**Metamorphosis imposes variable constraints on genome expansion**

**Article type:** Major Article

**Keywords**: genome size, constraint, development, comparative methods, life history

**Word Count**: 7,729

**Figures**: 2

**Tables**: 4

**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 ¾ a radical transformation of morphology ¾ 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 ¾ 3 to 40 times that of humans ¾ 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 undergo the most extensive and synchronous repatterning imposes the most severe constraint against genome expansion. Other types of metamorphosis that differ in extent and synchrony 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 766 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 by different mechanisms (Chippindale, et al. 2004; Mueller, et al. 2004; Wiens, et al. 2005; Bonett, et al. 2014).

Metamorphosis is a radical transformation of morphology during the life cycle that produces strikingly different, largely decoupled larval and adult forms (Moran 1994). During metamorphosis, depending on the taxon, organisms can be susceptible to: 1) potential performance handicaps (e.g. in accessing food and shelter or escaping predation), 2) increased energetic demands to power the destruction of larval and formation of adult structures, and 3) potential deformities resulting from genetic or environmental perturbations to the developmental systems underlying metamorphic repatterning. Thus, metamorphosis can be a vulnerable part of the life cycle, and its duration has been proposed as a target of natural selection. During metamorphosis, as during embryogenesis, salamanders undergo rapid cell division, differentiation, migration, and apoptosis (Alberch 1989). The dynamics of these cellular processes are slowed as genome/cell sizes increase (Horner and Macgregor 1983; Jockusch 1997). Selection to limit the time in metamorphosis would indirectly select for faster development, constraining genomes (and cells) to smaller sizes (Wake and Marks 1993; 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 ¾ including the presence or absence of metamorphosis ¾ 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 explore which specific metamorphic vulnerability ⎯ performance handicap, energetic demand, or risk of developmental deformity ⎯ could underlie selection to limit metamorphic duration in salamanders, which we contend is a key conceptual link between metamorphosis and genome size.

In this study, we build on this previous body of work, revisiting the hypothesis that the radical morphological repatterning associated with metamorphosis imposes evolutionary constraints on genome size in salamanders. We model characteristics that are likely to capture both energetic and developmental vulnerabilities that could place limits on the duration of metamorphosis: Does repatterning happen inside the egg, fueled solely by yolk stores? Does repatterning happen synchronously in a free-living organism, entailing more extensive structural destruction and rebuilding? We use life history and genome size data to inform stochastic models of trait evolution that explore how different metamorphic regimes interact with genome expansion. We combine these results with natural history data relevant to organismal performance during metamorphosis. More generally, we demonstrate the ability of OU-based stochastic models to identify trait evolution governed by constraint, expanding their use beyond classic scenarios of adaptive evolution.

*Life History Regimes Constraining Genome Size Evolution*

In the absence of any selection or constraint, genome size in salamanders is expected to expand by biased stochastic evolution. Overall mutation pressure in the clade leads to TE accumulation, as TE deletion rates are low and TE silencing is incomplete (Sun, Arriaza, et al. 2012; Frahry, et al. 2015; Madison-Villar, et al. 2016; Mohlhenrich and Mueller 2016). Salamanders’ enormous genomes are the cumulative result of unusually high levels of TE activity and retention (Sun, Shepard, et al. 2012; Sun and Mueller 2014; Keinath, et al. 2015; Liedtke, et al. 2018; Nowoshilow, et al. 2018).

The ancestral salamander life history included a larval growth stage followed by metamorphosis. Subsequent losses and regains of metamorphosis produced diverse life-history regimes that differ in their predicted effects on genome size evolution. We outline these predictions 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.

*Plethodontid 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.

*Non-plethodontid 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.

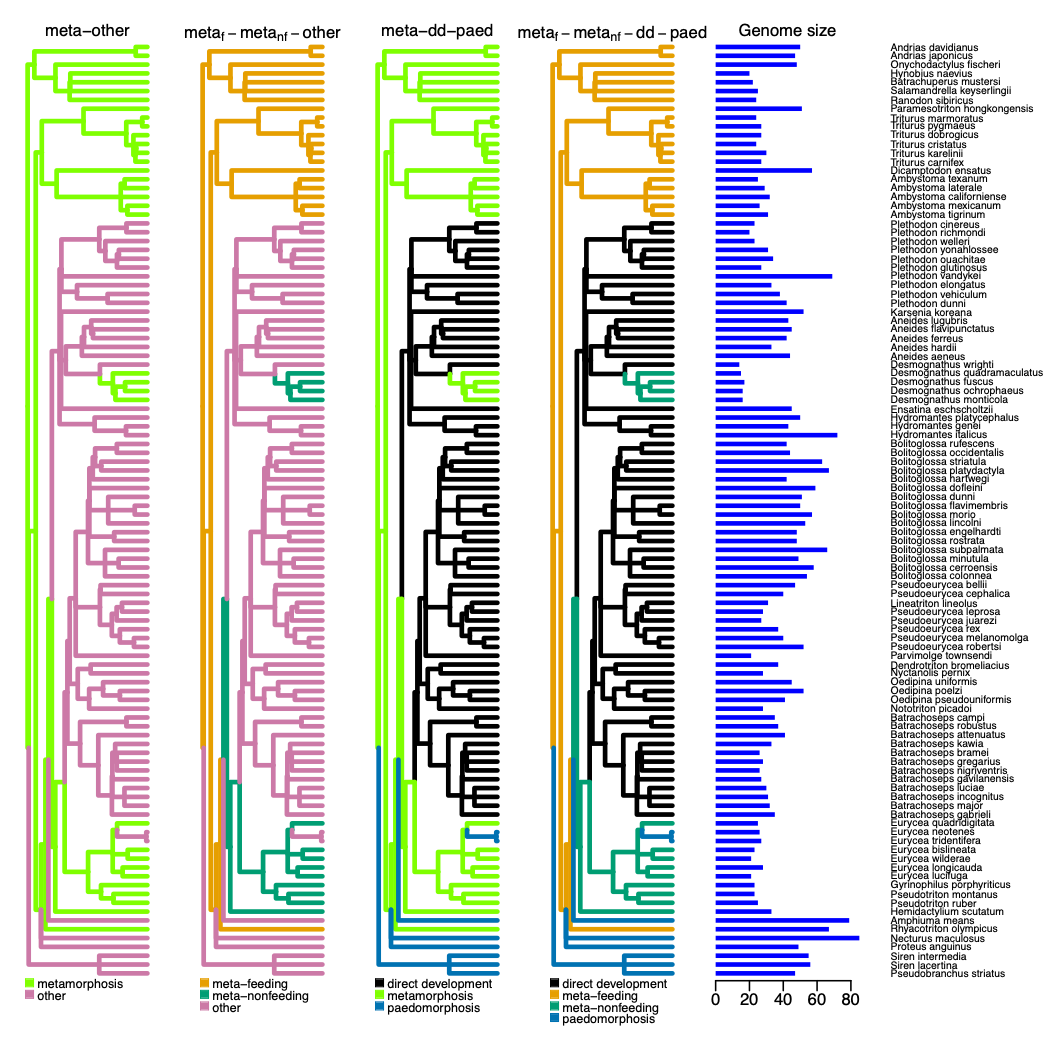


Figure 1. Alternative hypotheses for constraints imposed by development on genome size evolution in salamanders. On each phylogeny, alternative life history regimes are painted in different colors as indicated in each legend (see text). Genome sizes are shown on the right in pg (1 pg = 978 Mb).

**Methods**

*Taxon Sampling, Genome Size, and Phylogeny*

We analyzed genome size data for 118 species of salamanders (out of a total of 766 currently named extant species), encompassing all ten salamander families and 33 of 68 genera (Supplemental Table 1) (AmphibiaWeb 2021). The dataset includes all species that 1) have publicly available genome size data, and 2) are represented in the VertLife database for phylogeny subsampling (www.vertlife.org) (Jetz and Pyron 2018){Gregory, 2021 #2}. We transformed the data with natural logarithms prior to analysis. Our sampling includes representatives with diverse life histories: direct development, paedomorphosis, plethodontid metamorphosis (i.e. synchronous with more extensive remodeling), and non-plethodontid metamorphosis (i.e. asynchronous with less extensive remodeling). Hereafter, we distinguish between "plethodontid metamorphosis" and "non-plethodontid metamorphosis,” and use "metamorphosis" to indicate all species that undergo metamorphosis (both plethodontid and non-plethodontid). 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 sampled 1,000 ultrametric phylogenetic trees for our 118 species from the pseudo-posterior distribution of the VertLife database (VertLife.org) {Jetz, 2018 #746} and computed mean branch lengths using consensus.edges in phytools v 0.7-90 {Revell, 2012 #747}.

*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.

The multiple-rate BM model, introduced by O’Meara, et al. (2006), allows different σ values across different portions of a phylogenetic tree. We use this model to represent changes in the rate of stochastic evolution accompanying changes in constraint resulting from shifts in life history regime. Under this model, lineages evolving under different regimes may differ in variance.

OU models generalize the BM model by including terms that allow the mean to shift as well as allowing variance to narrow. They include a deterministic component of trait evolution that models the tendency of the trait to move toward an equilibrium value. Mathematically, the model for trait evolution expressed as a differential equation is

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where is the deterministic equilibrium for the trait at time *t* and is an evolutionary rate describing the *strength of the deterministic pull (e.g. selection)* towards that equilibrium along a branch of a tree. Hypotheses regarding trait evolution are specified by painting “regimes” on the tree to indicate where these parameters are expected to shift. The simplest OU models allow multiple equilibria leading to the evolution of differences in mean phenotype across regimes, so that varies with time, but selection strength and noise intensity are constant ( and ) (Hansen 1997; Butler and King 2004); for example, in the current study, ­­separate equilibria might exist for paedomorphs, metamorphosers, and direct developers. Further model extensions also allow the strength of the deterministic 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) *metap-metanp-other*: metamorphosis in plethodontids (*metap*) imposes constraints on genome size evolution that are distinct from those experienced by non-plethodontid salamanders undergoing metamorphosis (*metanp*). (5) *metap-metanp-paed-dd*: metamorphosing plethodontid species, metamorphosing non-plethodontid 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 (), and/or deterministic pull values () according to life history regime (Table 1). In all, we tested 21 models to explore life history constraints on genome size and model parameters (Supplementary Information; 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 (Supplementary Information). 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). 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 or 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-, single- 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).

Table 1. BM and OU models with single or multiple parameters used to fit the data. Numbers in parentheses specify (1) model parameters and notation, (2) parameters that remain constant across the phylogeny, (3) parameters that vary with shifts in life history regime, (4) OʻMeara (2006) model notation, and (5) notes for the model implementations and citations.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **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 *σi* |  | *σi* | BMS | Multiple-rate BM model of O’Meara et al. (2006) |
| Multiple equilibria | *σ* | *i* | OUM | OU model of Butler and King (2004) |
| Multiple equilibria and deterministic pull strengths | *σ* | *i*  *I* | OUMA | Multiple- model of Beaulieu et al. (2012). |
| Multiple equilibria and noise intensities |  | *σi*  *i* | OUMV | Multiple- model of Beaulieu et al. (2012) |
| Multiple equilibria, noise intensities, and deterministic pull strengths |  | *σi*  *i*  *i* | OUMVA | Full model of Beaulieu et al. (2012). |

*Model Comparison and Parameter Estimation*

We compared the fit of each of the models using the Akaike Information Criterion corrected for small sample size (AICc). 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 AICc 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,

*δ*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 ¾ 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: plethodontid and non-plethodontid metamorphosis, paedomorphosis, and direct development (*metap -metanp -paed-dd*; Table 2) under an OU model that allowed both equilibrium genome size and noise intensity to vary across these regimes (qi, si, a, Table 2). Note that models that allowed selection strength to vary produced fitting errors or nonsensical parameter estimates or likelihoods (Supplementary Information), so we exclude those results from Table 2. The three-regime *metap -metanp -other* (qi, si, a) and *meta-dd-paed* (qi, si, a) hypotheses also had some support (AICc 3).

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 multiple- version of the hypothesis, since these always had higher support than the single- versions.

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). Separating non-metamorphosing strategies into direct development and paedomorphosis (Fig. 2b) or separating metamorphosing strategies into plethodontid and non-plethodontid metamorphosis (Fig. 2c) only modestly improved explanatory power compared to the simpler *metamorphosis-other* hypothesis. However, including all four selective regimes significantly increased the explanatory power compared to any three-regime hypothesis (Fig. 2d,e), and allowing noise intensity to vary among regimes is strongly supported (Fig. 2f). Therefore, we have compelling evidence for a model of salamander genome size evolution that includes 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: s = Brownian motion; s*i* = Brownian motion with multiple noise intensities; q*i,* s*,* a = OU model with multiple equilibria; q*i,* s*i,* a = OU model with multiple equilibria and multiple noise intensities; q*i,* s*,* a*i* = OU model with multiple equilibria and multiple deterministic pull strengths; q*i,* s*i,* a*i* = OU model with multiple equilibria, noise intensities, and deterministic pull strengths.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| ΔAICc | Model | | | |
| OU Models | | BM Models | |
| Hypotheses |  |  |  |  |
| *metap -metanp -paed-dd* | **0** | 7.1 | 7.5 |  |
| *metap -metanp -other* | 2.5 | 6.9 | 9.5 |  |
| *meta-paed-dd* | 3.0 | 8.8 | 5.3 |  |
| *metamorphosis-other* | 4.2 | 7.5 | 7.6 |  |
| Brownian motion |  |  |  | 15.5 |

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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) plethodontid metamorphosis imposes a distinct constraint from metamorphosis in non-plethodontids; (D) plethodontid metamorphosis imposes a distinct constraint from non-plethodontid metamorphosis, after accounting for differences in non-metamorphosing strategies; (E) non-metamorphosing strategies impose unique constraints, after accounting for differences between plethodontid and non-plethodontid metamorphosis; (F) after identifying *metap-metanp-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. To help visualize what this best-fitting tells us about genome size evolution, we compared the distribution of observed genome sizes for species in each selective regime to the expected stationary distribution, given the model estimates of *θ*, *α*, and *σ* for each regime (Fig. 3). In particular, Ho and Ane (2013) showed that the stationary distribution of an OU process will be a normal distribution with mean *θ* and variance ; the stationary distribution thus provides a useful way of visualizing the model fit by capturing aspects of all parameters. It is apparent that genome size is under strong stabilizing selection in the direct development regime (which is by far the most common selective regime across the tree; Fig. 3a). This also appears to be true of genome size in the metamorphosing non-plethodontid salamander regime (although the peak of the genome size distribution is slightly smaller than the predicted mean; Fig. 3b). Genome size in the metamorphosing plethodontid regime, on the other hand, appears to be under directional selection towards smaller genome sizes, since almost all metamorphosing plethodontid salamanders have genome sizes that are larger than the expected mean (Fig. 3c). Conversely, genome size in the paedomorph regime appears to be under directional selection towards larger size (which we suspect is a fitting artifact produced by relatively unconstrained TE accumulation in these salamanders; Fig. 3d).

Table 3: Maximum likelihood parameter estimates and parametric bootstrap confidence intervals for the best-fitting model (*metap -metanp -dd-paed :* separate equilibrium values and noise intensities for lineages in the four life history regimes: dd = direct development, meta*np* = non-plethodontid metamorphosis, meta*p* = plethodontid metamorphosis, paed = paedomorphosis).

|  |  |  |  |
| --- | --- | --- | --- |
| Parameter |  | MLE | 95% CI |
| Deterministic pull strength |  | 1.27 | (0.429, 4.24) |
| Stochastic noise intensity | *dd* | 0.840 | (0.717, 1.08) |
|  | *meta\_np* | 0.587 | (0.411, 0.882) |
|  | *meta\_p* | 0.382 | (0.218, 0.560) |
|  | *paed* | 0.359 | (0.139, 0.675) |
| Equilibrium value | *dd* | 3.72 | (2.87, 4.58) |
|  | *meta\_np* | 3.66 | (3.39, 3.93) |
|  | *meta\_f* | 2.65 | (0.709, 3.39) |
|  | *paed* | 4.31 | (3.87, 5.03) |



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Figure 3. Comparison of the observed distribution of genome sizes for salamanders in each regime against the stationary distribution predicted by the best-fitting parameter set (Table 3). The stationary distribution is normal with mean and variance , where the values of and vary across regimes.

**Discussion**

*Biased stochastic expansion and life history constraints shape genome size.*¾

Overall, evolution of genome size in salamanders is well-described by a deterministic model with separate equilibria for each life history regime and moderate stochasticity. The model suggests that plethodontid salamanders that undergo metamorphosis are under the strongest selection to reduce genome sizes, whereas paedomorphic lineages that have lost metamorphosis are experiencing less constraint on genome size evolution. Non-plethodontid metamorphosers and direct developers do not appear to be experiencing any tendency towards either genome size contraction or expansion, but rather appear to be under fairly consistent stabilizing selection. Combined with previous results on genome expansion in salamanders, driven by TE insertions that outpace deletions, these results suggest that metamorphosis is actually acting to constrain genome size in most salamander species, including potentially driving the evolution of smaller genome sizes when metamorphic repatterning is more extreme (in plethodontid salamanders).

*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 ¾ and less dramatic ¾ 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). Plethodontid salamanders, however, are unable to feed during metamorphosis, requiring that they undergo the transformation using only stored energy reserves (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.*¾Lineages 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 around an equilibrium value that we interpret as a balance between TE accumulation and a constraint imposed by metamorphosis. Although the animals are able to feed, there are other ways in which fitness can be lowered during salamander metamorphosis; for example, metamorphosing individuals are less able to exploit stream habitat refugia than either larvae or adults, which increases their mortality (Lowe, et al. 2019). Our results indicate that feeding metamorphosis imposes a less severe constraint on genome size than non-feeding metamorphosis, and we infer that the constraint is mediated by vulnerabilities other than depletion of energetic stores.

*Genome size evolution in non-feeding metamorphosers.*¾Although other analyses have demonstrated a link between metamorphosis and genome size in salamanders (Wake and Marks 1993; Gregory 2002b; Sessions 2008; Bonett, et al. 2020), here we show that non-feeding metamorphosis imposes a substantial and distinct constraint, relative to feeding metamorphosis, as predicted if energetic vulnerability shapes the duration of metamorphosis. The deterministic trend toward smaller genome sizes within this regime is consistent with the imposition of a more severe constraint against genome expansion ¾ or, put another way, selection towards genome size reduction ¾¾ to shorten the duration of metamorphosis.

The evolution of non-feeding metamorphosis in plethodontids has been an important target of research because the phylogeny suggests that it evolved from a direct-developing ancestor(s), which necessitates the evolutionary reappearance of the “lost” larval stage (Chippindale, et al. 2004; Mueller, et al. 2004). This evolutionary transformation series was historically considered unlikely, although more recent work has revealed potential scenarios for regain of the larval stage (Bonett, et al. 2005; Bonett, et al. 2014). Under this scenario, in the direct-developing ancestral lineage(s), metamorphic repatterning steps were retained as part of the longer sequence of developmental events that occurred inside the egg (Kerney, et al. 2012). These changes were likely mediated by evolutionary changes in the timing of thyroid hormone activity and response (Rose 1995a, b; Bonett 2016). The re-evolution of (now non-feeding) metamorphosis reflected the insertion of a long, slow-growing larval growth phase back into ontogeny, followed by the synchronous occurrence of more drastic metamorphic repatterning events in the free-living organism (Rose 1995b, c; Beachy, et al. 2017). Metamorphic repatterning is more extreme in plethodontids than in other salamanders. Importantly, it involves a complete remodeling of the feeding apparatus, with the ceratobranchials (cartilaginous components of the tongue skeleton) replaced by new structures in the adult rather than remodeled from existing larval structures; this results in the inability to feed during metamorphosis (Alberch 1989; Rose 1995c; Deban and Marks 2002). Under the classical scenario, in contrast, this synchronization of metamorphosis and drastic remodeling of feeding structures would have evolved in a metamorphosing ancestor to produce non-feeding metamorphosis (Wake and Hanken 2004). Under either scenario, our results illustrate how phylogenetic comparative methods can reveal the evolutionary forces that have acted on genome size as lineages moved through the different life history regimes.

*Genome size evolution in direct developers.*¾In direct-developing lineages, some or all of the developmental steps of metamorphic repatterning occur inside the egg at the end of embryogenesis (Alberch 1989; Kerney, et al. 2012); these lineages show a weak deterministic trend towards genome size reduction. Because they are occurring in an embryo rather than a free-living organism that has undergone a growth period, the repatterning happens to a smaller number of cells in a smaller overall mass of tissue compared with metamorphosing lineages (Downie, et al. 2004). Thus, the energetic requirements for comparable developmental steps are lower in direct developers than in metamorphosers. On the other hand, the energy to fuel these steps comes from yolk stores which, although plentiful in direct developers, are still finite (Wake and Hanken 2004). Thus, we infer that direct development imposes a less severe constraint on genome size than does non-feeding metamorphosis, mediated by the potential for depletion of energy stores if the duration of metamorphic repatterning during embryogenesis is too long. We note that there is greater variation across direct developers in metamorphic repatterning than is modeled here. In some cases, the sequence of developmental events is shortened because the formation of larval structures is lost from ontogeny. In other cases, most or all events of embryogenesis and metamorphosis are retained but occur inside the egg (which allows for the possibility of re-evolution of metamorphosis; Alberch 1989). We would predict more severe constraints in these latter lineages. Although we treated both scenarios as a single category for simplicity, these two types of direct development may be different in their effects on genome size evolution and warrant more detailed study.

*Genome size evolution in paedomorphs.*¾Paedomorphic salamanders retain a larval body form throughout life and show a deterministic trend towards genome expansion. This trend is consistent with TE accumulation proceeding to higher overall levels, unchecked by any constraints imposed by metamorphic repatterning. However, we do not suggest that genome size is free from all constraints. The impacts of decreased surface-area-to-volume ratio that accompany increased cell size likely impose an upper limit on cell function that salamanders may well have reached (Chan and Marshall 2010); their cells are among the largest found in animals (Horner and Macgregor 1983). In addition, the duration of embryogenesis may well have an upper bound that constrains genome expansion at the extremely high end. In the past, huge cells have been proposed as adaptive because they coincide, at broad taxonomic levels, with low metabolic rates; salamanders and lungfishes have the lowest metabolic rates and the largest genomes/cells within vertebrates. This correlation led to the proposal that selection shaped an adaptive “frugal metabolic strategy” in these taxa (Szarski 1983; Olmo, et al. 1989). More recent analyses of the relationship between genome/cell size and metabolic rate, however, have failed to find a clear relationship (Licht and Lowcock 1991; Uyeda, et al. 2017; Gardner, et al. 2020). Thus, empirical evidence that huge genomes are a product of directional selection is currently lacking. Our results are more consistent with the relaxation of a constraint against genome expansion because of the extremely weak deterministic pull strength and strong stochastic noise parameters.

*Model complexity to capture the evolutionary process.*¾While the best model includes a deterministic pull parameter, its magnitude is miniscule. Yet our model selection results demonstrate that models with deterministic pull provide a huge improvement over any purely stochastic model (Table 2). One of the challenges of an OU model with a weak deterministic component is that the model parameters will be poorly defined (Cressler, et al. 2015). Intuitively, if the pull parameter () is important but close to zero, the equilibria (*)* can take on a wide range of values in combination with a range of values for the stochastic parameter and explain the phenotypic distribution equally as well. The difference between a purely stochastic (BM) model and one that has any degree of deterministic pull is that the variance of a BM model will grow unbounded over time (as variance of a trait undergoing a Brownian motion process is proportional to time), whereas the variance in a model with deterministic pull will not (variance ~) (Hansen and Martins 1996; Butler and King 2004). Thus, while the phenotype may explore a wide range of values in an OU model with very small , it will remain bounded. For paedomorphs, a weak deterministic pull allows the mean to wander, while a far-away equilibrium value captures a deterministic trend toward increase. In OU models, increasing deterministic pull strength influences the approach to the equilibrium, but also will tend to dampen stochastic effects (apart from the influence of ), so it is possible to have both weak deterministic pull and substantial noise in the stochastic process. This analysis demonstrates that deterministic pull can exert an important evolutionary influence, even if the magnitude of alpha is weak.

But how complex a model is necessary? One might suppose that stronger deterministic pull on one portion of the tree would support a rate shift in . However, all multiple a models performed poorly. This is consistent with extensive simulation results showing that, among the three basic parameters of the OU model, a is most poorly defined (Boettiger, et al. 2012; Ho and Ané 2013; Cressler, et al. 2015). Thus, even if a rate shift in a 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 q and perhaps s over the tree, with stronger deterministic trends accomplished by moving q to more extreme values. A shift in a is superlative, as changes in a and q are not independently identifiable. Beyond cases with weak deterministic pull, difficulty in identifiability of a may be a general problem for all comparative studies, as we do not know of a case as of yet where a multiple a 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 (), neutral (multiple ), or strongly detrimental (multiple ) 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.*¾Constraint 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.*¾Overall, 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 ¾ which vary enormously across life histories ¾ have also played an important role. We broaden the application of stochastic models of trait evolution to include constraint, and we highlight the challenges inherent in connecting model parameters and parameter values to complex biological phenomena.

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