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Body size divergence promotes post-zygotic reproductive isolation in centrarchids

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ABSTRACT

Question: Does morphological divergence accelerate the evolution of post-zygotic reproductive isolation?

Data incorporated: Estimates of divergence time between species, body size divergence, and hybrid embryo viability in the freshwater fish family Centrarchidae.

Method of analysis: We estimated the age of each node in the phylogeny using penalized likelihood, calibrated with multiple fossil dates. We then regressed the average body size and hybrid viability at each phylogenetic node against the node's age. Residuals from these regressions were compared to test for time-independent relationships between body size divergence and post-zygotic reproductive isolation.

Conclusions: Morphologically divergent species tend to experience stronger post-zygotic reproductive isolation than expected given their age. These results suggest that morphological divergence between species is associated with an accelerated accumulation of genetic incompatibilities, and highlight one potential avenue by which ecological divergence may facilitate speciation.

Keywords: genetic incompatibility, hybridization, reproductive isolation, speciation.

INTRODUCTION

In recent years, there has been extensive interest in the role of morphological divergence in speciation (Schluter, 2000; Coyne and Orr, 2004; Rundle and Nosil, 2005). Morphological divergence can facilitate reproductive isolation if the differences between populations reduce the likelihood of mating or the survival of hybrids. Pre-mating isolation can occur if morphological differences such as body size (McKinnon *et al.*, 2004) or colour (Boughman, 2001) affect mate choice. Morphological divergence can also cause 'extrinsic' post-zygotic barriers when hybrids are poorly adapted to either of the parents' ecological niches (Hatfield and Schluter, 1999). In contrast, relatively little attention has been given to the possibility that morphological differences can

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produce intrinsic post-zygotic isolation (Rundle and Nosil, 2005). Such isolation arises from genetic incompatibilities between species that are expressed regardless of the hybrid's environmental context.

Intrinsic genetic incompatibilities occur when two species undergo substitutions at loci that normally interact pleiotropically. For instance, a population with genotype AABB may split into two sub-populations, which diverge into genotypes aaBB and AAbb. Since the alleles a and b have never co-existed within a single individual, there is a chance that the alleles do not interact correctly during development and thus reduce hybrid fitness. This 'Dobzhansky-Müller' model (Dobzhansky, 1934) of reproductive isolation is silent as to the mechanism driving the substitution of these alleles – it could be drift or selection (Coyne and Orr, 2004; Welch, 2004).

Comparative studies have repeatedly found that reproductive isolation increases steadily with the divergence time separating pairs of populations or species (Coyne and Orr, 1989, 1997, 2004). The roughly clock-like accumulation of reproductive isolation implies that genetic incompatibilities are either largely neutral or result from a fairly constant selection pressure. However, there is usually some scatter in the relationship between measures of reproductive isolation and divergence time. While some of this scatter no doubt represents measurement error for either the isolation or divergence time estimates, it may also reflect instances of faster- or slower-than-average accumulation of incompatibilities. Factors that explain this residual variation can thus provide insight into processes that accelerate or constrain speciation (Funk *et al.*, 2002). For instance, sympatric pairs of *Drosophila* species show greater pre-zygotic isolation than allopatric pairs of the same age, providing evidence for reinforcement (Coyne and Orr, 1997). Reproductive isolation in *Drosophila* is also accelerated by faster-than-average allozyme divergence, suggesting that divergent natural selection on molecular traits facilitates speciation (Fitzpatrick, 2002). Finally, *Drosophila* species with larger hemizygous chromosomes exhibit stronger isolation than species with small X chromosomes of an equivalent age, confirming that Haldane's rule speeds up speciation (Turelli and Begun, 1997).

Rundle and Nosil (2005) recently advocated using this comparative approach to study the relationship between ecological divergence and intrinsic reproductive isolation. We report on one such analysis, using a clade of North American freshwater fishes (Centrarchidae) with 32 described species, including sunfish, crappies, rock basses, and black basses. We show that body size differences have a time-independent relationship with hybrid inviability. These results imply that morphological divergence does facilitate the evolution of intrinsic reproductive isolation.

METHODS

Phylogeny and divergence time

We sequenced seven genes [three mitochondrial gene regions (ND2, 16S, and a set of three tRNAs) and four nuclear genes (calmodulin intron 4, rhodopsin, S7 ribosomal protein intron 1, and *Tmo4C4*) for a total of >5500 bp] from between one and three individuals from each of the 32 described centrarchid species (Near *et al.*, 2004, 2005). We used MrBayes 3.0 (Ronquist and Huelsenbeck, 2003) to carry out a Bayesian phylogenetic analysis with 14 data partitions (by gene, and codon position where suitable) using optimal models of nucleotide evolution identified with likelihood ratio tests implemented by ModelTest 3.0 (Posada and

Crandall, 1998). The resulting phylogeny receives very strong posterior probability support (> 0.95 for nearly all nodes), and agrees closely with maximum parsimony trees with high bootstrap support [see Near *et al.* (2005) for more details on support indices].

We identified six centrarchid fossils that could be readily assigned to nodes and provide mutually consistent minimal age estimates to calibrate the molecular phylogeny (Near *et al.*, 2005). One of these fossil dates was fixed and the remainder were treated as minimal age constraints. The fixed fossil date was selected for its better performance in a jackknife analysis, though all six fossils yielded mutually consistent age predictions (Near *et al.*, 2005). We applied penalized likelihood (Sanderson, 2002), as implemented in the computer program r8s (Sanderson, 2003), to estimate branch lengths while permitting substitution rate heterogeneity. Bootstrapped data sets provided confidence intervals (Baldwin and Sanderson, 1998).

Divergence measures

We collected 130 published accounts of artificial hybridization experiments (see online appendix at: http://evolutionary-ecology.com/data/1974appendix.pdf) that report hybrid and control cross-hatching success for 37 pairs of species subtending 12 nodes of the phylogeny (Fig. 1). Non-independence of different species pairs was handled by treating

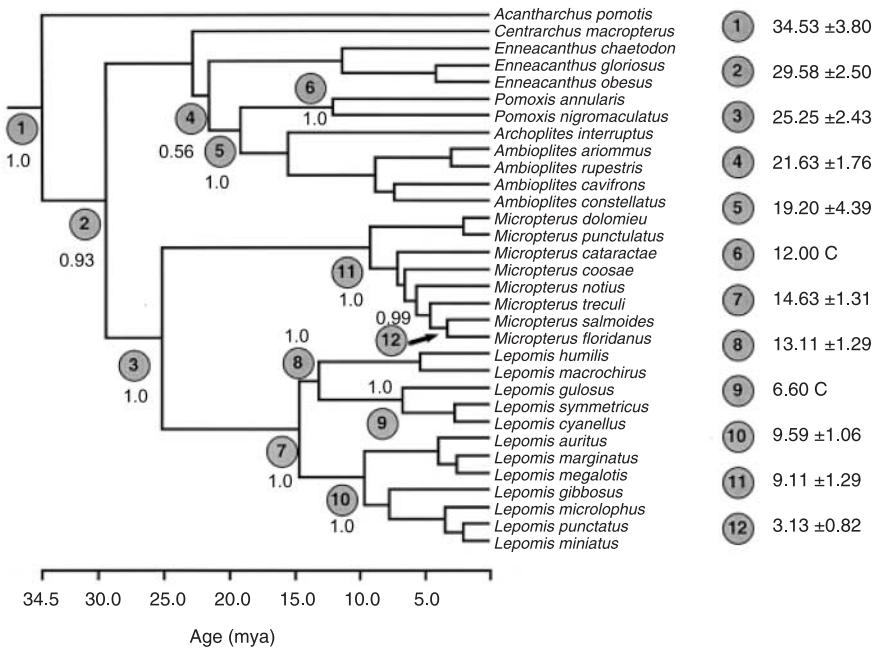

Fig. 1. A phylogeny of 32 species of centrarchids, based on DNA sequences of seven genes. Bayesian posterior probability support values are listed only for the nodes in this analysis (for more details on support levels, see Near *et al.*, 2005). Branch lengths are scaled to time (millions of years) using penalized likelihood to account for rate variation, and six fossil calibration points. Divergence times for nodes analysed in this study are listed to the right of the phylogeny, with standard errors derived from bootstrapping. Nodes with 'C' next to their age were used as fixed calibration points, and so do not have error estimates.

nodes as the level of replication, as described below. Hybrid viability was measured as the ratio of the percentage of hybrid eggs hatching into larvae, divided by the percentage of homospecific control crosses that hatch (Sasa *et al.*, 1998). The homospecific cross controls for potentially low egg viability resulting from the artificial fertilization procedures. Low hybrid viability relative to control crosses indicates genetic incompatibilities between species, so our index is inversely related to the strength of reproductive isolation.

In principle, measures of hybrid hatching rates can confound the effect of gametic isolation with hybrid inviability. However, we can state with confidence that gametic isolation does not contribute to our results, because fertilization rates are consistently high for interspecific and intergeneric crosses (Parker *et al.*, 1985a). For example, the fertilization rate between *L. gulosus* and other centrarchids does not decline with divergence time [86.4% in intraspecific crosses, 85.3% with *L. macrochirus*, 89.3% in crosses with *Micropterus*, and 87.7% with *Pomoxis* (Merriner, 1971a)]. Fertility of more than 90% is possible for all combinations of *Pomoxis*, *Micropterus*, and *Lepomis* that have been attempted (Merriner, 1971).

We chose to test whether body size is associated with accelerated hybrid inviability because it seems reasonable, *a priori*, that differences in body size might generate hybrid inviability due to conflicting developmental instructions from the two parents' genomes. Because centrarchids are indeterminate growers (and body size varies among populations), we used maximum standard length as a measure of body size for each species (Lee *et al.*, 1981). Body size disparity was measured as the absolute difference between a pair of species, standardized by the size of the smaller species.

Regression of divergence on time

The 37 species pairs do not provide independent estimates of divergence, because many of these pairs share evolutionary history (Felsenstein, 1985). To generate phylogenetically independent data points, we used the average hybrid viability (or size divergence) at each node instead of data on individual species pairs (Coyne and Orr, 1997), an extension of independent contrasts to pairwise comparisons. To calculate the mean divergence at a given node, we used a weighted averaging procedure that accounts for both phylogenetic topology and shared branch lengths of the different species pairs subtending a given node (Bolnick and Near, 2005). This averaging procedure weights species pairs in proportion to the percentage of their evolutionary history that is shared with other species pairs, and is equivalent to carrying out branch-length weighted independent contrasts (Felsenstein, 1985), with the distinction that the values being compared are a property of between-species comparisons rather than of individual species. Branch lengths were measured in absolute time (millions of years). An alternative node-averaging procedure outlined by Fitzpatrick (2002) yielded qualitatively similar results with equivalent significance levels.

We calculated both linear regression and Spearman correlations between node age and the node's mean body size difference or mean hybrid viability, with sequential Bonferroni correction to calculate significance levels. Both methods yielded equivalent results, so we report *P*-values from the regression. To estimate deviations from a clock-like relationship, we calculated the residuals for each node from regressions of body size disparity and hybrid viability against node age. To test whether body size disparity modifies the rate at which inviability evolves, we then regressed the residuals of body size on age, against the residuals of viability on age.

Residuals from linear regression may be unreliable if the true relationship is curvilinear. We therefore also carried out quadratic regression of size divergence and hybrid viability on node age, and used the residuals of these quadratic regressions to again test for an age-independent relationship between size divergence and hybrid viability. Because quadratic regressions did not fit the data significantly better than linear regressions (based on partial *F*-tests), and the residuals produced statistically equivalent results, we focus our presentation on the linear regression results.

In principle, an alternative way to test the hypothesis that body size has an age-independent relationship with hybrid viability would be to compare different species pairs across a single node in the phylogeny, thus controlling for divergence time. However, repeated use of particular species for crossing experiments means that none of the nodes in our data set contained enough independent species pairs to justify a within-node analysis.

RESULTS

Phylogenetic analysis of our seven gene sequences yielded a well-resolved phylogenetic hypothesis (Fig. 1). The branch lengths in Fig. 1 are scaled to time (in millions of years) based on our multiple fossil calibrations of a heterogeneous molecular clock (for more details, see Near *et al.*, 2005). Estimated divergence times are listed in Fig. 1 for all species pairs for which we have hybrid viability data.

Body size divergence between species increased with the length of time since their lineages diverged ($r = 0.86$, $t = 5.42$, $P < 0.001$, $r^2 = 0.73$; Fig. 2A), while the viability of their hybrids declined ($r = -0.85$, $t = -5.2$, $P < 0.001$, $r^2 = 0.75$; Fig. 2B). The hatching success of hybrid embryos declined approximately linearly from 100% (scaled relative to homospecific control crosses) for young taxa towards zero percent viability, although not even the most basal node in centrarchids had completely inviable hybrids (Fig. 2B). Quadratic regression did not improve the fit of the regression model to the data for any of these regressions (partial *F*-test, $P = 0.527$ and 0.866 respectively). Since both forms of divergence are correlated with node age, we next used residuals to remove the effect of node age and examine time-independent relationships between size and reproductive isolation.

Hybrid viability was as strongly correlated with body size disparity as it was with divergence time ($r = -0.90$, $P < 0.001$, compared with $r = -0.85$). To determine whether viability and divergence were correlated merely as a result of their shared dependence on time, we removed the confounding effect of age by calculating viability and disparity residuals in regressions on node age (Fitzpatrick, 2002). Residual variation in body size was negatively correlated with residual variation in hybrid viability ($t = -2.606$, $P = 0.026$, $r^2 = 0.404$; Fig. 3). Surprisingly, age had no size-independent relationship with genetic incompatibility: older species pairs were not more incompatible than expected given their body size ($P = 0.279$). Of course, this does not mean that divergence time has no effect on inviability, since time is surely the independent variable. Rather, time's effect on inviability may be fully explained through time's effect on size.

Comparing residuals in this manner may be compromised if age–size or age–viability relationships are not linear. For instance, the linear regression in Fig. 2B would predict that close relatives have negative body size differences, making the residuals biologically meaningless. We therefore re-analysed the data using residuals from quadratic regressions (which did not predict negative body size differences) and found equivalent results ($t = -2.65$, $P = 0.024$, $r^2 = 0.412$).

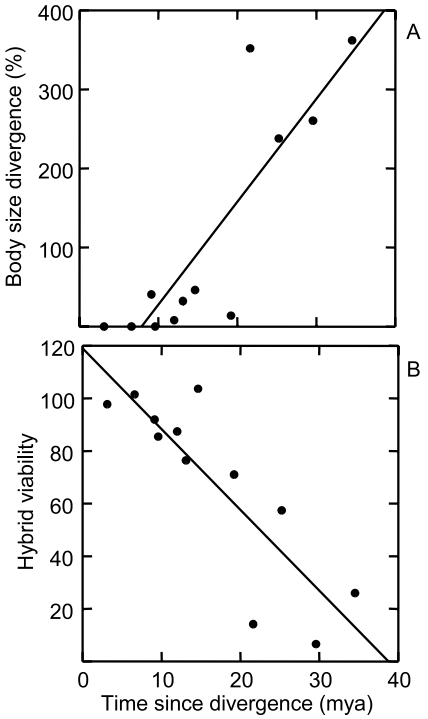

Fig. 2. Linear regression of (A) maximum body size disparity as a proportion of the body size of the smaller species, and (B) hybrid viability, measured as the percentage of hybrid eggs that hatch divided by the number of control homospecific cross eggs that hatch. Both measures were regressed against node age, measured in millions of years. Each data point represents the mean divergence at a node of the phylogeny.

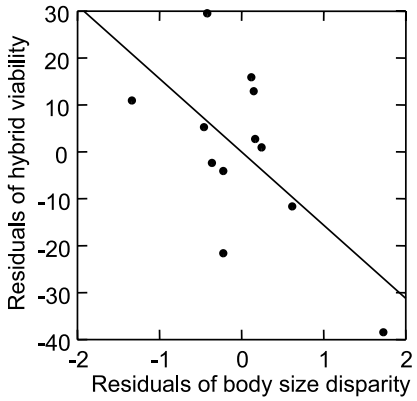

Fig. 3. Linear regression of the residuals of hybrid viability (with respect to divergence time) on the residuals of body size disparity (with respect to time).

Another possible weakness of our result is the high leverage of the point in the lower right corner of Fig. 3. This point represents lower hybrid viability than expected, given their divergence time, for species pairs with greatly differing body sizes (*Enneacanthus* and

Micropterus). The low hybrid viability was documented for two different interspecific crosses at this node, and so appears to be a real phenomenon. Repeating the regression twelve times, deleting a different data point for each repeat, we found that all regressions containing the high leverage point were significant, while the regression without that point was not ($P = 0.56$). Consequently, the regression result is driven by an accurate but outlying data point. Since this violates an assumption of regression, we re-analysed the data with a non-parametric Spearman's rank correlation, which is robust to non-normal data or high leverage points. Once again there was a significant relationship between hybrid viability residuals and body size residuals ($r_s = 0.51$, $P_{\text{one-tailed}} = 0.045$). This result is significant if we adopt a one-tailed test, which appears reasonable since there is no model of intrinsic isolation that would lead us to expect body size divergence to lessen genetic incompatibilities. However, even if we adopt a more conservative two-tailed test, the result is marginally significant ($P = 0.09$) and hence worth documenting.

DISCUSSION

We adopted a comparative approach to determine whether morphological divergence promotes reproductive isolation in a clade of freshwater fishes, Centrarchidae. We found that species pairs with greater body size differences produced less viable hybrids than would be expected given their divergence time. While this correlation is only weakly significant (Spearman's rank correlation, $r_s = 0.51$, $P_{\text{one-tailed}} = 0.045$, regression $P = 0.026$), it provides the first empirical support for an association between morphological disparity and intrinsic post-zygotic isolation. This correlation is expected if accelerated size divergence is associated with an increased rate of substitutions (Orr and Turelli, 2001), or if the genetic changes underlying size divergence have a direct impact on hybrid development and viability.

As with any phylogenetic comparative study, it is important to keep in mind that a significant correlation is not unambiguous evidence for causation: the correlation between size divergence and hybrid inviability may arise from mutual correlation with an unknown third variable (Price, 1997). Even if we accept a causal relationship, the direction of causation is not directly established: does body size divergence promote reproductive isolation, or vice versa? For example, one might argue that reproductive isolation is the independent variable, which permits greater size divergence by reducing rates of introgression. However, this effect is highly unlikely to explain the pattern documented here, because gene flow among centrarchids is also limited by pre-zygotic isolation and hybrid infertility, which generally eliminate gene flow regardless of hybrid viability (Bolnick and Near, 2005). Furthermore, any gene flow limiting size divergence after speciation would be expected to result in incongruent gene trees, whereas the four nuclear genes and three mitochondrial sequences yield consistent phylogenetic topologies (Near *et al.*, 2003, 2004, 2005). It is therefore reasonable to suggest that the causal relationship is that body size divergence modifies the rate at which hybrid inviability evolves between independently evolving lineages.

We suggest there are two potential mechanisms linking body size disparity and reproductive isolation. The first mechanism requires that body size evolution be driven largely by divergent selection. Such selection presumably arises during ecological divergence and niche shifts. Body size affects biomechanical performance such as locomotion and suction feeding (Wainwright and Shaw, 1999), energetic demands, and the size of prey an individual fish can swallow (Wainwright, 1996). Divergent selection on body size would accelerate the fixation of Dobzhansky-Mueller incompatibilities (Orr and Turelli, 2001; Welch, 2004).

Some evidence already exists that divergent selection facilitates intrinsic reproductive isolation. A number of individual 'speciation genes' have been identified that explain a large proportion of hybrid inviability or infertility (Ting *et al.*, 1998; Presgraves *et al.*, 2003; Barbash *et al.*, 2004). These genes consistently show the fingerprint of past natural selection, but in no instance is a speciation gene known to affect an ecologically important trait. Furthermore, such analyses are restricted to species pairs of model organisms with the requisite genetic toolkits. Additional evidence comes from artificial selection experiments, in which researchers apply divergent selection to laboratory populations and test for subsequent isolation (Rice and Hostert, 1993). For instance, de Oliveria and Corderion (1980) subjected populations of *Drosophila* to divergent selection for pH tolerance for 122 generations, and found hybrids had reduced fitness even under benign pH conditions. Analogous results were found in similar *Drosophila* experiments by Robertson (1966) and in Rice and Hostert's (1993) re-analysis of the data of Ringo *et al.* (1985), but not by Kilias *et al.* (1980). Finally, hybrid sterility occurs between populations of monkeyflowers (*Mimulus*) that inhabit divergent soil conditions [copper-contaminated or not (MacNair and Christie, 1983)]. While these studies confirm that divergent selection can facilitate genetic incompatibilities, there are still so few examples that Rice and Hostert (1993) concluded there was 'limited support for unconditional postzygotic isolation'.

The second possible mechanism linking size disparity to reproductive isolation is that body size differences could have a direct impact on hybrid viability, without regard to whether the size differences were selected for. For example, different-sized taxa might differ in the timing of gene expression during development, and hybrids have deleterious intermediate expression patterns. Alternatively, body size could be correlated with egg size and patterns of oocyte provisioning. If paternal alleles rely on particular patterns or levels of egg provisioning that are not available in a different species' eggs, hybrid fitness might suffer.

Three lines of evidence suggest that body size divergence has not evolved neutrally in centrarchids. First, a neutral model would suggest that recently diverged sister species are most likely to have similar body sizes. In reality, several closely related species exhibit large size disparity. Of three true sister species pairs in *Lepomis*, one pair includes the largest and second-smallest *Lepomis* species, another pair includes the second-largest and the smallest species, and the third pair includes the third-largest and third-smallest species. While reproductive isolation data are not available for these sister pairs for our analysis, the pattern suggests that body size divergence is a result of selection.

A second objection to this neutral explanation is that body size divergence is not associated with differences in the timing of gene expression, judging by allozyme expression during ontogeny. Even parental species with highly divergent body sizes (e.g. *Micropterus* and *Lepomis*) show similar timing of gene expression (Whitt *et al.*, 1977; Philipp *et al.*, 1983). Hybrid inviability occurs not because of mismatch in the timing of gene expression, but because hybrids exhibit delayed expression relative to either parent (Parker *et al.*, 1985a, 1985b). It thus appears that inviability results from divergence in the mechanisms of gene regulation, rather than when or where that regulation is applied to express genes. This is what one would expect if selection on body size (driving substitutions), rather than body size itself, were the cause of hybrid inviability.

Finally, egg size is not correlated with body size, and other workers have also found that egg size differences are not related to hybrid viability in centrarchids (Merriner, 1971b). Neither regression of raw species values nor independent contrasts found a significant effect of body

size difference on egg size ($P = 0.884$ and 0.926 respectively). While we cannot rule out a direct impact of neutral body size difference on inviability, these three lines of evidence make it appear less likely than the alternative explanation for our results – divergent selection.

One final alternative is that Dobzhansky-Mueller incompatibilities accrue not as a direct consequence of body size divergence, or of divergent selection on body size, but as a result of changes in the biology of the species brought about by size differences. In particular, body size may be associated with species ranges (Pyron, 1999), population structure, and effective population size, all of which might influence the substitution rate and accrual of incompatibilities (Welch, 2004). While this is certainly possible, it should result in a correlation between body size and isolation, rather than size disparity and isolation. For instance, imagine that larger species have smaller population sizes and so fix mildly deleterious mutations more quickly (Welch, 2004). A pair of large species (contrast = 0) will therefore accumulate inviabilities more quickly than a pair of small species (also with contrast = 0). Since this is not the pattern documented here, we feel this hypothesis is not the explanation for our particular results.

The patterns documented in this paper focus exclusively on hybrid inviability, which is probably not the driving force behind speciation in centrarchids. Post-zygotic isolation plays a relatively minor role in speciation in centrarchids, since most sister species pairs are too young (averaging 4 million years old) to have accumulated appreciable hybrid inviability, which begins to accumulate after a 6 million year lag (Bolnick and Near, 2005). It therefore appears that speciation is largely due to pre-zygotic isolation in centrarchids. Intrinsic post-zygotic isolation accumulates after speciation by other means, rather than playing a role in initial divergence. Nonetheless, natural hybridization does occur in centrarchids, so the post-zygotic isolation patterns described here play an important role in buttressing speciation should other isolating mechanisms weaken following environmental changes.

Previous studies have shown that morphological divergence between populations can contribute to speciation by promoting pre-mating isolation (Boughman, 2001; McKinnon *et al.*, 2004), or environmentally dependent post-zygotic isolation (Hatfield and Schluter, 1999). Here, we argue that morphological divergence can also contribute to intrinsic post-zygotic isolation, and provide weak but consistent evidence that it has done so in centrarchids. While both neutral and selected differences could explain this relationship, we argue that the former is unlikely in this system. To the extent that size disparity is the result of divergent selection, our results suggest that ecological divergence can contribute to all facets of reproductive isolation, not just pre-mating or extrinsic post-mating.

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