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# The evolution of large-scale body size clines in *Plethodon* salamanders: evidence of heat-balance or species-specific artifact?

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A major goal in macroecology is to determine how body size varies geographically, and explain why such patterns exist. Recently, a grid-cell assemblage analysis found significant body size trends with latitude and temperature in *Plethodon* salamanders, and support for the heat-balance hypothesis as a possible explanation for these trends. Here we demonstrate that the heat-balance hypothesis is unlikely to have generated this pattern, and that there is no overall body size trend with temperature in *Plethodon*. Using data from 3155 local *Plethodon* assemblages, we find no support for body size clines with latitude, and no relationship between body size and temperature. We also found that body size did not covary with elevation, in contrast to what was predicted by heat-balance. We then examined the various scenarios under which body size clines across grid-cell assemblages could evolve via heat-balance, and found that none were tenable in light of the existing data. Instead, a single, widely distributed species was responsible for the pattern across grid-cell assemblages. Finally, we examined why phylogenetic eigenvector regression does not account for phylogenetic non-independence among taxa, and should not be used to account for shared evolutionary history in assembly-level analyses. Assemblage-level patterns are a useful means of assessing biogeographic trends, and are an important complement to within-species and cross-species patterns. However, while the use of grid-cell assemblage approaches from digital databases is expedient, their results must be examined critically, and whenever possible, compared with data obtained from local species assemblages (particularly for ecological mechanisms that operate at the level of individuals). Finally, our results emphasize the importance of using corroborative data to evaluate alternative hypotheses, so that potential mechanisms that explain bioegeographic patterns are properly assigned.

Understanding how and why body size varies across the landscape has long fascinated ecologists and evolutionary biologists. Body size clines are found in a wide variety of taxa, and at various hierarchical scales (Gaston et al. 2008). Body size sometimes varies within species, where distinct sizes are exhibited in different populations across the landscape (Ashton et al. 2000, Belk and Houston 2002, Adams and Church 2008). Body size clines are also observed among species (Ray 1960, Blackburn and Ruggiero 2001), and at larger spatial scales, may vary across assemblages of species found in particular geographic locations (Blackburn and Hawkins 2004, Olalla-Tárraga and Rodríguez 2007). Such assemblage-level patterns are frequently identified by overlaying species' distributions on gridded maps, where co-occurring species in each grid-cell are identified (i.e. an assemblage; sensu Fauth et al. 1996), and patterns of mean body size across grid-cell assemblages are examined (Blackburn and Hawkins 2004, Olalla-Tárraga et al. 2006, Rodríguez et al. 2006, Diniz-Filho et al. 2007, Olalla-Tárraga and Rodríguez 2007). With the advent of large-scale geo-referenced databases, assemblage-level analyses at one or more spatial scales are becoming increasingly common.

In many endothermic vertebrates, body size tends to increase with latitude, a pattern termed Bergmann's rule (Bergmann 1847, Mayr 1956, Meiri and Dayan 2003, Blackburn and Hawkins 2004, Meiri and Thomas 2007). Several mechanisms have been proposed to explain Bergmann's clines (e.g. differential dispersal: Cushman et al. 1993; resource limitation: Rosenzweig 1968), but most explanations focus on temperature and heat conservation. Here, selection is thought to favor larger animals in cooler environments, as their surface-to-volume ratios make them better able to conserve heat (Bergmann 1847). Body size clines have also been observed in ectotherms (Ray 1960, Ashton 2002), with some groups displaying an increase in body size with latitude (e.g. turtles: Ashton and Feldman 2003; lizards: Olalla-Tárraga et al. 2006), and other groups showing a decrease in body size with latitude (e.g. salamanders: Olalla-Tárraga and Rodríguez 2007, but see Adams and Church 2008). Recent explanations for these patterns have also been based on temperature, following

the heat-balance hypothesis (Olalla-Tárraga and Rodríguez 2007). This hypothesis predicts that thermoregulating species will display increasing body size clines with cooler temperatures, in accordance with heat conservation. For thermoconforming species however, the hypothesis predicts that smaller body sizes are selectively advantageous in cooler environments, as greater surface-volume ratios allow for shorter heating times (Olalla-Tárraga and Rodríguez 2007).

We recently documented body size trends in *Plethodon* salamanders in eastern North America, at both the within-species and between-species levels (Adams and Church 2008). Our analyses revealed no evidence for body size clines with temperature or latitude at either of these hierarchical scales. By contrast, a re-analysis of this group using grid-cell assemblages and phylogenetic eigenvector regression found a negative relationship between mean body size and latitude, and a positive relationship between mean body size and mean temperature (Olalla-Tárraga et al. 2010). From this it was concluded that *Plethodon* salamanders do exhibit body size clines in support of the heat-balance hypothesis, albeit at a larger spatial scale than was previously examined (Olalla-Tárraga et al. 2010).

In this article, we provide additional tests of the heat-balance hypothesis in an effort to determine what processes may have generated the various body size trends observed in *Plethodon.* First we examine body size trends across local species assemblages at intermediate spatial scales and across elevation, and show that neither displays patterns consistent with the heat-balance hypothesis. We then describe why the heat-balance hypothesis is insufficient to explain the large-scale body size trend in *Plethodon,* and propose an alternative explanation that more succinctly explains this pattern (and the lack of patterns at other spatial scales). We also show why phylogenetic eigenvector regression does not account for phylogenetic non-independence among taxa, and should not be used to account for shared evolutionary history in assembly-level analyses. Some implications of our findings for use of grid-cell methods to identify ecogeographic trends, and for assigning causal processes to explain them, are discussed.

## Body size trends across local assemblages

Assemblage-level analyses examine the spatial distribution of body sizes in faunal assemblages across particular regions, where assemblages are the unit of analysis (Blackburn and Hawkins 2004, Olalla-Tárraga et al. 2006, 2010). Typically, grid-cell assemblages encompass relatively large regions (e.g. $20 \times 20$ km, $100 \times 100$ km, etc.), allowing biogeographic patterns at large spatial scales to be evaluated. Body size trends may also be present at intermediate spatial scales, such as across local assemblages obtained from distinct field localities. However, these patterns are less frequently evaluated, in part because this requires information on hundreds to thousands of actual local species assemblages in distinct geographic locations. Nevertheless, patterns in local assemblages are critically important for evaluating the potential processes that have generated patterns at larger spatial scales. The reason for this is simple. Some mechanistic processes operate on individuals. As such, large-scale patterns can only be observed from such processes if they emerge from patterns generated at lower levels. With respect to body size, mechanisms based on natural selection must necessarily operate on individuals, not on mean values found across species in an assemblage (because means across species are not inherited). Therefore, if an ecogeographic pattern at larger spatial scales is generated primarily by natural selection, a similar pattern should also be observed across assemblages at some lower spatial scale. Put another way, while some ecogeographic patterns are scale-dependent (Blackburn and Gaston 2002), not all mechanistic processes will generate scale-dependency.

The heat-balance hypothesis is a process based on natural selection: body size clines are obtained as a result of selection of individuals in their local climatic environment (Olalla-Tárraga and Rodríguez 2007, Olalla-Tárraga et al. 2010). Therefore, if this mechanism is responsible for patterns observed across mean body sizes in grid-cell assemblages, one may reasonably predict that similar patterns should be found between mean body size and temperature across assemblages at smaller geographic scales as well. Here we test this prediction by comparing patterns in grid-cell assemblages to those found in local assemblages, using an extensive dataset from over 3000 local assemblages of *Plethodon* salamanders.

For the grid-cell approach, we replicated the results reported in Olalla-Tárraga et al. (2010). First we generated a $110 \times 110$ km grid containing 319 grid-cells, and on this grid overlaid the geographic distributions of 44 species of *Plethodon* in eastern North America (using the same geographic range maps as in Olalla-Tárraga et al. 2010). The species present in each grid-cell were then determined, and the mean body size for each grid-cell assemblage was estimated from the $\log_{10}$ body sizes for all species present in that grid-cell. For the local *Plethodon* assemblages, we obtained the modal adult body size for each species in each of 3155 naturally-occurring *Plethodon* assemblages (from Adams 2007), using a dataset of 96 996 adult body size measurements from these localities (Adams and Church 2008). These assemblages represent local communities of *Plethodon* salamanders found in distinct geographic localities, and genetic studies from hundreds of these localities have documented the genetic differentiation among populations and species in many of these localities (Highton 1995, 1999, Highton and Peabody 2000). The average $\log_{10}$ body size among species in each local assemblage was then calculated. In addition, we calculated the overall mean annual temperature averaged across locations within each grid-cell (following Olalla-Tárraga et al. 2010) using climate data from the WorldClim database (Hijmans et al. 2005). We also obtained the mean annual temperature for each of the local assemblages. The range of annual mean temperatures in each grid-cell was also calculated. For both datasets, the relationship between body size and latitude and between body size and temperature were obtained and compared. All analyses were performed in R (R Development Core Team 2010).

Using the grid-cell assemblages, there was a strong negative relationship between mean body size and latitude (Fig. 1a), implying that larger mean body sizes were found in grid-cell assemblages at lower latitudes (Olalla-Tárraga et al. 2010). Across local *Plethodon* assemblages however, both the largest and the smallest mean body sizes were found for assemblages at middle latitudes, while assemblages intermediate in body size were found at lower

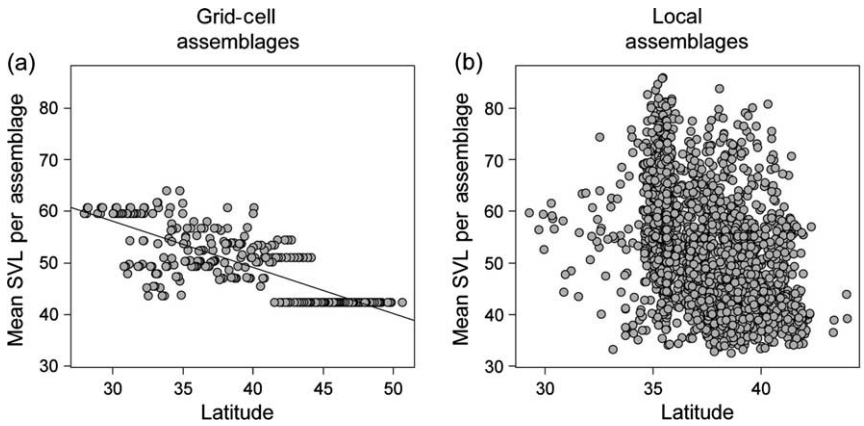



Figure 1. Body size trends across *Plethodon* assemblages across eastern North America (shown in un-logged units): (a) mean body size versus latitude for 319 grid-cell assemblages (110 × 110 km: following Olalla-Tárraga et al. 2010). (b) Mean body size versus latitude from 3155 local *Plethodon* assemblages.

latitudes (Fig. 1b). When temperature was considered, there was a strong positive relationship with body size in grid-cell assemblages ($R^2 = 0.6323$), but not in local *Plethodon* assemblages ($R^2 = 0.0057$). Thus, body size patterns across local assemblages were consistent with the lack of trends at both within-species and between-species levels, and were not consistent with what was predicted by the heat-balance hypothesis. This finding therefore weakens the support for the heat-balance model as a potential mechanism responsible for the body size clines across grid-cell assemblages, as similar patterns are not found at smaller spatial scales.

## Body size trends and elevation

Body size clines are frequently examined along latitudinal gradients, as temperate covaries with latitude (Olalla-Tárraga and Rodríguez 2007, Olalla-Tárraga et al. 2010). However, temperature also covaries with elevation, so any process requiring temperature gradients should also generate body size trends across elevation. Examining this prediction therefore provides an additional test of the heat-balance hypothesis, and its putative role in generating the latitudinal cline observed across *Plethodon* grid-cell assemblages. We tested this prediction at two spatial scales: across local assemblages and across larger grid-cell assemblages. For the large-scale analysis we obtained the mean elevation for each of the 319 grid-cell assemblages, and assessed the relationship between mean body size and mean elevation across these assemblages. Similarly, we obtained the elevation for each of the 3155 local assemblages, and assessed the relationship between elevation and mean body size at this intermediate spatial scale. In eastern North America, *Plethodon* salamanders are found from sea level to above 1850 m, providing a sufficient elevational range to test this prediction.

At both spatial scales, there was no clear relationship between body size and elevation, with most elevations displaying the full range of body sizes observed across assemblages (Fig. 2). In addition, at the highest elevations, intermediate body sizes were found, rather than small body sizes as predicted by the heat-balance hypothesis. Correspondingly, elevation explained only a small percentage of body size variation, particularly across grid-cell assemblages ($R^2 = 0.0827$). These results revealed that relative to elevation, body size did not display clinal variation consistent with the heat-balance hypothesis at either spatial scale. As such, these patterns do not provide support the heat-balance hypothesis as a mechanism driving body size variation in *Plethodon.*

## Reassessing the heat-balance hypothesis for *Plethodon*

Two major goals in macroecology are to document large-scale biogeographic trends and to identify the causal processes responsible for them (Brown and Maurer 1989). Considerable work over the past few decades has documented ecogeographic patterns in many taxa, and has explored the relationship between patterns at distinct hierarchical scales (reviewed in Gaston et al. 2008). However, while documenting ecogeographic patterns can be relatively straightforward, identifying the underlying processes responsible for them is more complicated. For the latter we advocate a strong inference approach (sensu Platt 1964), where multiple ecogeographic patterns are quantitatively examined to determine whether or not they are consistent with predictions derived from a putative mechanistic process.

For salamanders, the heat-balance hypothesis predicts that body size decreases in cooler climates as a result of selection (Olalla-Tárraga and Rodríguez 2007), implying that smaller organisms should be found in more northerly latitudes and at higher elevations. However, the empirical evidence in *Plethodon* does not support this hypothesis. Body size clines are not exhibited with either temperature or latitude within species or between species (Adams and Church 2008), nor are they found across local species assemblages. Additionally, no body size clines are evident with elevation in either local or grid-cell assemblages. In fact, the only body size cline observed in this genus is for large-scale grid-cell assemblage versus temperature and latitude. Taken together, these observations suggest that *Plethodon* salamanders generally lack any biogeographic

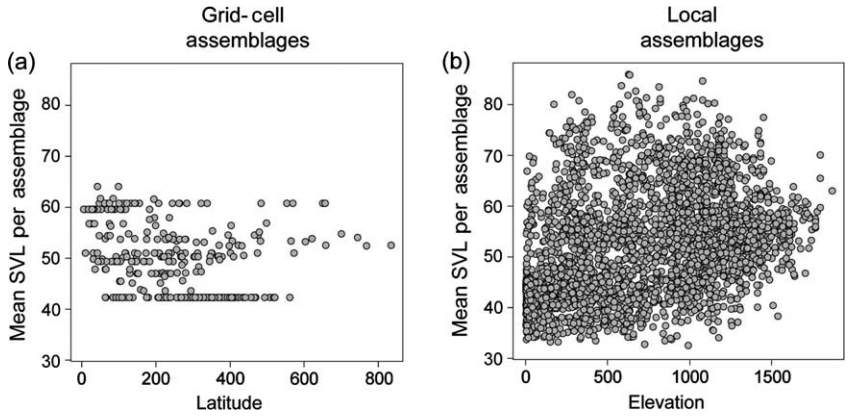



Figure 2. Body size trends across *Plethodon* assemblages across eastern North America (shown in un-logged units): (a) mean body size versus elevation for 319 grid-cell assemblages (110 × 110 km: following Olalla-Tárraga et al. 2010). (b) Mean body size versus elevation from 3155 local *Plethodon* assemblages.

trends in body size; an overall pattern that does not support the heat-balance hypothesis.

Beyond these observations however, it is also unlikely that the heat-balance hypothesis has generated the body size cline observed across grid-cell assemblages. To see why this is the case, consider from a biological perspective how ecogeographic clines could evolve by heat-balance. The heat-balance model is based on natural selection: body size clines evolve as a result of selection on individuals relative to their local climatic environment (Olalla-Tárraga and Rodríguez 2007, Olalla-Tárraga et al. 2010). But given that natural selection does not operate on means across species, there are only a few biological scenarios under which the heat-balance hypothesis could generate body size clines across large-scale grid-cell assemblages. One scenario is if populations of each species in each geographic region evolved in the direction predicted by heat-balance. This would result in a systematic decrease in body size in for all species in northerly localities, generating a lower mean body size among them, and the converse pattern in more southerly localities. However, this process would also generate within-species body size clines, a pattern which is not observed in *Plethodon* (Adams and Church 2008). Therefore, the body size trend observed across grid-cell assemblages could not have evolved under this scenario. A second possibility is if small species were found in northerly latitudes and larger species found in southerly latitudes, resulting in body size clines across grid-cell assemblages. However, this scenario would also result in latitudinal segregation of species by body size, which is not seen in *Plethodon* (most small and large *Plethodon* species widely overlap latitudinally: Highton 1995, Adams 2007). Further, this scenario would generate between species associations between body size and temperature, which are not observed (Adams and Church 2008). Thus, the heat-balance model could not have generated grid-cell assemblage patterns via this scenario. Finally, the large-scale assemblage-level patterns could evolve from patterns observed in assemblages at smaller spatial scales under this hypothesis. Yet, in *Plethodon,* patterns in local assemblages do not correspond what is observed in grid-cell assemblages, thus not supporting the heat-balance model as a possible explanation of the large-scale trends.

The result of this thought experiment is clear. In *Plethodon*, nearly all ecogeographic patterns display no systematic change in body size with latitude, elevation, or temperature. The only clinal pattern that is observed is found across large-scale grid-cell assemblages relative to latitude. However, this large-scale pattern was not generated by the heat-balance model, as other patterns predicted by this hypothesis are not observed, and the likely scenarios under which this large-scale pattern could have evolved are not supported by the evidence. Clearly, an alternative explanation for the body size trend across grid-cell assemblages is required.

## A possible explanation: geographic outliers

One possible explanation for the body size trend observed across grid-cell assemblages is that some geographically 'extreme' species biases the analyses at this spatial scale. Widely distributed species are known to unduly influence assemblage-level patterns of species richness (Gaston et al. 2008), and similar biases may exist for body size patterns as well (Meiri and Thomas 2007, Gaston et al. 2008). In eastern *Plethodon*, the most widely distributed species (*P. cinereus*) occupies more than half of the distribution of the entire genus (1.8 million $km^2$ out of 3.1 million $km^2$), and is the only species found in the northern-most 1.23 million $km^2$ of the genus' range. Furthermore, *P. cinereus* is a small salamander. The consequence is that nearly a third of all grid-cell assemblages for this genus are comprised of a single, small-bodied species, found in the northerly locations.

This issue was noted in Olalla-Tárraga et al. (2010), and a separate analysis excluding *P. cinereus* was presented to account for this potential bias. The results of this analysis are revealing. When *P. cinereus* was excluded from the analysis, temperature no longer had a significant effect on body size (Table S2 in Olalla-Tárraga et al. 2010). Consistent with this, we performed a regression of body size on temperature excluding *P. cinereus* and found a non-significant pattern ($R^2 = 0.004$; $\hat{\beta} = 0.029$, $T = 0.30$, $p = 0.76$). These analyses demonstrate that the statistical relationship between body size and temperature in grid-cell assemblages is driven by the presence of a single, small-bodied species with an expansive range and northerly distribution, and that the relationship between body size

and temperature is not a general attribute of *Plethodon* salamanders. Thus, the large-scale ecogeographic pattern can be explained not by heat-balance, but by the simple fact that a single, small bodied species lives in considerably cooler environments than does the rest of the genus (including other small-bodied *Plethodon*). Therefore, when examining all of the available evidence, the interesting biological question that emerges is not whether *Plethodon* conform to the heat-balance hypothesis, but rather what adaptations enable *P. cinereus* to live so far north.

## Considering phylogeny in ecogeographic studies

At larger spatial scales, ecogeographic clines are revealed by identifying the relationship between body size and temperature across species, or across species assemblages. Yet in both cases, shared evolutionary history must be considered, because the phylogenetic relationships among species generate statistical non-independence (Felsenstein 1985). One means of accomplishing this through phylogenetic generalized least squares (PGLS: Martins and Hansen 1997). PGLS is a standard least squares model that incorporates phylogenetic non-independence directly in the error term, rather than assuming independently distributed error among species. To estimate non-independence due to shared evolutionary history, one requires an explicit model of character evolution; the most common of which is Brownian motion (see discussion in Felsenstein 1985). With PGLS, different models of character evolution can be used (Brownian motion, Ornstein-Uhlenbeck, ACDC, etc. see Blomberg et al. 2003, Butler and King 2004), and the most widely-used comparative approach (phylogenetically independent contrasts) is a particular implementation of the PGLS method assuming a Brownian motion model of evolution (Garland and Ives 2000, Rohlf 2001).

With respect to body size clines in *Plethodon*, phylogenetically independent contrasts were previously used in cross-species analyses, revealing no relationship between body size and temperature (Adams and Church 2008). For within-species patterns, recently-developed analytical methods combining PGLS and meta-analysis can be utilized (Adams 2008, Lajeunesse 2009). For *Plethodon,* a phylogenetic meta-analysis of within-species patterns also reveals no significant relationship between body size and temperature ($\bar{E} = 0.2739$; $t = 1.373$; $p = 0.178$; $p_{rand} = 0.403$). Thus, for both within-species and cross-species analyses, a body size trend with temperature could not be distinguished from chance variation, when phylogeny was taken into consideration.

At the assemblage level, a phylogenetic eigenvector regression (PVR: Diniz-Filho et al. 1998) was used in Olalla-Tárraga et al. (2010) to incorporate phylogeny. PVR differs substantially from PGLS, in that it does not directly model phylogenetic non-independence or character evolution. Instead, it partitions variation in the extant species by regressing the dependent variable (e.g. body size) on a subset of eigenvectors obtained from a matrix of phylogenetic distances among species. Predicted values from this regression are considered the phylogenetic component (P) and all residual error variation from this regression is considered the species component (S). In Olalla-Tárraga et al. (2010) the first five (out of 43) eigenvectors were used, and body size was regressed on these to obtain the phylogenetic (P) and species (S) components. Significant relationships with temperature in both the P and S components of body size were found, and it was concluded that the latter represented species-specific adaptations to climatic condition (Olalla-Tárraga et al. 2010).

Unfortunately, there are several weaknesses that limit the utility of PVR. First, the number of eigenvectors one chooses to represent the phylogenetic component is arbitrary (described in Rohlf 2001). Therefore, a greater or lesser proportion of variation can be attributed to phylogeny simply by changing the number of eigenvectors included. For instance, if all eigenvectors were used, 100% of the variation among species would be attributed to phylogeny. In this case, there would be no species-specific effect, and as such one would conclude that *Plethodon* did not exhibit species-specific adaptations to climatic condition. This method also leads to the unintuitive result that when all eigenvectors are used, the phylogenetic component from PVR is identical to, and perfectly correlated with the original data. Such a result is nonsensical, as it implies that variation in the phylogenetic component of the data is identical to the same data for which phylogeny has not been taken into consideration.

Furthermore, unless all eigenvectors are utilized, PVR does not preserve the phylogenetic relationships among species. To show this distortion, we compared the original phylogeny of *Plethodon* (Wiens et al. 2006) to the tree as viewed from the phylogenetic component of PVR (in this case, the first five eigenvectors obtained from the phylogeny: Olalla-Tárraga et al. 2010). In contrast to the original phylogeny (Fig. 3a), the PVR tree contained shorter branches towards the tips of the phylogeny with many unresolved relationships among closely related species, as well as longer branches between higher nodes of the phylogeny (Fig. 3b). Moreover, comparing the actual phylogenetic distances between species to those estimated by PVR showed that evolutionary relationships were distorted at multiple levels of the phylogeny (Fig. 3c). This reveals that PVR does not partition variation between the species and higher levels of the phylogeny as was originally thought (Diniz-Filho et al. 1998), but rather cuts across different levels of the phylogeny in unpredictable ways. In fact, only when the phylogeny is perfectly balanced, and when one can identify the number of eigenvectors that match the desired level of the tree, could one possibly separate variation at the species level from variation at higher levels on the phylogeny with PVR (F. J. Rohlf pers. comm.).

Finally, partitioning variation in extant species using PVR does not in fact account for phylogenetic non-independence, because there is no explicit model of character evolution involved in the calculation of the phylogenetic (P) and species (S) components. As such, the evolutionary divergence due to phylogenetic non-independence as estimated from PVR differs from what is expected under various models of evolution, including Brownian motion, the evolutionary model most commonly implemented in phylogenetic comparative studies (Garland and Ives 2000, Rohlf 2001). To show this for *Plethodon,* we used PVR to obtain the phylogenetic component of body size (P), using both

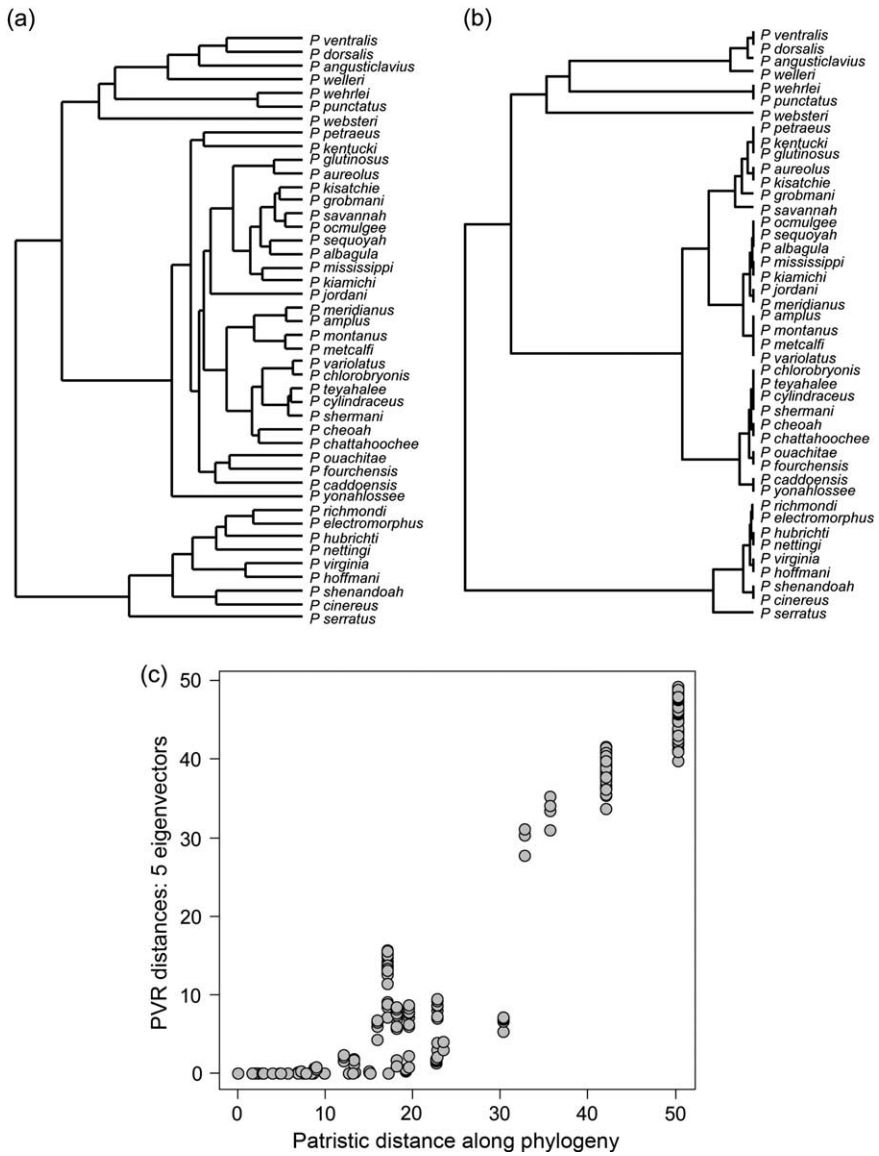



Figure 3. (a) Phylogenetic relationships among eastern *Plethodon* species (from Wiens et al. 2006). (b) Phylogenetic relationships as represented by the phylogenetic component of PVR, found from the first five eigenvectors of the matrix of patristic distances among species. (c) Plot of actual phylogenetic distances versus those estimated using PVR from this example. Phylogenetic analyses performed in R (R Development Core Team 2010), using the APE (Paradis 2006) and GEIGER (Harmon et al. 2008) packages.

5 eigenvectors and all 43 eigenvectors to represent the phylogeny. Pairwise Euclidean distances between species were then obtained, providing an estimate of the phylogenetic divergence in body size using PVR for each. Next we fit body size to the phylogeny using PGLS and several models of character evolution (Brownian motion, Ornstein–Uhlenbeck, and ACDC), and obtained estimates of ancestral states for all nodes of the phylogeny. For each pair of species, the expected shared evolutionary divergence along the phylogeny was found as the squared difference in body size from the root of the tree to their most recent common ancestor (Rohlf 2001). Comparing these revealed that phylogenetic divergence estimated with PVR was quite different from the observed evolutionary divergence along the phylogeny under Brownian motion, even when all eigenvectors were used (Fig. 4). In addition, phylogenetic divergence estimated with PVR also differed from that obtained from other models of character evolution, including an Ornstein–Uhlenbeck model and an ACDC (early-burst) model (results not shown). As such, the phylogenetic component of PVR does not capture the evolutionary divergence explained by Brownian motion, or other commonly implemented models of character evolution. These results imply that partitioning variation among extant species using PVR is not equivalent to incorporating phylogenetic non-independence through a structured error term in generalized least squares. Therefore, for assemblage-level analyses that wish to consider shared evolutionary history, PGLS and a population-level phylogeny for all species in all assemblages should be used, instead of PVR.

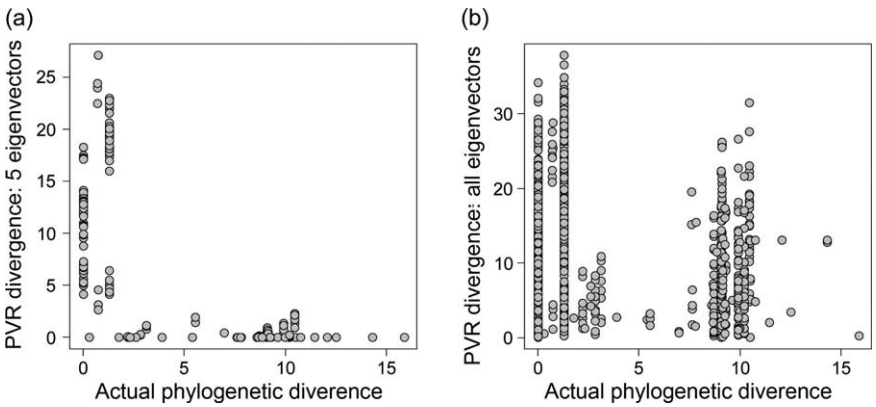



Figure 4. Pairwise phylogenetic divergence in body size among species. Evolutionary divergence along the phylogeny is found as the squared difference in body size from the root of the tree to the most recent common ancestor for each pair of species, using ancestral states estimated from a Brownian motion model of evolution and PGLS. Phylogenetic divergence estimated from PVR are based on pairwise distances in the phylogenetic component (P), using: (a) 5 eigenvectors to represent the phylogenetic component, (b) all eigenvectors to represent the phylogenetic component. Both divergence estimates are shown as distances to be in comparable units.

## Discussion

There are several important biological implications of our study. First, our study demonstrates that the empirical evidence does not support the heat-balance hypothesis for *Plethodon* (Olalla-Tárraga and Rodríguez 2007), and overwhelmingly shows a lack of ecogeographic variation in body size in this group. Specifically, no body size clines with temperature were observed within or between species (Adams and Church 2008), nor across 3155 local assemblages. Further, no body size clines were evident with elevation in either local or grid-cell assemblages, and when phylogeny was taken into consideration, patterns remained unchanged. These observations stand in direct contrast to predictions of the heat-balance hypothesis, which states that body size should decrease with cooler temperatures in salamanders (Olalla-Tárraga and Rodríguez 2007, Olalla-Tárraga et al. 2010). Most importantly, we examined the various scenarios under which body size clines across grid-cell assemblages could evolve via the heat-balance model, and found that none were tenable in light of the existing data. Thus, it is unlikely that the heat-balance hypothesis was responsible for the large-scale body size cline across grid-cell assemblages: the only significant trend observed in *Plethodon*. Taken together, all evidence therefore indicates that no evolutionary explanation is needed to explain the lack of biogeographic pattern in body size at any hierarchical scale, and that these thermoconforming, primarily nocturnal amphibians do not display patterns consistent with the heat-balance hypothesis (sensu Olalla-Tárraga and Rodríguez 2007).

Second, we demonstrated that phylogenetic eigenvector regression (PVR: Diniz-Filho et al. 1998) does not account for phylogenetic non-independence among species in a manner consistent with other phylogenetic comparative approaches. With the most direct approach, phylogenetic generalized least squares (PGLS), species non-independence due to shared evolutionary history is incorporated in the error term of the analysis (Martins and Hansen 1997, Garland and Ives 2000, Rohlf 2001), using a particular model of character evolution, such as Brownian motion. We showed that partitioning variation among extant species using PVR is not equivalent to this, and thus PVR does not capture character change in a manner consistent with typical models of evolution. We also showed that the phylogenetic trait divergence estimated by PVR is not equivalent to the observed evolutionary divergence along the phylogeny, and that the partitioning of variance cuts across different levels of the phylogeny, rather than partitioning species-level patterns from higher-level effects. Finally, using a different number of eigenvectors results in different levels of variation arbitrarily attributed to each component, leading to differing biological interpretations. From this we conclude that PVR has limited utility for phylogenetic comparative studies of body size evolution.

More generally, our results have important implications for understanding biogeographic patterns at various spatial scales. Scale is important in assessing macroecological trends (discussed in Blackburn and Gaston 2002, Gaston et al. 2008), but while within-species and cross-species patterns represent easily identifiable hierarchical levels, what constitutes an assemblage level pattern is less clear. Indeed, even if one uses a consistent definition to identify species assemblages, the meaning of 'assemblage' necessarily differs across spatial scales. For instance, if an assemblage is defined as all co-occurring species in a particular region (sensu Fauth et al. 1996), a local assemblage represents all sympatric species at a particular locality. By contrast, using the same operational definition for grid-cell assemblages, species may be found within a particular grid-cell (and thus 'co-occur' in that assemblage), but may never be locally sympatric. The latter certainly explains some of the disparity in species richness observed across local and grid-cell assemblages for *Plethodon*, where the maximum assemblage richness is 15 in grid-cell assemblages, but only five species are known to co-occur in any local assemblage (Highton 1995, Adams 2007). Also, many *Plethodon* species have parapatric distributions and are thus found in the same grid-cell region, but rarely co-occur in local assemblages. Other species are found on distinct mountains in adjacent regions, or at high or low elevations in the same mountain range (Highton 1995). These fine-scale distributional patterns are driven both by local environmental variation (e.g. montane versus lowland-dwelling species: Kozak and Wiens 2006, 2007, 2010), and interspecific competition (Adams 2007; see also Adams

et al. 2007, Adams 2010). Unfortunately, none of these biogeographic patterns are detectable from species distribution maps that tend to be generated as convex hulls at a spatial scale larger than these patterns can be identified. Thus, when examined with a grid-cell approach, these species are combined in a single, purportedly co-occurring assemblage that may not exist at smaller spatial scales. While the effects of such differences will be data-dependent, our results for *Plethodon* demonstrate that they can be substantial. We therefore urge caution in interpreting patterns obtained from grid-cell assemblages as evidence of local-scale phenomena (such as those derived from selection on traits of individuals), and recommend that such patterns be corroborated with data from actual field-collected, species assemblages to assess such effects at smaller spatial scales.

Our study also highlights the importance of distinguishing pattern and process, and the difficulties in attributing observed ecogeographic patterns to particular causal mechanisms. In the case of *Plethodon*, the large-scale body size cline across grid-cell assemblages was consistent with the heat-balance hypothesis (Olalla-Tárraga et al. 2010). But consistency alone is insufficient to conclude that heat-balance has generated this pattern; other corroborating data must also be examined. Indeed, using a strong-inference approach, we found that additional predictions of the heat-balance model were not supported empirically. Further, we showed that the observed pattern across grid-cell assemblages could not have evolved by any of the scenarios under which heat-balance could have generated it, as other patterns were not consistent with these scenarios. Thus, some alternative explanation for the pattern was required. Upon further examination, we found that ecogeographic pattern across grid-cell assemblages was the result of the presence of a single, widely-distributed, small-bodied species with an extreme northerly distribution (*P. cinereus*). When this species was removed, the patterns were no longer significant (for discussion of similar effects see Lennon et al. 2004, Gaston et al. 2008). This latter result significantly alters the biological inferences; away from general explanations across the genus (e.g. the heat-balance hypothesis: Olalla-Tárraga et al. 2010), and towards species-specific explanations (e.g. what adaptations enable *P. cinereus* to inhabit northerly locations?). Together, these results reveal the importance of corroborating grid-cell assemblage patterns with those found at other hierarchical scales, to test alternative mechanistic hypotheses that may have generated them.

Finally, our results emphasize the importance of considering both the proximate and ultimate components of hypothesized causal scenarios before such mechanisms are proposed as explanations for observed ecogeographic patterns. Explicitly defining the level at which a hypothesized mechanism operates is critical for determining whether it is logically and biologically plausible, and whether or not it can be expected to generate the observed patterns. Frequently, our mechanistic hypotheses are couched in natural selection. However, for selection-based mechanisms, one must carefully consider how they could generate patterns at larger spatial scales, since selection operates on individuals (the level at which genetic inheritance occurs). Clearly, some broad-scale biogeographic patterns have been generated by selection. But in such cases, there must be a direct link between patterns observed at large spatial scales, and patterns manifest at lower levels of biological organization. By comparing patterns across spatial and hierarchical scales, we may more critically evaluate these alternative hypotheses, and generate a greater understanding of the causal processes responsible for ecogeographic patterns.

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