evolve toward an "optimum" size with respect to one or more of these correlated traits. However, the context-sensitive nature of associations with these factors belies a strongly adaptive explanation for genome size. For example, metabolic rate and genome size are correlated in some vertebrate clades, but not in others, and genome size provides no overall explanatory power for basal metabolic rate across vertebrates (Licht and Lowcock 1991; Gregory 2002a; Smith, et al. 2013; Uyeda, et al. 2017; Gardner, et al. 2020). Genome size evolution may instead evolve nearly neutrally until some threshold value is reached, beyond which fitness is

impacted (Gregory 2002b). This process is more aptly described as governed by constraints. As far as we are aware, the constraint model has never been formally tested within a phylogenetic comparative framework for a univariate trait.

Recent years have seen tremendous improvement in our understanding of the mechanistic processes by which genome size evolves. Variation in genome size can reflect the accumulation of many types of sequences, from simple repeats to increases in ploidy (Elliott and Gregory 2015; Pasquesi, et al. 2018; Carta, et al. 2020), such that the majority of the genome is non-coding or “junk” DNA. In vertebrate animals, genome size is strongly determined by the accumulation of transposable elements (TEs), sequences that replicate and spread throughout host genomes (Sotero-Caio, et al. 2017; Shao, et al. 2019). TEs are also deleted by mutations introduced during replication, recombination, and DNA repair (Michael 2014; Vu, et al. 2017). In the absence of selection or constraint on genome size, the background process for genome evolution is stochastic, with genome size increasing if TE insertions outpace deletions. TE activity is often neutral at the cellular and organismal levels, with most individual insertions and deletions missing functional regions of the genome and resulting in negligible fitness consequences (Arkhipova 2018). Non-coding DNA can therefore accumulate until genome size crosses a threshold where it begins to impact fitness through a correlated trait (e.g. cell size or developmental rate).

While the notion of constraint on genome size is conceptually appealing, there are few comparative methods that can detect constraint and possibly distinguish it from adaptation. Whether by correlation of phylogenetically independent contrasts

(Felsenstein 1985) or by phylogenetic autocorrelation (Cheverud, et al. 1985), phylogenetic comparative methods that focus primarily on the mean or "location" of the phenotype are primed to detect adaptive evolution, as selection is expected to move the phenotype toward an optimum (Simpson 1953; Lande 1980). For example, testing for a correlation between traits after independent contrasts (Felsenstein 1985) may reveal whether, on average, a phenotype covaries across species with some other trait, with the relationship maintained presumably as a result of adaptive evolution. Phylogenetic autocorrelation (Cheverud, et al. 1985) and phylogenetic regression (Grafen and Hamilton 1989) use regression approaches to separate phylogenetic and environmental (adaptive) effects on variation of the mean phenotype. Ancestral character state reconstruction methods can infer the mean phenotype of interest along nodes of the tree and are used to explore evolution in relation to some other factor, but vary in the underlying models employed (Huey and Bennett 1987; Maddison 1991; Schluter, et al. 1997). How mean-focused methods enable the detection of constraint, however, is unclear. Methods that model both the mean and variance of a stochastically evolving phenotype, such as Ornstein-Uhlenbeck-based methods (Hansen 1997; Butler and King 2004; O'Meara, et al. 2006; Beaulieu, et al. 2012), may open the door to exploring constraint by revealing whether a stochastically spreading phenotype evolves by selection, or by weak selection bounding the phenotype consistent with the presence of constraint, or by no selection at all.

Salamanders are an ideal group to test hypotheses of selection and constraint on genome size. They have both the largest genomes and the largest range of variation in genome size among vertebrates. Genome sizes range from 9 Gb – 120 Gb across the

763 extant species (Decena-Segarra, et al. 2020; AmphibiaWeb 2021; Gregory 2021), reflecting varying levels of TE accumulation. Salamanders also have exceptional life-history diversity, in particular, with metamorphosis lost, modified, and regained throughout their evolutionary history (Wiens, et al. 2005; Bonett, et al. 2014). Metamorphosis has been hypothesized to shape genome size evolution because of the effects that genome (and therefore cell) size has on the rate of development (Wake and Marks 1993; Gregory 2002b). Because metamorphosis can be a vulnerable part of the life cycle, its duration has been proposed as a target of natural selection. Selection to limit the time in metamorphosis would indirectly select for faster development, constraining genomes (and cells) to smaller sizes (Gregory 2002b; Bonett, et al. 2020).

Several studies have linked life history to genome size in salamanders, with smaller (albeit still enormous relative to other taxa) genome sizes associated with metamorphosis (Wake and Marks 1993; Gregory 2002b; Sessions 2008; Bonett, et al. 2020). Bonnett (2020) used phylogenetic comparative methods to demonstrate that salamander genome size is better explained by models that account for differences in life history — including the presence or absence of metamorphosis — as opposed to variation in ecology or habitat stability. However, this study was largely focused on the effects of facultative loss of metamorphosis on genome size evolution; thus, it did not explore the concept of evolutionary constraint versus adaptation. In addition, it did not examine the connection between energetic vulnerability and metamorphic duration, which we propose as a key conceptual link between metamorphosis and genome size.

116 In this study, we build on this previous body of work, revisiting the hypothesis that
117 the radical morphological repatterning associated with metamorphosis imposes evolu-
118 tionary constraints on genome size in salamanders. In particular, we model characteris-
119 tics that are likely to place energetic limits on the duration of metamorphosis: Does re-
120 patterning happen inside the egg, fueled solely by yolk stores? Does repatterning hap-
121 pen in an organism that is free-living, but unable to feed? We use life history and ge-
122 nome size data to inform stochastic models of trait evolution that explore how different
123 metamorphic regimes interact with genome expansion. More generally, we demonstrate
124 the ability of OU-based stochastic models to identify trait evolution governed by con-
125 straint, expanding their use beyond classic scenarios of adaptive evolution.
126
127 *Life History Regimes Constraining Genome Size Evolution*
128
129 In the absence of any selection or constraint, genome size in salamanders is expected
130 to expand by biased stochastic evolution. Overall mutation pressure in the clade leads
131 to TE accumulation, as TE deletion rates are low and TE silencing is incomplete (Sun,
132 Arriaza, et al. 2012; Frahry, et al. 2015; Madison-Villar, et al. 2016; Mohlhenrich and
133 Mueller 2016). Salamanders' enormous genomes are the cumulative result of unusually
134 high levels of TE activity and retention (Sun, Shepard, et al. 2012; Sun and Mueller
135 2014; Keinath, et al. 2015; Liedtke, et al. 2018; Nowoshilow, et al. 2018).
136 The ancestral salamander life history included a larval growth stage followed by
137 metamorphosis. Metamorphosis is a radical transformation of morphology during the
138 life cycle that produces strikingly different, largely decoupled larval and adult forms

(Moran 1994). During metamorphosis, as during embryogenesis, salamanders undergo rapid cell division, differentiation, migration, and apoptosis (Alberch 1989). The dynamics of these cellular processes are affected by genome size and cell size; as genome/cell sizes increase, developmental rates throughout ontogeny — from embryogenesis through metamorphosis — slow down (Horner and Macgregor 1983; Jockusch 1997). Metamorphosis has been lost and regained from the life cycle numerous times by different mechanisms (Chippindale, et al. 2004; Mueller, et al. 2004; Wiens, et al. 2005; Bonett, et al. 2014), producing multiple life history regimes that could shape genome size evolution. We outline these regimes, and their predicted effects on genome size evolution, below and in Figure 1:

Paedomorphosis: In paedomorphic species, some or all stages of metamorphic repatterning are lost, and organisms reach sexual maturity retaining largely larval traits (Gould 1977). Because there is no selection to limit time in metamorphosis, there is no associated constraint on genome size. Thus, paedomorphs are expected to most closely reflect the background condition of stochastic genome expansion.

Direct development: In direct-developing species, the larval growth stage is eliminated, and embryogenesis and metamorphosis are integrated into a single sequence of developmental events that takes place inside the egg (Alberch 1989; Rose 2014). This entire sequence must be fueled by yolk provisioned by the mother. Thus, we might expect selection to limit time in metamorphosis, imposing a constraint on genome size.

Non-feeding metamorphosis: Within the salamander family Plethodontidae, metamorphic repatterning events happen relatively synchronously in a free-living organism. During this metamorphosis, the organisms are unable to feed because of the replacement

of cartilaginous elements associated with the change from suction to projectile feeding (Rose 1995a; Deban and Marks 2002). The entire process must be fueled by energy reserves built up during the larval stage. Thus, we might expect selection to limit time in metamorphosis, imposing a constraint on genome size.

Feeding metamorphosis: In non-plethodontid salamanders, metamorphic repatterning events happen in a free-living organism that is able to feed throughout the transformation. Thus, we would not expect selection based on energy demands to limit time spent in metamorphosis. There are, however, other ways in which metamorphosis can increase vulnerability that might translate into selection on metamorphic duration, constraining genome size.

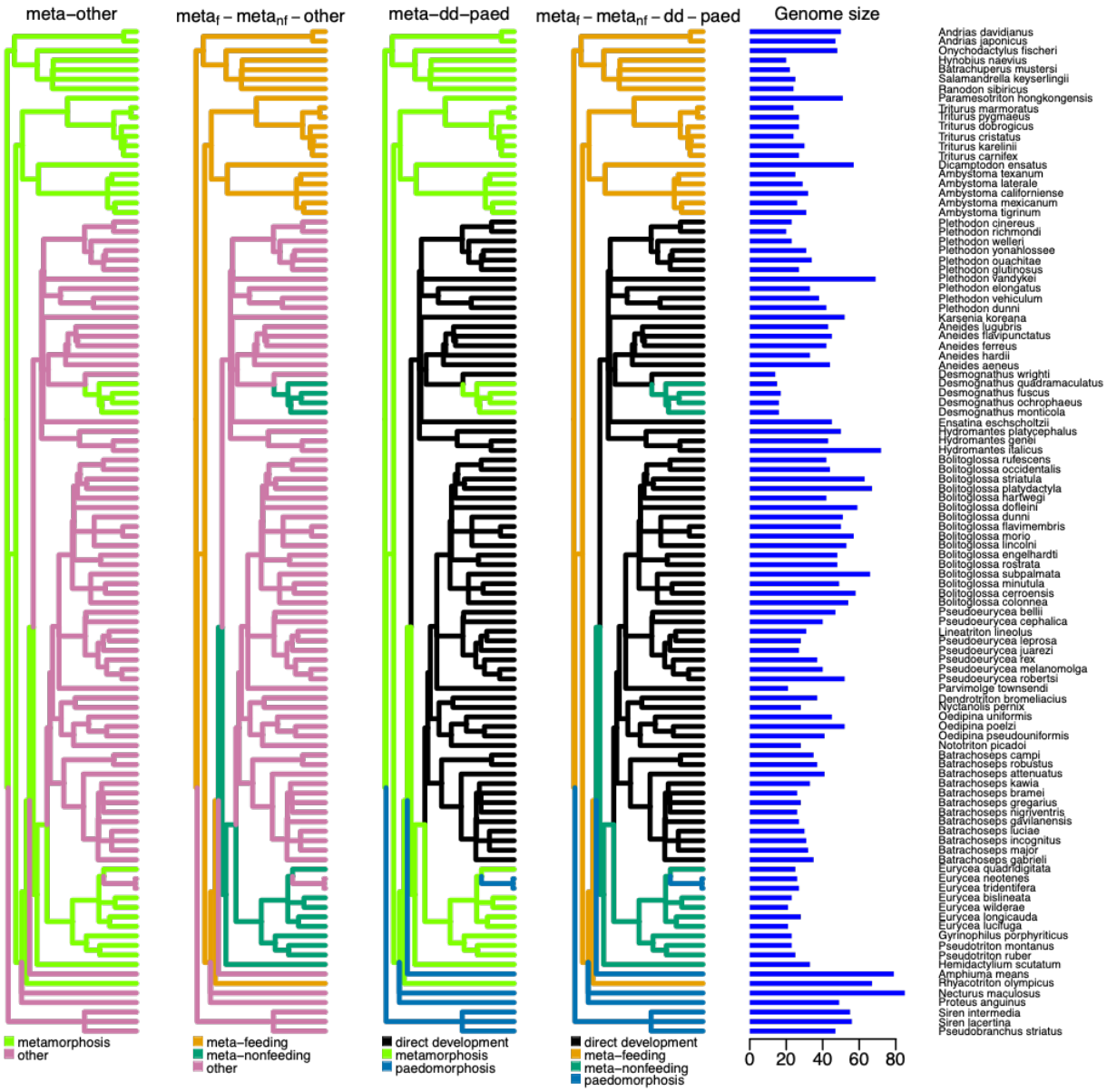

Figure 1. Alternative hypotheses for constraints imposed by development on genome size evolution in salamanders. On each phylogeny, alternative life history regimes are painted in different colors as indicated in each legend (see text). Genome sizes are shown on the right in pg (1 pg = 978 Mb).

Methods

Taxon Sampling, Genome Size, and Phylogeny

Genome size data were available for 106 species of salamanders (out of a total of 763 currently named extant species), encompassing all ten salamander families and 35 of 68 genera (Supplemental Table 1) (AmphibiaWeb 2021; Gregory 2021). We transformed the data with natural logarithms prior to analysis. Our sampling includes representatives with diverse life histories: direct development, paedomorphosis, feeding metamorphosis, and non-feeding metamorphosis. Hereafter, we distinguish between "feeding" and "non-feeding metamorphosis", and use "metamorphosis" to indicate all species which undergo metamorphosis, whether feeding or non-feeding. Several lineages are facultative paedomorphs, which retain the ability to undergo metamorphosis under certain conditions. We coded these taxa as metamorphic, reflecting the experience of constraint, even if only occasionally, and supported by recent work examining the evolutionary impacts of facultative paedomorphosis (Bonett, et al. 2020).

We used a previously published phylogeny (Mueller, et al. 2008; Pyron and Wiens 2011; Vieites, et al. 2011; Zheng, et al. 2011) and estimated branch lengths on this topology using sequence data for two mitochondrial genes – cytochrome-b and 16S obtained from GenBank. Additional Sanger sequences were collected as necessary at the Colorado State University sequencing core facility or the Joint Genome Institute following (Mueller, et al. 2004) (Supplemental Table 1). Loci were aligned using MUSCLE

(Edgar 2004). Branch lengths were estimated for each locus independently and then averaged, weighted by gene length. Branch lengths were estimated using RAxML (Stamatakis 2006) using the best-fit (AIC) model of nucleotide substitution in ModelTest 3.6 (Posada and Crandall 1998), with Cyt-b partitioned by codon. The resulting tree was ultrametricized using penalized likelihood implemented in r8s with the truncated Newton algorithm and cross validation to select the optimal smoothing parameter value (Sanderson 2003).

Models of Genome Size Evolution

We modeled genome size evolution using both Brownian motion (BM) and Ornstein-Uhlenbeck (OU) models of evolution (Hansen 1997; Butler and King 2004; O'Meara, et al. 2006; Beaulieu, et al. 2012). As these models have been described previously, we cover them only briefly here. The BM model is the simplest stochastic model used in comparative analysis and has a single rate parameter σ, which can be thought of as a stochastic noise intensity parameter shared by all taxa and describing the magnitude of the independent random walks of the trait evolving along the branches of the phylogeny. This model predicts that across lineages, there will be no change in mean phenotype, but variance will grow through time as lineages wander over trait space, and the expected variance between two lineages will be proportional to the time since their divergence. Conceptually, the BM model may be a good candidate for purely stochastic genome size evolution if increases occur as frequently as decreases.

225 The multiple-rate BM model, introduced by O'Meara, et al. (2006), allows differ-
226 ent σ values across different portions of a phylogenetic tree. We use this model to rep-
227 resent changes in the rate of stochastic evolution accompanying changes in constraint
228 resulting from shifts in life history regime. Under this model, lineages evolving under dif-
229 ferent regimes may differ in variance.
230
231 OU models generalize the BM model by including terms that allow the mean to
232 shift as well as allowing variance to narrow. They include a deterministic component of
233 trait evolution that models the tendency of the trait to move toward an equilibrium value.
234 Mathematically, the model for trait evolution expressed as a differential equation is
235
236 $$dX(t) = \alpha(\theta(t) - X(t)) + \sigma dB(t),$$
237
238 where $\theta(t)$ is the deterministic equilibrium for the trait at time *t* and α is an evolutionary
239 rate describing the *strength of the deterministic pull (e.g. selection)* towards that equilib-
240 rium along a branch of a tree. Hypotheses regarding trait evolution are specified by
241 painting "regimes" on the tree to indicate where these parameters are expected to shift.
242 The simplest OU models allow multiple equilibria leading to the evolution of differences
243 in mean phenotype across regimes (Hansen 1997; Butler and King 2004); for example,
244 in the current study, separate equilibria might exist for paedomorphs, metamorphosers,
245 and direct developers. Further model extensions also allow the strength of the determin-
246 istic pull and the stochastic noise intensity to vary across regimes (Beaulieu, et al.

2012), as might be consistent with the lifting or imposition of constraints on genome size evolution.

We formalized five hypotheses for how life history regime could constrain genome size evolution in salamanders: (1) Brownian motion: Genome size evolves by purely stochastic evolutionary processes with neither constraint nor bias. (2) *metamorphosis-other*: Metamorphosis imposes a constraint on genome expansion that is distinct from the other life histories. (3) *meta-paed-dd*: Metamorphosis, direct development, and paedomorphosis each impose distinct constraints on genome expansion. (4) *meta$_f$-meta$_{nf}$-other*: Feeding metamorphosis (*meta$_f$*) and non-feeding metamorphosis (*meta$_{nf}$*) each impose constraints that are distinct from those experienced by salamander species with other life histories. (5) *meta$_f$-meta$_{nf}$-paed-dd*: non-feeding metamorphic species, feeding metamorphic species, paedomorphic species, and direct-developing species each experience different levels of constraint on genome expansion.

These five biologically-inspired hypotheses are specified by fitting evolutionary models that allow their parameters to vary with life history regime. The simplest form allows the equilibrium to vary with life history regime while assuming the rates of evolution are constant across the phylogeny. Additional subhypotheses fit evolutionary models that vary stochastic noise intensity values (σ), and/or deterministic pull values (α) according to life history regime (Table 1). In all, we tested 21 models to explore life history constraints on genome size and model parameters (Table 2). We fit all of the models with the character state of the node at the base of the plethodontid lineage defined as metamorphosing as well as direct-developing (Bonett, et al. 2014), and found that the choice of the basal character state made no qualitative difference to our conclusions. In

addition, we made a series of pairwise comparisons to further assess the degree of influence of feeding metamorphosis, paedomorphosis, direct development, and non-feeding metamorphosis on genome size evolution in combinations of increasing complexity (Table 3).

We fit models of evolution using the software package OUwie (Beaulieu, et al. 2012) and compared the multiple optimum OU models with results fitted in OUCH (Butler and King 2004; King and Butler 2009). We modified OUwie to fit models with regime changes that occur at the nodes, as in OUCH, rather than the default behavior of OUwie which places regime changes mid-way along the branch between a clade of interest and its ancestor. We recognize that while either choice can be argued to be arbitrary, assuming that the regime change occurred simultaneously with the origin of the clade that bears the phenotype as indicated in Figure 1 seems reasonable. All analyses were conducted in the R statistical computing environment (R Core Team 2020). We note here that OUwie has different options for dealing with the root state, $X(0)$. Absent any information about the phenotype deep in the tree, this parameter is often very difficult to estimate in OU models (Cressler, et al. 2015). One alternative is to assume that the value of $X(0)$ is distributed according to the stationary distribution of an OU process, which eliminates this parameter by absorbing the variance into the covariance matrix implied by the phylogeny itself (Ho and Ané 2013). However, OUwie does not currently support this approach for OU models with multiple α or σ parameters, and as far as we know, the mathematical modifications for these models have not been worked out. Because the option to estimate the value of the root node as in the earlier implementations

(Hansen 1997; Butler and King 2004); and specified in OUwie by setting root.station=FALSE) is available for all of the models of interest, we used it for all model fitting. However, as we show in the Supplementary Materials, the parameter estimates of a single-α, single-σ OU model fit assuming the root is in the stationary distribution are very different from the parameter estimates obtained by fitting the root state, although model selection conclusions hold. This large difference in the parameter values reinforces a general point in phylogenetic comparative hypothesis testing, which is that parameter estimation is often more fraught than hypothesis testing, and as such, parameter estimates should be interpreted with caution (Beaulieu, et al. 2012; Ho and Ané 2013; Ho and Ané 2014; Cressler, et al. 2015; Cooper, et al. 2016).

When fitting OU models, there are datasets for which it is possible to recover an extremely low value of α (weak deterministic pull), with equally extremely large θ (equilibrium values). This combination can reasonably be interpreted as weak selection, or a biased nearly-random walk. Rather than look only at these parameters in isolation, we considered whether it may be more informative to determine their combined effect on trait evolution. To this end, we calculated the value of $\alpha(X - \theta)$ for each life history regime to estimate any "deterministic trend" imposed by the best-fitting model, where X is the average genome size of species in that regime at the end of the evolutionary process.

311 Table 1. BM and OU models with single or multiple parameters used to fit the data.
312 Numbers in parentheses specify (1) model parameters and notation, (2) parameters that
313 remain constant across the phylogeny, (3) parameters that vary with shifts in life history
314 regime, (4) O'Meara (2006) model notation, and (5) notes for the model implementa-
315 tions and citations.
316

Models	**Uniform**	**Variable with Regime**	**O'Meara Notation**	**Notes**
(1)	(2)	(3)	(4)	(5)
Single noise intensity σ	σ		BM1	Classic BM model of (Felsenstein 1985)
Multiple noise intensities σ_i		σ_i	BMS	Multiple-rate BM model of O'Meara et al. (2006)
Multiple equilibria $\theta i, \sigma, \alpha$	σ α	θ_i	OUM	OU model of Butler and King (2004)
Multiple equilibria and deterministic pull strengths $\theta i, \sigma, \alpha i$	σ	α_i θ_i	OUMA	Multiple-α model of Beaulieu et al. (2012).
Multiple equilibria and noise intensities $\theta i, \sigma i, \alpha$	α	σ_i θ_i	OUMV	Multiple-σ model of Beaulieu et al. (2012)

Multiple equilibria, noise intensities, and deterministic pull strengths $\theta i, \sigma i, \alpha i$		σ_i α_i θ_i	OUMVA	Full model of Beaulieu et al. (2012).

317
318

Model Comparison and Parameter Estimation

We compared the fit of each of the models using the Akaike Information Criterion corrected for small sample size (AIC_c). We further performed model selection bootstrap analysis (phylogenetic Monte Carlo; (Boettiger, et al. 2012) for targeted comparisons of the models in Table 3 to assess the strength of evidence for different hypotheses as well as the power to detect differences in model support. Model selection bootstrap analysis is necessary because AIC_c differences can favor more complicated models, even when a simpler model is correct (Boettiger, et al. 2012). For each comparison, we computed the observed likelihood difference,

$$\delta_{obs} = -2 (\log L_0 - \log L_1),$$

where L_0 is the likelihood of the simpler model and L_1 is the likelihood of the more complex model.

Determining whether δ_{obs} is significantly different from a null expectation requires an approximate *p*-value — the probability of observing δ_{obs} *if the simpler model were true*. That is, we need to compare the value δ_{obs} to the distribution of δ values under the simpler model. To create this distribution, we generated 500 datasets by simulating the simpler model at its MLE parameter estimates; we then fit both the simpler and more complex models to each simulated dataset and computed the values of δ, producing a null distribution of δ under the simpler model. We compared the observed value of δ to this null distribution to calculate an approximate *p*-value.

Power conveys the (desirable) probability of rejecting the simpler model when the more complex model is true. To estimate power, we generated 500 datasets by simulating the more complex model at its MLE parameter estimates; we then fit the two models

and computed the values of δ. The fraction of these δ values that are greater than the 95% quantile of the distribution generated under the simpler model (described above) gives an estimate of power. All data and code necessary to carry out the analysis in this manuscript can be found at https://github.com/claycressler/genomesize and in Supplemental Material.

Results

The best-fitting model for salamander genome size evolution accounted for four regimes: both non-feeding and feeding metamorphosis, paedomorphosis, and direct development (*meta$_f$-meta$_{nf}$-paed-dd*; Table 2) under an OU model that allowed both equilibrium genome size and noise intensity to vary across these regimes (θ_i, σ_i, α, Table 2). An identical 4-regime model with only a single noise intensity fit nearly as well (Table 2). Additionally, the three-regime *meta$_f$-meta$_{nf}$-other* (θ_i, σ_i, α) hypothesis provided some explanatory power. These three models were far superior to the remaining models.

Overall, the addition of multiple α values resulted in worse model fit relative to a uniform α value, whereas the addition of multiple σ values relative to a single σ both improved and worsened model fit, depending on the hypothesis (Table 2). We present the results with the character state of the node at the base of the plethodontid lineage defined as metamorphosing. In the Supplementary Materials, we show that defining this node as direct-developing has no effect on the evolutionary conclusions we draw here.

The model selection bootstrap analysis allows us to quantify the degree of improvement in explanatory power provided by moving between specific hypotheses (Figure 2). Note that for each comparison, we used the version of the OU model that had the lowest AICc (Table 2), so comparisons involving *metamorphosis-other* and *meta-paed-dd* used the single-σ model, whereas comparisons involving *meta$_f$-meta$_{nf}$-paed-dd* and *meta$_f$-meta$_{nf}$-other* used the multiple-σ model.

Based on these results, we can reject any purely stochastic hypothesis for genome size evolution, as a model that allows for separate equilibrium values for metamorphosers was far superior to any purely neutral model (Table 2; Figure 2A). Specifying distinct equilibrium values for non-feeding and feeding metamorphosis substantially improves the explanatory power of any model (Figure 2C, D). In particular, the separation of metamorphosis into feeding and non-feeding categories provided far greater improvement of the model than subdividing the "other" category into direct development and paedomorphosis (compare the results in Figure 2C to those in Figure 2B, and Figure 2D to 2E). Allowing distinct noise parameters for each regime slightly improves the fit of the OU models with separate equilibria for non-feeding and feeding metamorphosis (Table 2), although the improvement over the single-σ model is not significant (Comparison [6], Table 3; Figure 2F). Therefore, the best model included distinct equilibrium values for direct development, paedomorphosis, feeding metamorphosis, and non-feeding metamorphosis.

Table 2. Model comparison statistics. Best models (interrogated by bootstrap, Table 3) indicated in bold. Model parameterizations are indicated by: σ = Brownian motion; σ_i = Brownian motion with multiple noise intensities; θ_i, σ, α = OU model with multiple equilibria; θ_i, σ_i, α = OU model with multiple equilibria and multiple noise intensities; θ_i, σ, α_i = OU model with multiple equilibria and multiple deterministic pull strengths; θ_i, σ_i, α_i = OU model with multiple equilibria, noise intensities, and deterministic pull strengths.

ΔAIC_c	Model					
	OU Models				BM Models	
Hypotheses	$\theta i, \sigma i, \alpha$	$\theta i, \sigma, \alpha$	$\theta i, \sigma i, \alpha i$	$\theta i, \sigma, \alpha i$	σi	σ
meta$_f$-meta$_{nf}$-paed-dd	**0**	**1.1**[a]	7.5	8.1	12.9	
meta$_f$-meta$_{nf}$-other	1.4[b]	3.6	6.1	8.1	11.1	
meta-paed-dd	6.0	3.4	10.7	7.9	11.4	
metamorphosis-other	5.8	4.1	8.0	6.3	9.5	
Brownian motion						31.8

[a] *meta$_f$-meta$_{nf}$-dd-paed* with single or multiple noise parameters σ are compared in Figure 2F.

[b] *meta$_f$-meta$_{nf}$-other* is compared against the best model via bootstrap in Figure 2E.

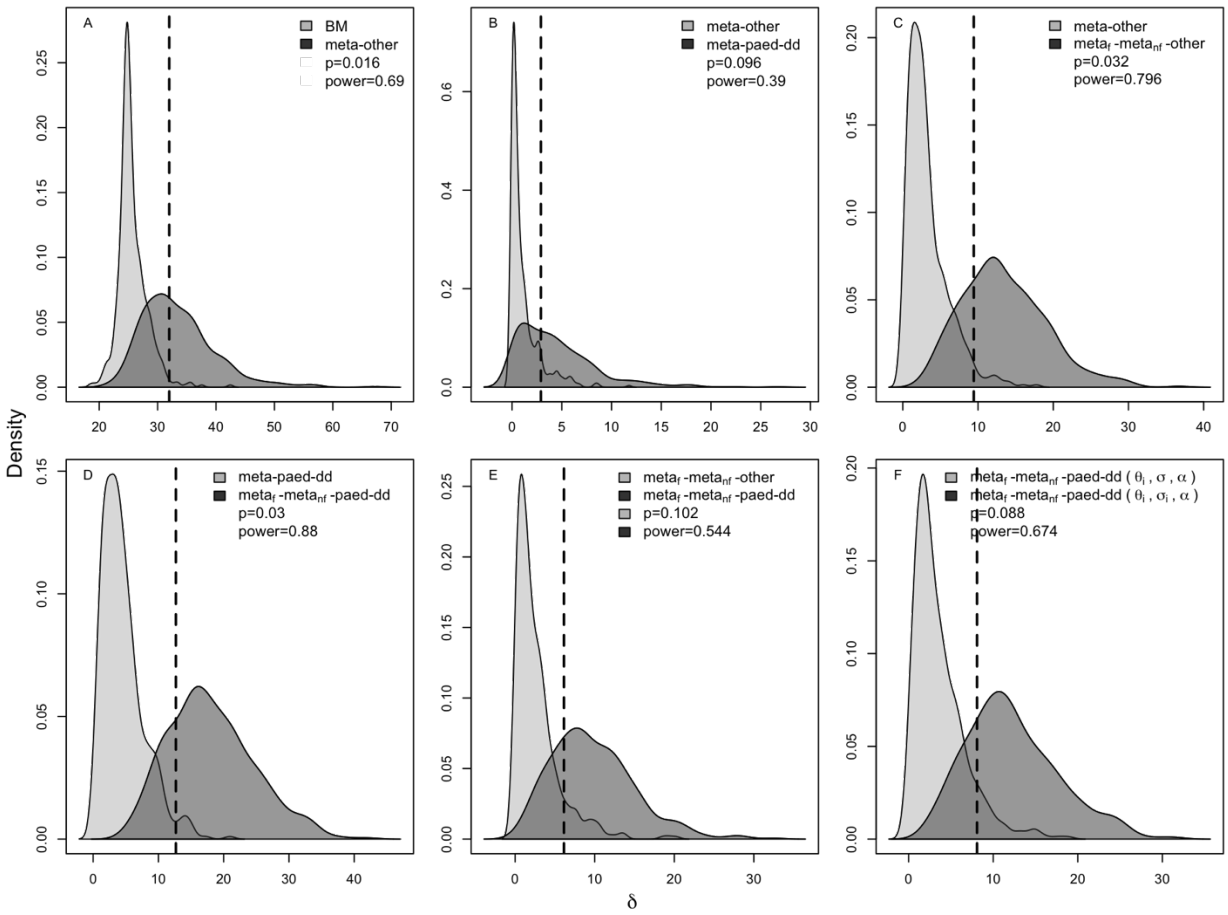

Figure 2. Bootstrap distributions of the likelihood difference (δ) calculated by generating 500 datasets under each of two competing models at their MLE parameter estimates, refitting the two models, and computing δ. The light gray region shows the probability density of the parameter when the data is generated by the simpler model; the dark gray region shows the density when the data is generated by the more complex model. The dashed line gives the observed value (δ_{obs}) from fitting the actual genome size data. The reported *p*-value is the fraction of the light gray distribution that lies to the right of δ_{obs}; the power is the fraction of the dark gray distribution that lies to the right of the 95th percentile of the light gray distribution. Each panel evaluates the support for a different hypothesis: (A) metamorphosis imposes a constraint on genome expansion; (B) there are distinct constraints imposed by the different non-metamorphosing strategies, direct development and paedomorphosis; (C) non-feeding metamorphosis imposes a distinct constraint from feeding metamorphosis; (D) non-feeding metamorphosis imposes a distinct constraint from feeding metamorphosis, after accounting for differences in non-metamorphosing strategies; (E) non-metamorphosing strategies impose unique constraints, after accounting for differences between feeding and non-feeding metamorphosis; (F) after identifying *meta$_f$-meta$_{nf}$-paed-dd* as best-fitting, this comparison tested whether fitting each regime with a distinct stochastic noise intensity improved fit to the data.

Parameter values for each regime estimated under the best-fitting model are presented in Table 3. Direct-developers, non-feeding metamorphosers, paedomorphs, and feeding metamorphosers have broadly overlapping stochastic noise intensity (σ) values. Deterministic pull strength (α) takes an extremely small value, and the equilibrium values (θ) take extreme values both large and small. Although the deterministic pull strength parameter is vanishingly small, nevertheless, the models that account for metamorphosis are far superior to a purely stochastic model (Table 2). We note that the extreme nature of the parameter estimates is a consequence of the handling of the root state (see Supplementary Materials).

Table 3: Maximum likelihood parameter estimates and parametric bootstrap confidence intervals for the best-fitting model (*meta$_f$-meta$_{nf}$-dd-paed* $\theta i, \sigma i, \alpha$*:* separate equilibrium values and noise intensities for lineages in the four life history regimes: dd = direct development, meta$_{nf}$ = non-feeding metamorphosis, meta$_f$ = feeding metamorphosis, paed = paedomorphosis).

Parameter		MLE	95% CI
Deterministic pull strength	α	1.05e-7	(1.01e-7, 1.7)
Stochastic noise intensity	σ_{dd}	0.365	(0.308, 0.545)
	σ_{meta_nf}	0.355	(0.252, 0.525)
	σ_{meta_f}	0.187	(0.108, 0.283)
	σ_{paed}	0.249	(0.112, 0.464)
Equilibrium value	θ_{dd}	-3.77e5	(-2.67e6, 1.99e6)
	θ_{meta_nf}	3.62	(3.32, 3.90)
	θ_{meta_f}	-5.55e6	(-7.62e6, 3.28)
	θ_{paed}	4.41e6	(3.96, 6.29e6)

There is evidence of a deterministic trend $\alpha(X - \theta)$ towards small genome size in salamanders that undergo non-feeding metamorphosis as well as a trend towards large genome size in paedomorphic salamanders (Table 4). In contrast, genome sizes in direct developing and feeding metamorphosing salamanders show no such trends. The range of values for the strength of the overall deterministic trend (Table 4) is much greater than the range of values for stochastic noise intensity (Table 3).

Table 4: Estimates of the deterministic trend in the best-fitting model for lineages evolving in each life history regime, based on the average genome size (log-scale) of salamanders in each regime. Genome sizes (in pg) are also shown in parentheses.

Regime	Average log genome size X	Deterministic trend $\alpha(\theta - X)$
Direct development	3.66 (38.9 pg)	-0.0396
Feeding metamorphosis	3.46 (31.8 pg)	1.68e-8
Non-feeding metamorphosis	3.07 (21.5 pg)	-0.583
Paedomorphosis	3.89 (48.9 pg)	0.463

Discussion

Biased stochastic expansion and life history constraints shape genome size.— Overall, evolution of genome size in salamanders is well-described by a weakly deterministic model with separate equilibria for each life history regime and moderate stochasticity. An increased level of constraint in lineages that evolved non-feeding metamorphosis, as well as a decreased level of constraint in lineages that have lost metamorphosis, exert the greatest deterministic influences on the evolution of genome size. The strength of the deterministic trend towards genome expansion for paedomorphs is roughly the same as the strength of the deterministic trend towards genome reduction for non-feeding metamorphosers; both are an order of magnitude stronger than the trend for direct-developers. Feeding metamorphosers have no deterministic trend (Table 4). Taken together, these results, and the observed spread of genome sizes within regimes, suggest that genome size evolution is driven by stochasticity with a bias toward increase — likely representing TE insertions that outpace deletions — constrained to varying degrees by metamorphic repatterning, when selection acts to limit metamorphic duration.

Metamorphosis as a vulnerable stage of the life cycle in amphibians

Metamorphosis has been posited to exert evolutionary pressure for reduced genome size in amphibians to speed development during a vulnerable life stage. This hypothesis comes primarily from evidence in frogs. Metamorphosing frogs experience higher predation levels because they can neither swim nor hop effectively (Wassersug and Sperry

1977; Arnold and Wassersug 1978). In addition, frogs are unable to feed during metamorphosis, at a time when their energetic requirements can nearly double as compared to the period immediately preceding metamorphosis (Orlofske and Hopkins 2009; Wright, et al. 2011). Despite being homologous and retaining broad similarities at the transcriptomic, hormonal, and organismal levels (Sanchez, et al. 2018), salamander metamorphosis is different — and less dramatic — than frog metamorphosis. The process takes much longer in salamanders; timescales are on the order of weeks to months rather than days (Norman 1985; Downie, et al. 2004; Vladimirova, et al. 2012; Sanchez, et al. 2018), suggesting little, if any, time pressure. Metamorphosing salamanders do not experience compromised locomotion and are thus not more vulnerable to predation as are frogs (Landberg and Azizi 2010). In addition, metamorphosing salamanders do not have higher energetic requirements compared to non-metamorphosing individuals of the same species (Vladimirova, et al. 2012). Some salamanders, however, are unable to feed during metamorphosis, requiring that they undergo the transformation using only stored energy reserves (i.e., non-feeding metamorphosers) (Deban and Marks 2002)). Direct developers undergo the transformation inside the egg, fueled only by yolk stores. Genome size constraints for these two life history regimes likely reflect energetic vulnerability that is not relevant for the other two life histories considered here: feeding metamorphosis and paedomorphosis.

Genome size evolution in feeding metamorphosers.—Lineages that undergo metamorphosis, but are able to feed throughout the process, show no deterministic trend in genome size evolution. Rather, trait evolution is described by moderate stochastic noise

499 around an equilibrium value that we interpret as a balance between TE accumulation
500 and a constraint imposed by metamorphosis. Although the animals are able to feed,
501 there are other ways in which fitness can be lowered during salamander metamorpho-
502 sis; for example, metamorphosing individuals are less able to exploit stream habitat re-
503 fugia than either larvae or adults, which increases their mortality (Lowe, et al. 2019).
504 Our results indicate that feeding metamorphosis imposes a less severe constraint on
505 genome size than non-feeding metamorphosis, and we infer that the constraint is medi-
506 ated by vulnerabilities other than depletion of energetic stores.
507
508 *Genome size evolution in non-feeding metamorphosers.*— Although other analyses
509 have demonstrated a link between metamorphosis and genome size in salamanders
510 (Wake and Marks 1993; Gregory 2002b; Sessions 2008; Bonett, et al. 2020), here we
511 show that non-feeding metamorphosis imposes a substantial and distinct constraint, rel-
512 ative to feeding metamorphosis, as predicted if energetic vulnerability shapes the dura-
513 tion of metamorphosis. The deterministic trend toward smaller genome sizes within this
514 regime is consistent with the imposition of a more severe constraint against genome ex-
515 pansion — or, put another way, selection towards genome size reduction —— to
516 shorten the duration of metamorphosis.
517 The evolution of non-feeding metamorphosis in plethodontids has been an im-
518 portant target of research because the phylogeny suggests that it evolved from a direct-
519 developing ancestor(s), which necessitates the evolutionary reappearance of the “lost”
520 larval stage (Chippindale, et al. 2004; Mueller, et al. 2004). This evolutionary transfor-

521 mation series was historically considered unlikely, although more recent work has re-
522 vealed potential scenarios for regain of the larval stage (Bonett, et al. 2005; Bonett, et
523 al. 2014). Under this scenario, in the direct-developing ancestral lineage(s), metamor-
524 phic repatterning steps were retained as part of the longer sequence of developmental
525 events that occurred inside the egg (Kerney, et al. 2012). These changes were likely
526 mediated by evolutionary changes in the timing of thyroid hormone activity and re-
527 sponse (Rose 1995a, b; Bonett 2016). The re-evolution of (now non-feeding) metamor-
528 phosis reflected the insertion of a long, slow-growing larval growth phase back into on-
529 togeny, followed by the synchronous occurrence of more drastic metamorphic repattern-
530 ing events in the free-living organism (Rose 1995b, c; Beachy, et al. 2017). Metamor-
531 phic repatterning is more extreme in plethodontids than in other salamanders. Im-
532 portantly, it involves a complete remodeling of the feeding apparatus, with the cerato-
533 branchials (cartilaginous components of the tongue skeleton) replaced by new struc-
534 tures in the adult rather than remodeled from existing larval structures; this results in the
535 inability to feed during metamorphosis (Alberch 1989; Rose 1995c; Deban and Marks
536 2002). Under the classical scenario, in contrast, this synchronization of metamorphosis
537 and drastic remodeling of feeding structures would have evolved in a metamorphosing
538 ancestor to produce non-feeding metamorphosis (Wake and Hanken 2004). Under ei-
539 ther scenario, our results illustrate how phylogenetic comparative methods can reveal
540 the evolutionary forces that have acted on genome size as lineages moved through the
541 different life history regimes.
542

543 *Genome size evolution in direct developers.*—In direct-developing lineages, some or all
544 of the developmental steps of metamorphic repatterning occur inside the egg at the end
545 of embryogenesis (Alberch 1989; Kerney, et al. 2012); these lineages show a weak de-
546 terministic trend towards genome size reduction. Because they are occurring in an em-
547 bryo rather than a free-living organism that has undergone a growth period, the repat-
548 terning happens to a smaller number of cells in a smaller overall mass of tissue com-
549 pared with metamorphosing lineages (Downie, et al. 2004). Thus, the energetic require-
550 ments for comparable developmental steps are lower in direct developers than in meta-
551 morphosers. On the other hand, the energy to fuel these steps comes from yolk stores
552 which, although plentiful in direct developers, are still finite (Wake and Hanken 2004).
553 Thus, we infer that direct development imposes a less severe constraint on genome
554 size than does non-feeding metamorphosis, mediated by the potential for depletion of
555 energy stores if the duration of metamorphic repatterning during embryogenesis is too
556 long. We note that there is greater variation across direct developers in metamorphic re-
557 patterning than is modeled here. In some cases, the sequence of developmental events
558 is shortened because the formation of larval structures is lost from ontogeny. In other
559 cases, most or all events of embryogenesis and metamorphosis are retained but occur
560 inside the egg (which allows for the possibility of re-evolution of metamorphosis; Alberch
561 1989). We would predict more severe constraints in these latter lineages. Although we
562 treated both scenarios as a single category for simplicity, these two types of direct de-
563 velopment may be different in their effects on genome size evolution and warrant more
564 detailed study.
565

566 *Genome size evolution in paedomorphs.*—Paedomorphic salamanders retain a larval
567 body form throughout life and show a deterministic trend towards genome expansion.
568 This trend is consistent with TE accumulation proceeding to higher overall levels, un-
569 checked by any constraints imposed by metamorphic repatterning. However, we do not
570 suggest that genome size is free from all constraints. The impacts of decreased sur-
571 face-area-to-volume ratio that accompany increased cell size likely impose an upper
572 limit on cell function that salamanders may well have reached (Chan and Marshall
573 2010); their cells are among the largest found in animals (Horner and Macgregor 1983).
574 In addition, the duration of embryogenesis may well have an upper bound that con-
575 strains genome expansion at the extremely high end. In the past, huge cells have been
576 proposed as adaptive because they coincide, at broad taxonomic levels, with low meta-
577 bolic rates; salamanders and lungfishes have the lowest metabolic rates and the largest
578 genomes/cells within vertebrates. This correlation led to the proposal that selection
579 shaped an adaptive “frugal metabolic strategy” in these taxa (Szarski 1983; Olmo, et al.
580 1989). More recent analyses of the relationship between genome/cell size and meta-
581 bolic rate, however, have failed to find a clear relationship (Licht and Lowcock 1991;
582 Uyeda, et al. 2017; Gardner, et al. 2020). Thus, empirical evidence that huge genomes
583 are a product of directional selection is currently lacking. Our results are more con-
584 sistent with the relaxation of a constraint against genome expansion because of the ex-
585 tremely weak deterministic pull strength and strong stochastic noise parameters.
586
587 *Model complexity to capture the evolutionary process.*—While the best model includes a
588 deterministic pull parameter, its magnitude is miniscule. Yet our model selection results

589 demonstrate that models with deterministic pull provide a huge improvement over any
590 purely stochastic model (Table 2). One of the challenges of an OU model with a weak
591 deterministic component is that the model parameters will be poorly defined (Cressler,
592 et al. 2015). Intuitively, if the pull parameter (α) is important but close to zero, the equi-
593 libria (θi) can take on a wide range of values in combination with a range of values for
594 the stochastic parameter σ and explain the phenotypic distribution equally as well. The
595 difference between a purely stochastic (BM) model and one that has any degree of de-
596 terministic pull is that the variance of a BM model will grow unbounded over time (as
597 variance of a trait undergoing a Brownian motion process is proportional to time),
598 whereas the variance in a model with deterministic pull will not (variance ~$\sigma^2/2\alpha$)
599 (Hansen and Martins 1996; Butler and King 2004). Thus, while the phenotype may ex-
600 plore a wide range of values in an OU model with very small α, it will remain bounded.
601 For paedomorphs, a weak deterministic pull allows the mean to wander, while a far-
602 away equilibrium value captures a deterministic trend toward increase. In OU models,
603 increasing deterministic pull strength influences the approach to the equilibrium, but
604 also will tend to dampen stochastic effects (apart from the influence of σ), so it is possi-
605 ble to have both weak deterministic pull and substantial noise in the stochastic process.
606 This analysis demonstrates that deterministic pull can exert an important evolutionary
607 influence, even if the magnitude of alpha is weak.

608 But how complex a model is necessary? One might suppose that stronger deter-
609 ministic pull on one portion of the tree would support a rate shift in α. However, all multi-
610 ple α models performed poorly. This is consistent with extensive simulation results

showing that, among the three basic parameters of the OU model, α is most poorly defined (Boettiger, et al. 2012; Ho and Ané 2013; Cressler, et al. 2015). Thus, even if a rate shift in α existed, there is probably little power to detect it. In this study, we find that with multiple free parameters, we can readily capture shifts in the evolutionary process with variable θ and perhaps σ over the tree, with stronger deterministic trends accomplished by moving θ to more extreme values. A shift in α is superlative, as changes in α and θ are not independently identifiable. Beyond cases with weak deterministic pull, difficulty in identifiability of α may be a general problem for all comparative studies, as we do not know of a case as of yet where a multiple α model was superior.

What this analysis clearly illustrates is the importance of including relevant biologically-informed hypotheses without over-parameterizing the evolutionary model. Isolating the evolutionary "signal" from the "noise" in this dataset was most strongly aided by including relevant hypotheses for variation in constraint, particularly by specifying shifts in metamorphosis and non-feeding metamorphosis along the evolutionary history of salamanders. When looking at adding parameters to the evolutionary model, the effects were variable with strongly positive (α), neutral (multiple σ), or strongly detrimental (multiple α) effects on explanatory power. We note that the harm from overparameterizing is not only significantly poorer model scores, but at least in this dataset, the order of the hypotheses changed. It is possible that these significantly worse-fit models leak variation from some of the regime categories to the extraneous parameters. It is critical for model testing, therefore, to include a test of all of the meaningful biological

hypotheses with a range of parameterizations including models with fewer parameters.

Exploring evolutionary constraints with the comparative method.—Constraint has long been argued as a necessary component of the evolutionary toolbox, as workers realized that selection alone is insufficient to explain macroevolutionary shifts in phenotype (Alberch 1980; Gould 1980; Cheverud 1984). The concept of constraint is paradoxically both simple: "evolutionary constraints are restrictions or limitations on the course or outcome of evolution" and wide-reaching, including genetic, selective, developmental, and functional constraints (Arnold 1992). Many fields have attempted to quantify constraints at various levels of biological organization using the notion of limitation. Genetic constraints have been identified as limitations on genetic variation (Kirkpatrick and Lofsvold 1992), constrained responses to selection (Cheverud 1984), or multivariate correlations that are antagonistic to the direction of selection (Etterson and Shaw 2001). All of these mechanisms allow the phenotype to change in ways that are not directly adaptive. Developmental canalization, decisions in the developmental program which narrow the phenotypic possibilities in some traits later in ontogeny, illustrate the potentially creative force of constraint when coupled with modularity by opening opportunities for the larger jumps in phenotype that become possible by evolutionary changes in modules (Wagner 1988; Wagner and Altenberg 1996; Jones, et al. 2018). Here, bounds to evolution clearly extend the range of possible phenotypic evolution. In terms of broader comparative study, modularity in multivariate shape evolution has been studied via patterns of multivariate covariation, especially for skeletal features (Parsons, et al. 2012; Jones, et al. 2018). However, progress in applying concepts of constraint on single characters

has been hampered by a lack of precision in the concept of constraint and how it could be detected.

Adaptation is typically envisioned as an evolutionary process whereby phenotypes evolve in response to the strong pull toward an optimum, which tends to concentrate phenotypes about one or more optima after sufficient time has proceeded. On the other hand, an evolutionary process bounded by constraint would be dominated by stochastic evolution for much of the range of a phenotype, appearing as random evolution with a substantially larger — but bounded — variance. Only when the phenotype crosses a threshold would selection come into play, and this may occur due to correlated selection on another linked trait. Thus, the evolutionary "driving force" can simply be stochastic change with limits imposed by constraints. Our results support this theoretical model as an explanation for genome size evolution in salamanders. More generally, our results show that OU models, which incorporate both the change in the mean as well as variance of the phenotype, can be used to distinguish between trait evolution explained by a strong pull toward an optimum versus weak selection with bounded variance. Phylogenetic comparative methods have by and large focused on explaining shifts in mean phenotype, and have thus lent themselves well to studying adaptation, convergent evolution, and parallelism. By diving deeper into the exploration of stochastic models with opportunities for varying selection and noise, we can make great progress in understanding the action of constraint in shaping evolution.

Salamanders as a model for linking organismal traits to genome biology.—Much remains to be learned about how selection on organismal traits translates into changes in

genome size, and this represents a gap in our knowledge of genome biology and evolution that studies of salamanders can help to fill. For example, our results suggest that salamanders that undergo non-feeding metamorphosis can be leveraged as a model system to distinguish between possible mechanisms of genome reduction. Variation in genome size is introduced into a population by TE insertions and deletions. Selection could, in principle, sort among these genome size variants, even as the fitness consequences of individual TE loci are typically miniscule and effectively neutral (Lynch 2007; Arkhipova 2018). In addition, selection could sort among differences in TE control machinery including the pathways that underlie TE silencing and deletion, which could yield variants with greater differences in TE composition and fitness (Mueller 2015; Parhad and Theurkauf 2019; Parhad, et al. 2020). Comparing TE control machinery in non-feeding metamorphosers versus other life history regimes (e.g. paedomorphs) could identify the mechanisms underlying genome reduction.

Conclusion.— Overall, our study shows that the evolution of a life history that includes non-feeding metamorphosis exerts a unique influence on the evolution of genome size, imposing the most severe constraints of any salamander life history strategy. This result suggests that selection to shorten the duration of metamorphosis because of energetic vulnerability has indirectly selected for faster development, constraining cells and genomes to smaller sizes. We show that genome size evolution is shaped by strong stochastic forces that are not widely variable across the salamander clade, but that deterministic forces — which vary enormously across life histories — have also played an im-

700 portant role. We broaden the application of stochastic models of trait evolution to in-
701 clude constraint, and we highlight the challenges inherent in connecting model parame-
702 ters and parameter values to complex biological phenomena.
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