

# Temperature-dependent oxygen limitation and the rise of Bergmann's rule in species with aquatic respiration

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Bergmann's rule is the propensity for species-mean body size to decrease with increasing temperature. Temperature-dependent oxygen limitation has been hypothesized to help drive temperature–size relationships among ectotherms, including Bergmann's rule, where organisms reduce body size under warm oxygen-limited conditions, thereby maintaining aerobic scope. Temperature-dependent oxygen limitation should be most pronounced among aquatic ectotherms that cannot breathe aerially, as oxygen solubility in water decreases with increasing temperature. We use phylogenetically explicit analyses to show that species-mean adult size of aquatic salamanders with branchial or cutaneous oxygen uptake becomes small in warm environments and large in cool environments, whereas body size of aquatic species with lungs (i.e., that respire aerially), as well as size of semiaquatic and terrestrial species do not decrease with temperature. We argue that oxygen limitation drives the evolution of small size in warm aquatic environments for species with aquatic respiration. More broadly, the stronger decline in size with temperature observed in aquatic versus terrestrial salamander species mirrors the relatively strong plastic declines in size observed previously among aquatic versus terrestrial invertebrates, suggesting that temperature-dependent oxygen availability can help drive patterns of plasticity, micro- and macroevolution.

**KEY WORDS:** body size, ecological constraint, latitudinal cline, life history, macroevolution, temperature-size rule (TSR).

## Introduction

Biologists have long struggled to understand patterns in the evolution of body size (Bergmann 1847; Cope 1896; Uyeda et al. 2011). Environmental temperature is often directly or indirectly implicated in body-size evolution (Smith et al. 1995; Lawson and Weir 2014; Clavel and Morlon 2017), which is not surprising as temperature affects the rate of nearly all biological processes. But for ectotherms in particular, there is a key observation that may prove valuable in developing more general hypotheses of body-size evolution: when ectotherms are reared under cool conditions, they grow slowly and mature at a larger body size than under warm conditions (Atkinson and Sibly 1997). This pattern, called the temperature-size rule (TSR), has been observed in over 80% of cases in which relationships between rearing temperature and adult size have been examined under laboratory conditions

(Atkinson 1994). The TSR is therefore one of the most predictable and widespread patterns of phenotypic plasticity. If the generality of the TSR is taken as evidence of a strong and pervasive agent of selection that shapes body-size plasticity, then it is possible that a hypothesis explaining the TSR might also apply to body-size evolution at higher levels of biological organization in a wide variety of ectotherms.

At the proximate level, the TSR can be attributed to phenotypic plasticity in the sizes and/or numbers of some types of cells, organs, and repeated structures (Van Voorhies 1996; Zwaan et al. 2000; Azevedo et al. 2002; Blanckenhorn and Llaurens 2005), which affects whole-organism body size (Atkinson et al. 2006). At the ultimate level, several explanations for the TSR have been debated (Atkinson and Sibly 1997; Angilletta et al. 2004), but a more recent literature emphasizes how oxygen uptake

and transport can set limits on body size in warm environments (Atkinson et al. 2006; Forster et al. 2012; Verberk and Atkinson 2013; Horne et al. 2015). For ectotherms, metabolic oxygen consumption increases more quickly with temperature than does rate of oxygen diffusion in the oxygen uptake and transport systems that ultimately supply the organism (Woods 1999). At high temperature, ectotherms can reduce the sizes of some cell types, the number of cells, the size of organs, and ultimately whole-organism size, thereby adopting a more favorable ratio of absorptive surface area to oxygen demand (Atkinson et al. 2006; Verberk and Atkinson 2013). Indeed, there is strong evidence among ectotherms that large adult size and cell size occur when oxygen availability per se is high (Harrison and Haddad 2011; Heinrich et al. 2011), and that low temperature increases the benefits of large size by virtue of decreased metabolic rate and hence a further increase in the ratio of oxygen supply to demand (Frazier et al. 2001). The ultimate explanation for a reduction in body size under warm conditions was originally described as MASROS, or “Maintain Aerobic Scope and Regulate Oxygen Supply” (Atkinson et al. 2006). At its core, MASROS emphasizes a phenomenon that results in plastic reductions in body size under warm oxygen-limited conditions, and in the present study, we refer this phenomenon more generally as “temperature-dependent oxygen limitation.”

The extent to which temperature-dependent oxygen limitation occurs may depend upon modes of respiration, and on whether oxygen is being taken up in an aquatic or terrestrial environment. Forced air convection over respiratory surfaces, as occurs in air-breathing species with lungs, can help overcome the limitations associated with oxygen diffusion, as forced convection greatly accelerates oxygen delivery (Angilletta and Dunham 2003; Klok et al. 2004). Yet, most aquatic species respire using gills and by cutaneous oxygen uptake, both of which are more reliant on passive diffusion. Further, oxygen solubility in water is highly temperature dependent, consequently oxygen should be more limiting in aquatic than terrestrial systems (Woods 1999; Makarieva et al. 2005; Atkinson et al. 2006; Verberk et al. 2011; Verberk and Atkinson 2013). Meta-analyses of temperature—body size reaction norms have shown that reaction norm slopes are more strongly negative in aquatic compared to terrestrial species, thereby supporting a role for temperature-dependent oxygen limitation in driving the TSR (Forster et al. 2012; Horne et al. 2015).

Although the TSR formally describes norms of reaction to temperature (Atkinson 1994), interspecific variation in body size sometimes mirrors the TSR as well. For instance, James’s Rule (Blackburn et al. 1999) describes the propensity for mean body size of populations of the same species to decrease with temperature or increase with latitude. Although James originally described the pattern for birds (James 1970), subsequent research has shown that some ectotherm species also follow the rule. Body size in pop-

ulations of some fishes, aquatic insects, and *Drosophila*, for example, typically becomes smaller at low latitudes (Zwaan et al. 2000; Rypel 2014; Horne et al. 2015). For the well-studied *Drosophila*, a genetic basis partly underlies the latitudinal clines (James et al. 1997; Partridge and Coyne 1997), and smaller adult sizes evolve under warm conditions in the laboratory (Partridge and French 1996). James’s rule is more common in aquatic compared to terrestrial insects (Horne et al. 2015), and the strength of James clines in insect species is proportional the strength of the species’ phenotypic plasticity of body size (i.e., the TSR) observed under laboratory conditions. It is therefore possible that temperature-dependent oxygen limitation helps drive both the TSR and James’s rule, at least among aquatic species (Horne et al. 2015).

At the interspecific level, Carl Bergmann (Bergmann 1847) first noticed the propensity for species mean body size of endotherms to become smaller in warm environments (Blackburn et al. 1999), and Bergmann’s rule has since been described in some ectotherms (Timofeev 2001; Verberk et al. 2011; Thatje and Hall 2016). It is possible that Bergmann’s rule in ectotherms is related to oxygen availability. For example, variation in atmospheric oxygen concentration over the last 500 million years is positively associated with the evolution of large size in a variety of air-breathing animals (Berner et al. 2007; Harrison et al. 2010), underlining a potential role for oxygen availability in macroevolution. Among ectothermic species that exist today, oxygen availability will be relatively similar and independent of temperature in species that respire aerially, compared to species that respire in water, where oxygen availability is strongly temperature dependent (Woods 1999; Atkinson et al. 2006; Verberk and Atkinson 2013). This suggests that Bergmann’s rule will be more common among ectothermic species that respire in water. Indeed, the largest species of copepods are found in the most oxygenated regions of the world’s oceans and seas, which also happen to be found in cooler regions of the globe (Chapelle and Peck 1999), and variation in temperature per se is negatively correlated with variation in body size of other aquatic crustaceans as well (Timofeev 2001; Thatje and Hall 2016). There is, therefore, strong evidence to suggest that oxygen availability can drive the macroevolution of body size in ectotherms, and there is growing evidence that the temperature-dependence of oxygen availability in aquatic systems may drive Bergmann’s rule.

Yet, a role for temperature-dependent oxygen limitation in driving Bergmann’s rule is not clear, as no study has linked the interaction of temperature and oxygen availability to the evolution of size at the species level. A study of this nature is challenging, as it would necessarily require incorporating many estimates of species-mean body size to examine how body size changes as a function of both temperature and oxygen availability, all in a phylogenetically explicit context. Amphibians are unique among vertebrates in their diverse modes of respiration, complex

life history transitions, and lifestyles. Here, we emphasize that amphibians are an underexploited system in which to explore body-size evolution in the context of oxygen availability and temperature; further, a large phylogeny of amphibians has recently become available (Pyron and Wiens 2013). Drawing from a large database on amphibian body size and species-specific temperature data, we test the prediction that body size of aquatic species with branchial and/or cutaneous respiration should exhibit a stronger negative response to environmental temperature than aquatic species that respire aerially, and than species that inhabit terrestrial environments where oxygen availability is less temperature dependent. Our analyses support the hypothesis that temperature-dependent oxygen limitation plays an important role in generating Bergmann rule in these aquatic ectotherms, especially those where oxygen cannot be assimilated using forced air convection over respiratory surfaces.

## Materials and Methods

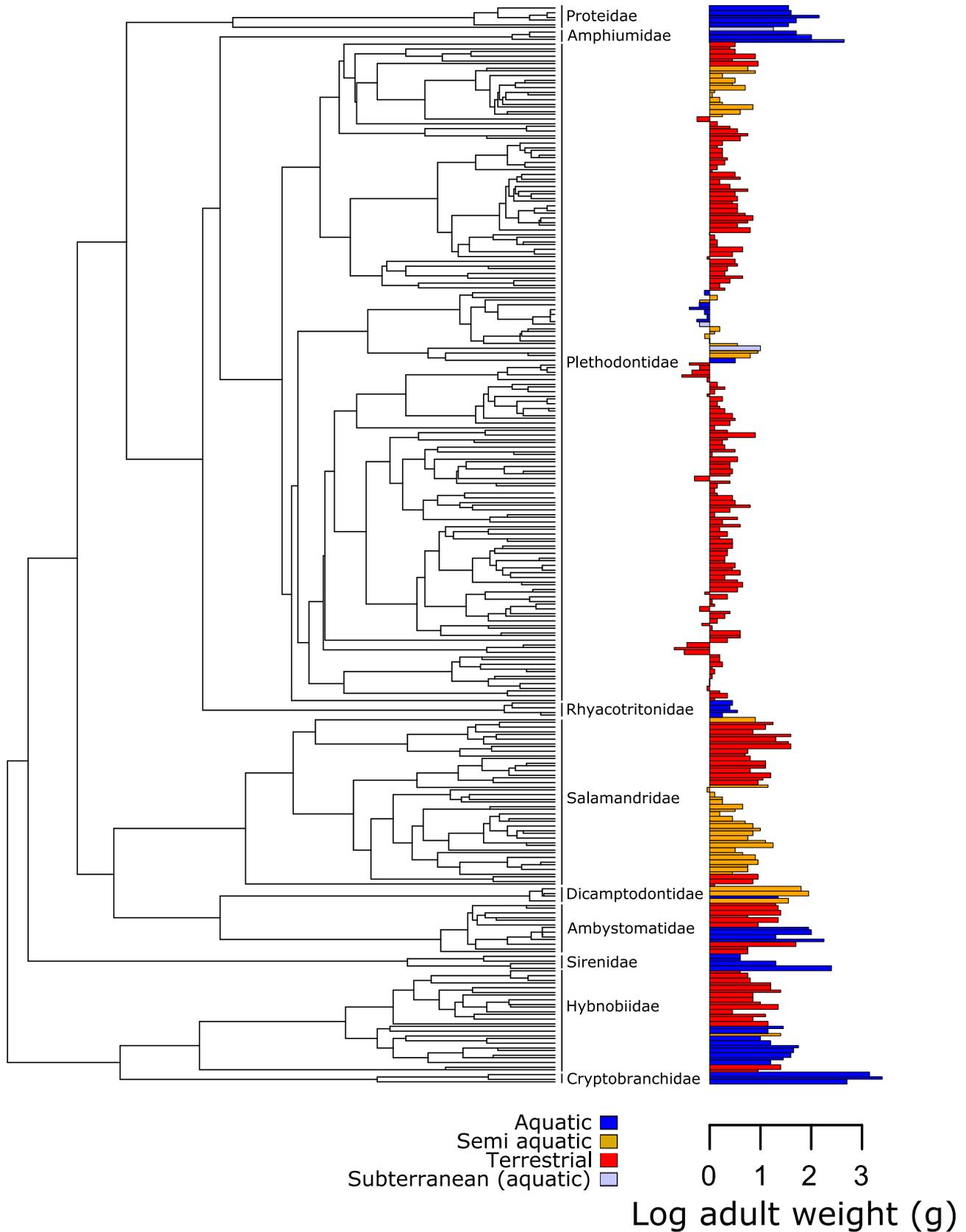
We compiled a database for adult size (SVL) and adult weight (g) of salamanders. For SVL data, we relied on previous compilations (Adams et al. 2009; Eastman 2010; De Lisle and Rowe 2013; Trochet et al. 2014), but estimates of SVL and body weight were also compiled from the primary literature and online databases. When both male and female SVL data were available, the average of the two sexes was used; otherwise SVL data were generally reported as a single species-wide estimate. Few direct estimates of body weight were available for target species. For all Plethodontidae, Ambystomatidae, and Salamandridae, we estimated weight from SVL based on family-specific SVL-mass regressions (Table S1). Weight of Hynobiidae and Rhyacotritonidae was estimated from Ambystomatidae and Plethodontidae regressions, respectively, as body shapes are similar. For obligate paedomorphic salamanders (e.g., Proteidae, Cryptobranchidae), we obtained direct estimates of adult weight from the primary literature, as these families are species poor and have peculiar body shapes.

We classified the habitat of salamander species as aquatic, semiaquatic, or terrestrial, relying primarily on species descriptions in Petranka (1998) and Sparreboom (2014). We classified aquatic species as those that are rarely, if ever, found outside of water; relatively few species met this criterion (Fig. 1). Aquatic species included obligate paedomorphs such as the Cryptobranchidae, Proteidae, Amphiumidae, and Sirenidae. Other aquatic species are the inducible-obligate Paedomorphs (Wakahara 1996), as is found in some *Eurycea* sp. (Plethodontidae), *Ambystoma* sp. (Ambystomatidae), and *Dicamptodon* (*Dicamptodon*), but these species were classified as aquatic only if paedomorphy was described by Petranka (1998), Lanoo (2005), or AmphibaWeb (2016), as near-ubiquitous or prevalent through-

out the species' range, or if natural metamorphs were considered rare. Aquatic species also included all four species of torrent salamanders (Rhyacotritonidae), as well as a few members of Hynobiidae (i.e., *Ranodon sibiricus*, all *Liua* sp., *Batrachopus* sp., and *Paradactylodon* sp.). Finally, we also included the plethodontid salamander *Stereochilus marginatus* in our analysis as an aquatic species, given its overwhelmingly aquatic lifestyle (Bruce 1971; Means 2000). All cave-dwelling paedomorphic species were excluded from our analysis in the main text, as we assumed a priori that fundamental differences in surface versus subterranean environments (e.g., pH, food availability) may affect patterns of body size evolution; however, we were able to identify the necessary life history and temperature data for three cave-dwelling species that are aquatic and lungless (Plethodontidae: *Eurycea troglodytes*, *Gyrinophilus palleucus*; Proteidae: *Proteus anguinus*), and we perform a supplementary analysis that includes these species.

Species were classified as terrestrial provided that the adult life stage is spent entirely on land. This includes aquatic breeding species that are highly terrestrial as adults, which includes some members of *Ambystoma* (Ambystomatidae), *Hybnobius* (Hynobiidae), *Tylototriton*, and *Taricha* (Salamandridae). Most other terrestrial species were members of the family Plethodontidae, as well as a few members of Salamandridae (*Echinotriton* sp., *Lyciasalamandra* sp., *Salamandra* sp.). Finally, the semiaquatic group included all species that are strongly associated with permanent or semipermanent bodies of water, but that have a significant terrestrial component to their lifestyle. This included most or all members of *Eurycea*, *Desmognathus*, *Pseudotriton* (Plethodontidae), as well as truly amphibious members of the Salamandridae (e.g., *Lissotriton* sp., *Neurergus* sp., *Paramesotriton*, etc.), and all *Dicamptodon* sp. that could not be classified as aquatic (Fig. 1). We note that species classified as semiaquatic likely vary widely in their level of aquatic activity; however, the aquatic and terrestrial classifications represent each end of a continuum, so classifications for aquatic and terrestrial species should be biologically precise.

We determined whether each species (or group of species) is able to breathe aerially, by noting the presence of lungs and/or whether lungs are well developed. All species in Plethodontidae lack lungs and therefore oxygen uptake is exclusively cutaneous or branchial. Similarly, all species in Rhyacotritonidae and Cryptobranchidae, all species of the genus *Nectrurus* (Proteidae), and a few members of Hynobiidae (*Ranodon sibiricus*, *Onychodactylus* sp.) are lungless or feature vestigial lungs, and therefore respiration is branchial and cutaneous. For example, *Nectrurus* (Proteidae) and *Cryptobranchus* (Cryptobranchidae) feature vestigial lungs, and at least 89% (and usually >93%) of all oxygen obtained is branchial or cutaneous even under very warm conditions (Ultsch 2012). All other salamander species not mentioned above feature well-developed lungs and can breathe aerially. Even the



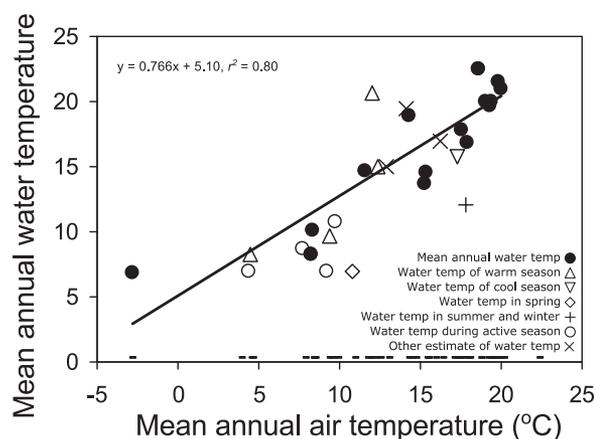
**Figure 1.** Phylogeny of 273 species used in PGLS analyses. Bar graph illustrates log adult mass (g) for each species. Species that are terrestrial as adults are in red, aquatic species are blue, semiaquatic species in orange, and subterranean species are light blue.

Sirenidae and Amphiumidae feature well-developed lungs, and respiration is primarily aerial despite their completely aquatic habitat: for instance, when *Siren lacertina* is allowed to consume aerial oxygen, its oxygen consumption increases up to 340% over oxygen consumed while forcibly submerged, and large specimens of this species cannot survive forcible submergence for prolonged periods (Ultsch 1976). Similarly, *Ambystoma* feature lungs that develop during very early life (Burggren and Pinder 1991), and ventilate aerially as adults (Whitford and Hutchison 1965). More broadly, each treatment group in our analysis (terrestrial, semi-aquatic, and aquatic species) was represented by members with lungs, and members without lungs. This allows us to test for interactions between mode of respiration, temperature, and habitat.

### ENVIRONMENTAL AND GEOGRAPHIC DATA

Data on range size (km<sup>2</sup>) and environmental temperature were collected by Sodhi et al. (2008). In brief, Sodhi et al. (2008) extracted range size data for nearly 6000 amphibian species from the Global Amphibian Assessment (The World Conservation Union Conservation International (CI) and NatureServe 2006); we use a subset of these range size data herein. Air temperature data were used as estimates of environmental temperature for semiaquatic and terrestrial species. Air temperature data were obtained from Sodhi et al. (2008), which were estimated following Hijmans et al. (2005) and are based on average climatic data, interpolated from weather stations located on a 30 arc second resolution grid within the geographic range of each species. Temperature data represent mean daily air temperature (interpolated from weather stations within each species' range) averaged within each month, then across all months in the year, thereby creating a single estimate of air temperature per species.

We used mean annual water temperature as an estimate of environmental temperature for aquatic species. For each of the 40 aquatic species, we surveyed the literature for estimates of mean annual water temperature. In general, we obtained mean monthly water temperature data for one or two populations in a species' range, and we averaged water temperature across all months in a year, then (if necessary) across studies. A few studies also presented estimates of monthly temperature from representative months spanning an entire year (e.g., October, January, April, and July), which we averaged to produce a mean annual temperature. Many studies provided temperature data only for part of the year, and these data were not used as they would likely be biased upward or downward relative to mean annual temperature, depending on the season in which they were measured. The exception is that a few species inhabited thermally stable aquatic environments (e.g., spring-fed lakes or streams), in which case we accepted partial temperature estimates as representations of mean annual temperature. In total, we obtained mean annual water temperature data for 17 surface-dwelling species, and for the



**Figure 2.** Relationship between water temperature and air temperature. Black circles represent cases where a reliable estimate of mean annual water temperature was obtained, and the regression line was estimated using only the data represented by black circles. Other points represent a variety of estimates of water temperature from various seasons; these data are depicted for completeness but were not used in this study. Dark ticks on the x-axis represent the range of mean annual air temperature data experienced by the 40 aquatic species in the analysis, demonstrating that a representative range of mean annual water temperatures was sampled to construct the regression.

remaining 23 species either no water temperature data could be found, or only partial estimates were available. To be able to include all 40 surface-dwelling aquatic species in our analysis, we performed a linear regression between mean annual air temperature (from Sodhi et al. 2008) and mean annual water temperature. We found that air temperature was a good predictor of water temperature ( $r^2 = 0.80$ ), and therefore used the regression equation, water temperature = 0.766 (air temperature) + 5.10 to predict mean annual water temperature for the 23 aquatic species where no water temperature was available (Fig. 2). Our direct estimates of water temperature were used in our analyses for the 17 species with available water temperature data.

### STATISTICS

Our analyses included up to 270 species (Fig. 1), representing all 10 taxonomic families and up to 38% of the ca. 700 species in the order Caudata; the number of species reflects the availability of a full suite of data for each candidate species on range size, environmental temperature, body size, habitat, and whether the species is present in the phylogeny (Pyron and Wiens 2013). We used phylogenetic least squares regression (PGLS) to estimate the relationship between temperature and size traits. All PGLS analyses were performed in the R environment (R Development Core Team 2016), primarily by implementing the packages *ape* (Paradis et al. 2004), *phytools* (Revell 2012), and *geiger* (Harmon et al. 2008). PGLS regression parameters and

phylogenetic signal were estimated simultaneously, using Pagel's  $\lambda$  (Pagel 1999), and models were fit using maximum likelihood. Dependent variables were centered (i.e., the mean was subtracted), and continuous explanatory variables were centered then divided by 2 SDs (Gelman 2008) so that effect sizes of explanatory variables could be meaningfully compared (Schielzeth 2010).

Local adaptation to temperature might give rise to geographic variation in body size within a species' range. This is potentially problematic, as each datum in the present study represents a species-wide estimate of body size, and in many cases it is not clear where within the species range the data come from. Therefore, annual air temperature averaged across a species' geographic range is not necessarily representative of the temperature experienced by the population(s) from which the size data were obtained. The same argument applies to our estimates of water temperature, where annual water temperature estimated from one or two populations within the species' range may not be directly associated with our body size estimates. To help overcome this problem, we assume that the extent of variation in temperature experienced across a species' range is proportional to the geographic range size of the species. It follows that our size data is well matched to mean annual temperature data for species with small geographic range sizes, but becomes relatively uncoupled as range size increases. We therefore focus on species with small range sizes to ensure a match between size data and temperature data, and we analyze the data in two different ways. First, Hagget et al. (1965) emphasize how measures of areal magnitude become readily interpretable when  $\log_{10}$  transformed and standardized with respect to a reference measure of areal size (e.g., in the case of Hagget et al., the total area of the earth's surface). Following the philosophy of Hagget et al., we perform a PGLS with observations weighted by  $\log_{10}$  range size. To create statistical weights based on range size, we subtracted the maximum observed ( $\log$ ) range size observed in the dataset of 270 species from the ( $\log$ ) range size of each species, thereby providing greater statistical weight to species with small range sizes. The statistical weights ranged from a value of 6.6 to 0.1, where 0.1 was arbitrarily assigned to the species with the largest range size; median weight was 2.7. This method of statistical weighting is conceptually similar to the conventional method where observations are weighted by sample size, as observations with a greater sample size (or in this case, a smaller range size) are given more emphasis as they are expected to be more accurate. Next, given that our primary interest is in the three-way interaction between habitat (aquatic, semiaquatic, terrestrial), mode of respiration (lungless, or lungs present), and temperature, we use information theory (Burnham and Anderson 2002) to explore support for the three-way interaction term, given a range of alternative model formulations. We tested all possible model combinations given the three variables of interest, with the expectation that body size of species that

are both aquatic and lungless will decline relatively strongly with temperature. This resulted in 18 candidate models, culminating in the most complicated model with the three-way interaction.

We also use a complementary method to overcome the problem of geographic range size. We perform a supplemental analysis where we focus on species with range sizes of less than 50,000 km<sup>2</sup>, that is, excluding species where range size is large and where size and temperature are more likely to vary considerably within species. This analysis excluded lungless semiaquatic species, as variation in temperatures experienced by semiaquatic species at this spatial scale was only 2.3°C, precluding any meaningful estimate of the slope of body size over temperature. As a result, mode of respiration was no longer fully crossed with habitat, precluding a test of the three-way interaction between habitat, temperature, and mode of respiration. We therefore created five treatment groups with our data: aquatic species without lungs, aquatic species with lungs, semiaquatic species with lungs, terrestrial species without lungs, and terrestrial species with lungs. We used frequentist statistics to test the prediction that body mass of aquatic species without lungs would exhibit a stronger temperature—size response than other the treatment other groups, simply by testing the statistical significance of the interaction between temperature and treatment group in a GLM.

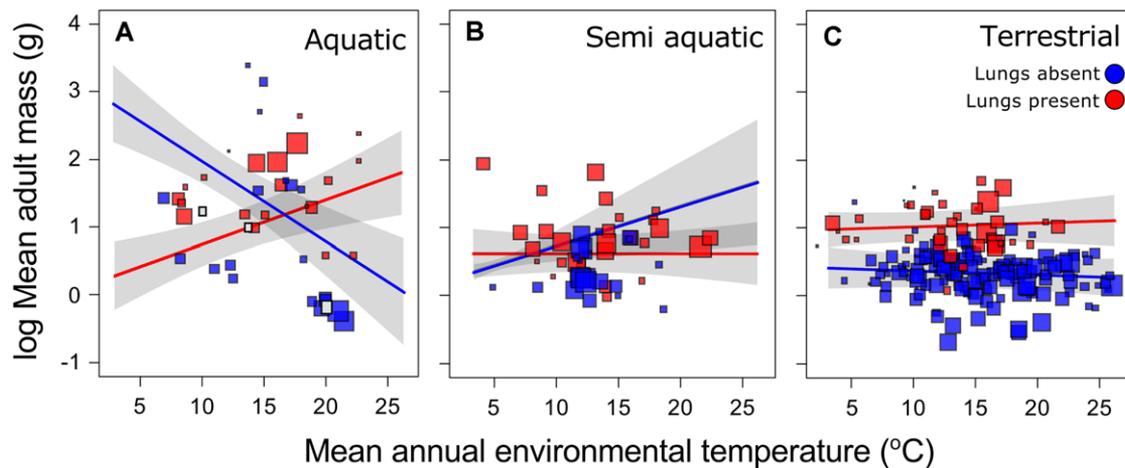
## Results

We estimated body size for 270 salamanders that vary in habitat and mode of respiration ( $n = 20$  lungless and aquatic,  $n = 20$  aquatic with lungs,  $n = 23$  lungless and semiaquatic,  $n = 28$  semiaquatic with lungs,  $n = 138$  lungless and terrestrial,  $n = 42$  terrestrial with lungs). We used model selection in a weighted PGLS framework to explore support for the interaction between mode of respiration, temperature, and habitat. Of the 18 candidate models, the model featuring the three-way interaction between environmental temperature, mode of respiration, and habitat was overwhelmingly supported ( $w_i = 1.0$ , Table 1). As predicted, the interaction suggested that body size of aquatic species without lungs declined relatively strongly with temperature (Fig. 3; Table 2).

This result was robust to different methods of controlling for geographic range size, to the inclusion of cave-dwelling species, and to the metric of temperature used. For instance, we added the three cave-dwelling species to the data, which increased our sample size of lungless aquatic species from 20 to 23, or by 15%. Here, we found that the model featuring a three-way interaction was overwhelmingly supported for these 273 species ( $w_i = 1.0$ ; Tables S2 and S3), suggesting that cave-dwelling aquatic species follow a similar temperature—body size relationship as surface-dwelling species (Fig. 3A). Further, using mean annual air temperature for all 270 species (rather than using water temperature

**Table 1.** Model rankings of top six candidate models (of 18 total models) predicting variation in (log) adult body weight of salamanders that differ in habitat (aquatic, semiaquatic, terrestrial), mode of respiration (lungs present, or lungs absent), with mean annual environmental temperature as a covariate (water temperature for aquatic species, air temperature for semiaquatic and terrestrial species).

Rank	Model formulation	$k$	$\Delta AICc$	$w_i$	Log-likelihood
1	Habitat $\times$ TempMn $\times$ Lungs	14	0	1.0	-118.8
2	Habitat $\times$ TempMn + Habitat $\times$ Lungs + TempMn $\times$ Lungs	12	17.1	0.0	-129.6
3	Habitat $\times$ Lungs + TempMn $\times$ Lungs	10	17.3	0.0	-131.8
4	Habitat $\times$ Lungs	8	23.8	0.0	-137.2
5	Habitat $\times$ TempMn + Habitat $\times$ Lungs	11	25.3	0.0	-134.7
6	Habitat $\times$ Lungs + TempMn	9	25.4	0.0	-137.0



**Figure 3.** Interaction plot for the model that best predicted variation in log adult weight from mode of respiration (lungs present, red; lungs absent, blue), adult habitat (aquatic, semi-aquatic, terrestrial), and mean annual temperature. Mean adult mass in relation to (A) water temperature for aquatic species, (B) air temperature for semi-aquatic species, (C) air temperature for terrestrial species. Pale blue squares in panel (A) are lungless salamanders that are aquatic and live in caves; cave-dwelling salamanders are plotted for comparison, but not featured in the analysis (see Tables S2 and S3 for analysis including these three species). Prediction lines represent interactions between variables and mean annual temperature, plotted by habitat type for clarity. Each datapoint is one species, with the size of the datapoint inversely proportional to log geographic range size. In each panel, fits pass through the phylogenetic means, not the data means. Confidence intervals were estimated in R with the *effects* package and should be considered approximate.

**Table 2.** Parameter estimates from the best candidate model exploring how body size of salamanders is predicted by habitat (aquatic habitat is the reference category), mode of respiration (no lungs is the reference category), and mean annual environmental temperature.

Parameter	Value	LCI	UCI	$\lambda$
Intercept	0.811	0.488	1.13	0.95
Habitat (terrestrial)	-1.11	-1.47	-0.753	
Habitat (semiaquatic)	-0.820	-1.12	-0.520	
TempMn	-1.14	-1.63	-0.652	
Lungs (yes)	-0.366	-0.797	0.064	
Habitat (terrestrial) $\times$ TempMn	1.09	0.567	1.60	
Habitat (semiaquatic) $\times$ TempMn	1.14	0.611	1.67	
Habitat (terrestrial) $\times$ Lungs (yes)	1.06	0.585	1.54	
Habitat (semiaquatic) $\times$ Lungs (yes)	0.415	-0.052	0.883	
TempMn $\times$ lungs (yes)	1.79	1.16	2.41	
Habitat (terrestrial) $\times$ TempMn $\times$ lungs (yes)	-1.67	-2.35	-1.00	
Habitat (semiaquatic) $\times$ TempMn $\times$ Lungs (yes)	-1.42	-2.14	-0.697	

for aquatic species and air temperature for semiaquatic and terrestrial species) resulted in two competing models (cumulative  $w_i = 0.70$ ), the best-supported of which ( $w_i = 0.39$ ) featured the three-way interaction, with lungless aquatic species showing a relatively strong, negative, temperature-size response (Table S4 and S5). Finally, restricting our analysis to surface-dwelling species with range sizes of up to 50,000 km<sup>2</sup> resulted in a sample size of 153 species ( $n = 13$  lungless and aquatic,  $n = 10$  aquatic with lungs,  $n = 0$  lungless and semiaquatic,  $n = 12$  semiaquatic with lungs,  $n = 111$  lungless and terrestrial,  $n = 19$  terrestrial with lungs), but the same patterns were apparent despite the substantially smaller dataset: using a frequentist approach, body size of species that are lungless and aquatic declined with environmental temperature significantly more strongly than in semiaquatic and terrestrial species (Fig. S1; Table S6). In sum, all methods of data analysis suggested that body size of aquatic species without lungs declined with temperature relatively strongly compared to semiaquatic and terrestrial species.

## Discussion

The present study provides comparative phylogenetic support to the hypothesis that oxygen limitation can play a role in generating Bergmann's rule in ectotherms, primarily those where respiration depends strongly on diffusive processes. We found evidence that species mean body size of aquatic salamanders that respire cutaneously and/or bronchially declines relatively strongly with increasing environmental temperature, compared to body size of aquatic salamanders that can breathe aerially, and compared to body size of semiaquatic and terrestrial salamanders. This result was supported regardless of which metric of environmental temperature was used in the analysis (mean air temperature, or mean air/water temperature), and the pattern was consistent when incorporating a small sample of cave-dwelling species, and when we analyzed all available species or only species with small range sizes.

The MASROS hypothesis (Maintain Aerobic Scope and Regulate Oxygen Supply) suggests that metabolic rate increases more rapidly with temperature than oxygen diffusivity, and as a result, gas exchange systems with a diffusional step likely have difficulty maintaining oxygen supply in warm environments (von Bertalanffy 1960; Woods 1999; Atkinson et al. 2006). Rate of ventilation of respiratory tissues and surfaces will increase with temperature to supply oxygen to tissues, but these behaviors too require oxygen, and so cannot be increased or sustained indefinitely. Thus, a plastic reduction of body size in warm aquatic environments effectively increases the ratio of absorptive surface area relative to potential oxygen consumption, such that scope for aerobic activity is maintained. Limitations associated with oxygen diffusion can be partly overcome with forced air convection

over respiratory surfaces, as occurs in air-breathing species with lungs, as forced convection greatly accelerates oxygen delivery (Angilletta and Dunham 2003; Klok et al. 2004). In water, however, oxygen solubility is temperature dependent, and because water is viscous, the energetic costs of respiration and ventilation are higher than in air (Pauly 2010; Verberk and Atkinson 2013). Evidence of temperature-dependent oxygen limitation should be especially apparent where oxygen availability is temperature dependent, as in water, and for species that cannot uptake oxygen using forced air convection, as in lungless salamanders. We therefore suggest that the negative relationship between temperature and body size of salamanders that are both aquatic and lungless arises because of temperature-dependent oxygen limitation (Fig. 3). If this interpretation is correct, then we have demonstrated that Atkinson et al.'s (2006) MASROS hypothesis may extend well beyond patterns of phenotypic plasticity among ectotherms, such that temperature-dependent oxygen limitation can also help explain the rise of Bergmann's rule in aquatic vertebrates.

A second finding in the present study is that body size of salamanders that are not aquatic and lungless either increases with environmental temperature, or shows little correlation with temperature (Fig. 3). Similarly, previous studies have shown that the TSR, James's rule, and Bergmann's rule are frequently absent or reversed for terrestrial ectotherms (Ashton and Feldman 2003; Adams and Church 2008; Pincheira-Donoso et al. 2008; Forster et al. 2012; Horne et al. 2015), possibly because the ability to ventilate aerially should alleviate the energetic cost of oxygen uptake (Atkinson et al. 2006; Pauly 2010). The increase in size of the lunged aquatic salamanders with temperature might arise because foraging activity is not restricted by moisture in aquatic and semiaquatic environments, whereas foraging in terrestrial environments often is (Jaeger 1980; Bruce et al. 2000). Air-breathing aquatic larvae may therefore be able to grow more rapidly during early life compared to their terrestrial counterparts. Relatively rapid early growth in warm aquatic environments, in the absence of strong oxygen limitation, may then select for a relatively larger size at metamorphosis and maturity, as less time would be spent at small body sizes where individual survival prospects are low (Rowe and Ludwig 1991).

The present study is correlative, and we argue that oxygen availability is associated with body-size evolution without any direct estimates of oxygen (see also Forster et al. 2012; Horne et al. 2015). In a similar study, correlative evidence in support of temperature-dependent oxygen limitation as a driving mechanism of the TSR was provided by Forster et al. (2012), who demonstrated that aquatic species show stronger body-size plasticity in response to temperature than terrestrial species. Further, Horne et al. (2015) demonstrated that the strength of a clade's plasticity in response to temperature mirrors the strength and

direction of James's Rule, with aquatic clades overwhelmingly exhibiting negative temperature–size responses, suggesting that temperature-dependent oxygen relationships affect plasticity (i.e., the TSR) and microevolution (i.e., James's rule) in a similar manner. Although our study remains correlative, ours improves on the methods of Forster et al. (2012) and Horne et al. (2015) by moving beyond a contrast of aquatic and terrestrial environments, in that we compared temperature–size responses of aquatic and terrestrial species in a fully factorial design that accounts for aquatic versus aerial respiration in a phylogenetically explicit context. Nevertheless, alternative explanations exist for the decline in body size of lungless aquatic salamanders with temperature. For instance, many of the lungless aquatic species used in our contrasts inhabit streams or rivers, whereas most other lunged aquatic salamanders inhabit lakes (Petranka 1998; Sparreboom 2014). As a result, the aquatic groups compared in our contrasts differ not only in mode of respiration, but also in habitat features that may otherwise influence life histories. We therefore caution that this work remains correlational, and that our results can be interpreted in ways that are independent of temperature-dependent oxygen limitation.

The small, lungless, aquatic *Eurycea* species that we analyzed are endemic to small ponds and lakes in the southern United States (Petranka 1998). These paedomorphic species have small range sizes, and as can be seen from Figure 3A (the cluster of large squares in the bottom-right corner), are therefore important drivers of the negative relationship between body size and temperature. The aquatic habitats inhabited by these species are thermally stable, fed by aquifers, and are between 18.9°C and 21.5°C regardless of time of year. One interpretation of our results, then, is simply that ecological constraints on size exist in species that inhabit smaller ponds, and these small *Eurycea* by chance happen to inhabit warm environments, thereby driving the negative relationship between body size and temperature for lungless, aquatic species. We cannot discount this possibility, however, we note two lines of evidence that favor the oxygen-limitation interpretation. The first is simply that a mean temperature of *ca.* 20°C is greater than the mean temperature of the environments inhabited by almost all other aquatic species (except three members of the air-breathing Amphiumidae and Sirenidae at 22.1–22.6°C). Second, and relatedly, the thermal stability of the environments inhabited by the small *Eurycea* indicates that these species always experience a temperature of about 20°C during activity. This temperature is, in fact, exceptionally high relative to the temperatures experienced by many large paedomorphic species during activity. For instance, the genus *Necturus* comprises large paedomorphic salamanders, and the five *Necturus* species in our dataset experience mean annual water temperatures between 14°C and 18°C. However, the actual temperature experienced by these salamanders is much lower, as they are active only during coolest months of the year, generally when water temperature is between 7°C

and 9°C, and either aestivate or are inactive during summer when water temperature is warm (Braswell and Ashton 1985; Petranka 1998; Brenes and Ford 2006; Beattie et al. 2017). A constant water temperature of 20°C therefore appears to be an extreme temperature to sustain activity among lungless, aquatic species, and we argue these extreme temperatures have played a role in the evolution of small size among the paedomorphic *Eurycea*.

Most research on temperature-dependent oxygen limitation has been performed on invertebrates, primarily arthropods (Verberk et al. 2016), single-celled organisms (Forster et al. 2012), or organisms with a fixed number of cells (Czarnoleski et al. 2015; Walczyńska et al. 2015). Taxonomic bias, and a strong bias toward arthropods in particular, is reflected in all existing meta-analyses (Forster et al. 2012; Klok and Harrison 2013; Horne et al. 2015), and it is therefore valuable to explore temperature-dependent oxygen limitation in a vertebrate system.

To our knowledge, our study is the most comprehensive examination of how temperature-dependent oxygen limitation might drive the macroevolution of adult size, both in terms of sample size ( $n > 200$ ), taxonomic coverage (38% of salamander species, 100% of families represented), and in its phylogenetically explicit nature. More broadly, perhaps the most remarkable result of our study is that a signature of temperature-dependent oxygen limitation withstood a phylogenetically explicit test across the diversity of amphibian lifestyles and habitats (Wells 2010). Given that the TSR is widely observed at both the adult and at the embryonic, juvenile, and larval stages (Atkinson et al. 2001), future research using amphibians can leverage their bewildering array of ontogenetic differences, reproductive modes, habitats, and mechanisms of larval and adult oxygen uptake (Ultsch et al. 1999) to produce nuanced tests of how the interaction of temperature and oxygen influences body-size evolution at all life stages, and ultimately how adaptation to temperature and oxygen availability affects life-history trajectories.

#### AUTHOR CONTRIBUTIONS

NR conceived this work, collected the data, and performed the analysis. NR and LR wrote the manuscript.

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#### DATA ARCHIVING

Data available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.8k28p34>.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

**Table S1.** Interspecific regressions used to convert  $\log(\text{SVL, mm})$  to  $\log(\text{adult weight, g})$ .

**Table S2.** Model rankings of top six candidate models (of 18 total models) predicting variation in (log) adult body weight of salamanders that differ in habitat (aquatic, semi aquatic, and terrestrial), mode of respiration (lungs present, or lungs absent), with mean annual environmental temperature as a covariate.

**Table S3.** Parameter estimates from the best candidate model exploring how body size of salamanders is predicted by habitat (aquatic habitat is the reference category), mode of respiration (no lungs is the reference category), and with mean annual environmental temperature as a covariate.

**Table S4.** Model rankings of top six candidate models (of 18 total models) predicting variation in (log) adult body weight of salamanders that differ in habitat (aquatic, semiaquatic, terrestrial), mode of respiration (lungs present, or lungs absent), with mean annual air temperature as a covariate.

**Table S5.** Parameter estimates from the best candidate model exploring how body size of salamanders is predicted by habitat (aquatic habitat is the reference category), mode of respiration (no lungs is the reference category), and mean annual air temperature.

**Table S6.** Model describing variation in log adult body weight as an interaction between treatment group (combinations of habitat type and mode of respiration) using mean annual environmental temperature as a covariate.

**Figure S1.** Variation in adult body size predicted by the interaction between salamander habitat (A, aquatic; S, semiaquatic; T, terrestrial), mode of respiration (L, lungs present; NL, no lungs) and mean annual environmental temperature.