

# Sex-specific evolution during the diversification of live-bearing fishes

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**Natural selection is often assumed to drive parallel functional diversification of the sexes. But males and females exhibit fundamental differences in their biology, and it remains largely unknown how sex differences affect macroevolutionary patterns. On microevolutionary scales, we understand how natural and sexual selection interact to give rise to sex-specific evolution during phenotypic diversification and speciation. Here we show that ignoring sex-specific patterns of functional trait evolution misrepresents the macroevolutionary adaptive landscape and evolutionary rates for 112 species of live-bearing fishes (Poeciliidae). Males and females of the same species evolve in different adaptive landscapes. Major axes of female morphology were correlated with environmental variables but not reproductive investment, while male morphological variation was primarily associated with sexual selection. Despite the importance of both natural and sexual selection in shaping sex-specific phenotypic diversification, species diversification was overwhelmingly associated with ecological divergence. Hence, the inter-predictability of mechanisms of phenotypic and species diversification may be limited in many systems. These results underscore the importance of explicitly addressing sex-specific diversification in empirical and theoretical frameworks of evolutionary radiations to elucidate the roles of different sources of selection and constraint.**

Theory posits that natural selection is a dominant force in shaping functional traits, maximizing performance in a given environment<sup>1</sup>. Diversification across macroevolutionary adaptive landscapes should therefore drive evolution of lineages towards naturally selected adaptive peaks<sup>2</sup>, and reproductive isolation can emerge as a by-product of adaptation (ecological speciation<sup>3</sup>), giving rise to adaptive radiations of ecologically distinct species<sup>4,5</sup>. Natural selection consequently can play a dual role, shaping both phenotypic and species diversification. Macroevolutionary analyses typically assume that divergent selection is parallel for the two sexes, yet female and male reproductive biology are fundamentally different<sup>6</sup>. The resulting sex-specific selection<sup>7,8</sup>, constraints<sup>9,10</sup> and antagonistic co-evolution<sup>11</sup> can displace one or both sexes from their phenotypic optima and, as a result, sexual dimorphism has been documented in diverse taxa. The consequences of sex differences are routinely considered on intraspecific and microevolutionary scales<sup>12,13</sup>, and empirical studies in yeast and fruit flies have demonstrated that sexual selection can impede evolution towards new adaptive peaks<sup>14,15</sup>. In contrast, we routinely ignore sex differences in empirical studies of macroevolutionary diversification and instead analyse only a single sex or sex-averaged phenotypes that may not exist in nature. A number of studies have documented sexual dimorphism in functional phenotypic traits from small to broad phylogenetic scales<sup>16–19</sup> and concluded that sex-specific evolution may significantly bias inferences of macroevolutionary patterns<sup>16</sup>. Nonetheless, we lack an understanding of how sex-specific evolution affects the macroevolutionary adaptive landscape<sup>2</sup>, and what sources of selection shape sex-specific phenotypic responses on these scales.

The evolution of variation in female reproductive investment<sup>20</sup> and associated shifts in the extent of pre-copulatory sexual selection on males<sup>21</sup> in live-bearing fishes of the family Poeciliidae provides an excellent system to address questions about the interactions of context- and sex-specific selection on phenotypic diversification

and speciation. The extent of reproductive investment across poeciliids spans a continuum from species that produce large, well-provisioned eggs with no post-fertilization provisioning (lecithotrophy) to species that produce small eggs that increase in size throughout gestation (matrotrophy). Associated with variation in matrotrophy, species vary in pre-copulatory female mate choice, resulting in sexual selection on males<sup>21</sup>. Species with stronger pre-copulatory selection are characterized by males exhibiting courtship and having shorter intromittent organs that require female cooperation during copulation<sup>21</sup>. Species with weaker pre-copulatory selection are characterized by coercive mating tactics and longer intromittent organs that facilitate forced copulation. Across poeciliids, sexual selection has been widely studied in the context of the evolution of exaggerated male traits, the maintenance of variation in coloration, fluctuating asymmetry, body size and male courtship behaviour<sup>22</sup>.

Here we evaluate the relative importance of multiple sources of selection on sex-specific functional diversification and speciation using data on body shape, sexual selection and maternal investment together with environmental data from 70,000+ occurrence records for 112 species. Body shape influences locomotor performance in the aqueous environment<sup>23</sup> and serves as a cue for female mate choice<sup>24,25</sup>. Analyses of body shape variation thus facilitate testing how natural and sexual selection interact to drive phenotypic diversification on broad phylogenetic scales. The largest phylogenetic comparative dataset on poeciliids to date enabled an integrative approach incorporating: (1) SURFACE analyses to test for sex-specific adaptive landscapes, (2) determination of explicit sources of natural and sexual selection shaping major axes of functional trait variation for each sex, (3) comparison of the relative contribution of the same sources of selection to species diversification across 14 genera.

## Results

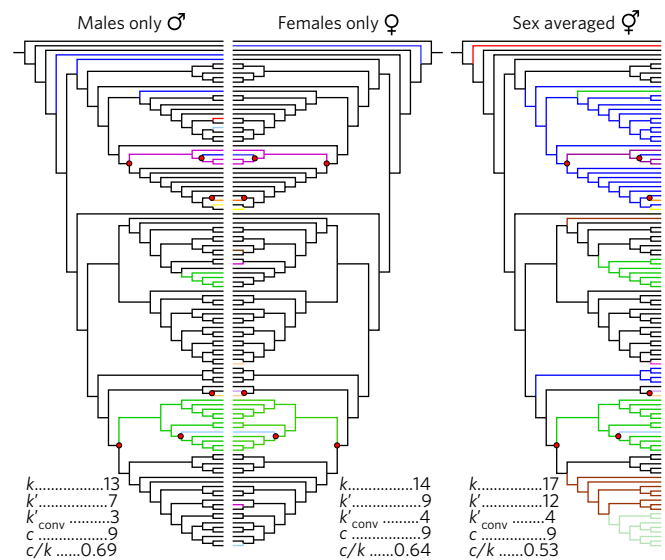
Considering that males and females of a species share most of their genome and inhabit the same environments, their phenotypic

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evolution in response to natural selection should occur in parallel. However, sexual selection on males or reproductive constraints in females may cause sex-specific evolutionary dynamics. We compared adaptive landscapes using the two primary axes of body shape variation (Supplementary Fig. 1) to identify unique phenotypic peaks and convergent peak shifts for females, males and sex-averaged phenotypes across the phylogeny. Analyses of sex-specific phenotypic optima indicated that, despite similar numbers of adaptive peaks and peak shifts, males and females had significantly different adaptive landscapes (Fig. 1). The location of adaptive peak shifts was discordant between the sexes for 71% of shifts (Fig. 1; Supplementary Table 1), and the adaptive landscape analysis using sex-averaged morphology did not accurately reflect evolutionary patterns within the family. Sex-averaged analyses placed nearly half of all species on incorrect adaptive peaks and sometimes erroneously placed entire genera on convergent or unique adaptive peaks (for example, *Gambusia*; Fig. 1). It is important to note that discordance between male and female adaptive landscapes could also be influenced by measurement uncertainty, which is particularly relevant for highly polymorphic species. For example, morphological polymorphisms in poeciliids have been associated with alternative male mating strategies<sup>26</sup>, and our sampling may not have adequately represented sex-specific mean morphologies for polymorphic species. However, even when sex-specific morphological variation within species was adequately sampled, current implementations of Ornstein–Uhlenbeck models for the detection of evolutionary shifts cannot account for measurement uncertainty in comparative datasets.

Significant biases were also observed when examining rates of evolution along the major morphological axes using phylogenetic explicit maximum likelihood analyses. Likelihood ratio tests (LRT) between a model of equal versus unequal evolutionary rates indicated that phenotypic evolution was faster in males compared with females along both phylogenetic principal component axis (pPC) 1 (LRT = 1,806.9;  $P < 0.001$ ) and pPC2 (LRT = 1,704.4;  $P < 0.001$ ; Supplementary Table 2). Using sex-averaged phenotypes resulted in an underestimation of male rates and overestimation of female rates along pPC1, and an underestimation of both male and female evolutionary rates along pPC2 (Supplementary Table 2). These sex-specific evolutionary dynamics may arise via two non-mutually exclusive scenarios. First, sexual selection on males in the form of female choice or competition for mates may displace males from naturally selected adaptive peaks and accelerate phenotypic evolution. Second, evolution of female body shape related to reproductive biology may impose constraints on female locomotion<sup>27,28</sup>, and the resulting trade-offs could decelerate evolutionary rates in females.

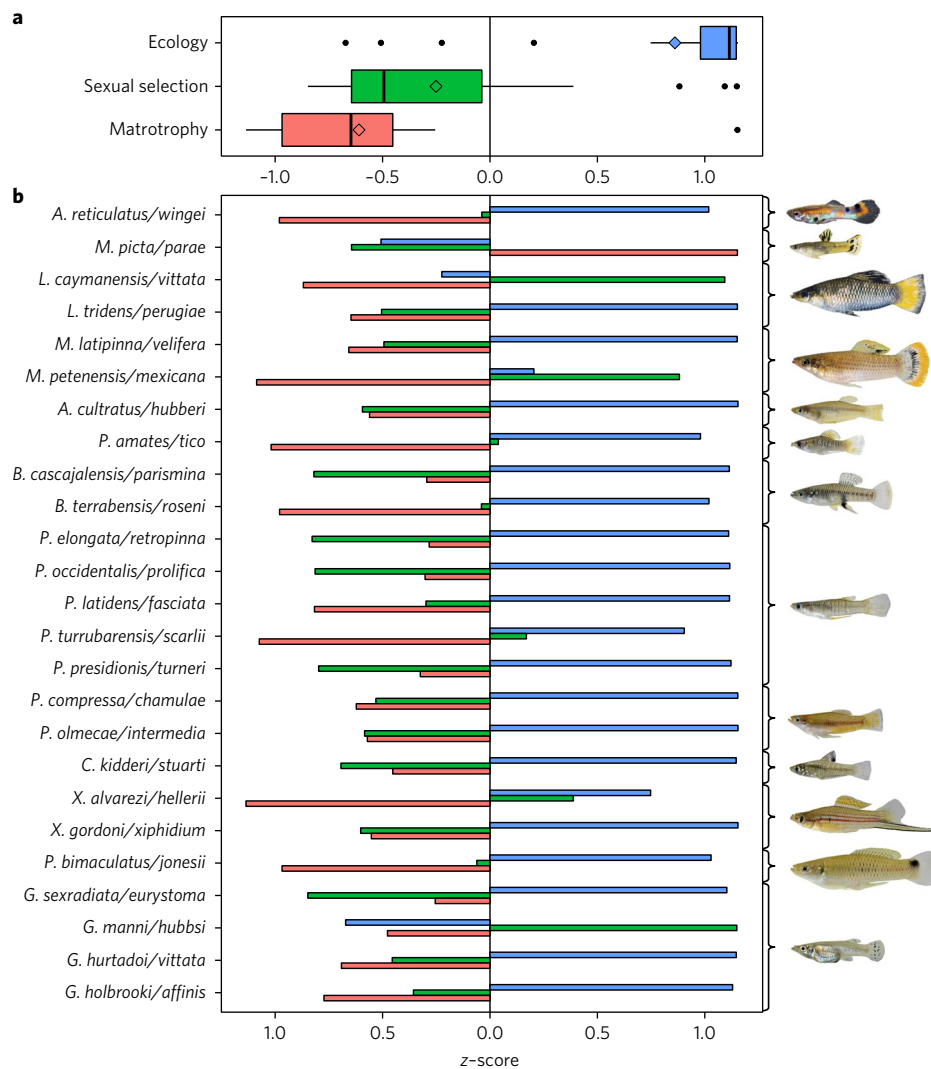
Drivers of sex-specific evolution can be disentangled by contrasting associations of natural and sexual selection with variation in male and female body shape. Sex-specific selection was assessed by generating a sexual selection index (SSI) for males, which reflects variation in morphological traits known to influence male mating tactics and female mating decisions (that is, determining male fitness). The SSI was quantified by the degree of sexual dimorphism in fin size and body shape, and reflected a continuum from species characterized by long gonopodia and coercive mating tactics to species with short gonopodia and courtship. Our SSI was significantly correlated (Pearson correlation:  $r^2 = 0.28$ ,  $P < 0.05$ ; phylogenetic generalized least squares (PGLS):  $t = 2.9$ ,  $P < 0.01$ ) with another recently described index of sexual selection for poeciliids based on behavioural traits<sup>21</sup>. In addition, we described sex-specific selection on females by using a matrotrophy index, which summarizes the extent of post-fertilization maternal investment within species. Variation in natural selection was characterized by quantifying each species' ecological niche based on spatially referenced occurrence data and climatic and hydrological variables that can influence



**Figure 1 | Results of adaptive landscape analyses for male, female and sex-averaged body shape data using the best scoring tree.** Parameter summaries include  $k$  (number of adaptive peak shifts),  $k'$  (number of adaptive peaks),  $k'_{\text{conv}}$  (number of convergent adaptive peaks),  $c$  (number of convergent peak shifts) and  $c/k$  (convergence fraction). Within each tree, unique adaptive peaks are indicated by different colours and convergent peaks are represented by the same colour arising multiple times across the phylogeny. Note that branch/peak colours are not comparable across the three trees. Replication of these analyses across 100 trees and 100 bootstrap replicates per tree confirmed the results, indicating ~70% of peak shifts were discordant between males and females (see Supplementary Table 1). Red circles indicate peak shifts that were shared between males and females, leading to only six peak shifts consistently seen in males and females that were correctly identified using sex-averaged body shape.

adaptive evolution in functional traits (Z. W. Culumber and M. Tobler, manuscript in preparation)<sup>29</sup>. PGLS analysis was then used to evaluate the drivers of variation along the major axes of male and female body shape that could generate different adaptive landscapes. As expected, there were contrasting patterns of selection between the sexes. The major axes of male body shape variation were strongly associated with sexual but not natural selection (pPC1 with SSI:  $\beta_{106,112} = 0.02$ ,  $P < 0.0001$ ; pPC2 with SSI:  $\beta_{106,112} = 0.03$ ,  $P < 0.0001$ ; Supplementary Table 3). In contrast, natural selection was the only significant predictor of the major axes of female body shape (pPC1 with niche pPC2:  $\beta_{88,94} = 0.0026$ ,  $P = 0.01$ ; pPC2 with niche pPC1:  $\beta_{88,94} = 0.0018$ ,  $P = 0.03$ ). There were no correlations between the extent of matrotrophy and either axis of female body shape (pPC1:  $P = 0.18$ ; pPC2:  $P = 0.56$ ). Despite previous hypotheses and some evidence of reproductive constraints within species<sup>27,28,30</sup>, we found no evidence that matrotrophy is related to female body shape variation on a macroevolutionary scale. Instead, the ecological niche was associated with female body shape variation. Although the specific climatic and hydrological variables used to characterize the ecological niche may not be the immediate sources of selection affecting female body shape, they seem to reflect the variety of biotic and abiotic environmental variables that have documented effects on poeciliid body shape<sup>28,31</sup>. In addition, our results confirm that sexual selection drives and accelerates male trait evolution in poeciliids<sup>26,32</sup>. Consequently, sex-specific functional diversification on a macroevolutionary scale results from interactions between natural and sexual selection, and is entirely consistent with microevolutionary studies that link specific sources of selection to trait evolution.

But what drives species diversification when there are pronounced patterns of sex-specific evolution? Natural and sexual selection as



**Figure 2 | Relative contributions of different mechanisms on species diversification.** In both panels, negative z-scores indicate low relative importance of a variable, whereas positive scores indicate variables with the strongest divergence with species pairs. **a**, Comparison of divergence in ecology, sexual selection and matrotrophy between multiple sister species across the family indicated disproportional effects of ecological niche differentiation. Boxes cover the first to third quartile of the data; vertical black lines indicate the median. Filled dots indicate outliers and open diamonds indicate the mean for each variable. **b**, Variation in the relative importance of divergence across mechanisms in all species pair comparisons revealed few instances where divergence in sexual selection or maternal investment had large contributions. Pictures on the right are representative species of each genus included in this analysis (from the top: *Acanthophaeus reticulatus*, *Micropoecilia picta*, *Limia perugiae*, *Mollienesia mexicana*, *Alfaro cultratus*, *Phallichthys amates*, *Brachyrhaphis olomina*, *Poeciliopsis turrubarensis*, *Priapella compressa*, *Carlhubbsia kidderi*, *Xiphophorus hellerii*, *Pseudoxiphophorus bimaculatus* and *Gambusia eurystoma*).

well as female reproductive biology have all been implicated in driving speciation in poeciliids and other groups<sup>32</sup>. Ecological speciation theory postulates that reproductive isolation arises as a by-product of divergent selection driving adaptive evolution<sup>3</sup>. Speciation by sexual selection may arise through divergence in mate choice and secondary sex traits, which provides an avenue for behavioural and mechanical pre-mating isolation<sup>22</sup>. Finally, the viviparity-driven conflict hypothesis suggests that divergence in maternal investment between lineages should generate post-mating reproductive isolation through hybrid inviability<sup>33</sup>. We quantified the extent of divergence in maternal investment and natural and sexual selection in 25 species pairs across 14 genera (Supplementary Fig. 2). There was a significant difference in the extent of divergence among the different reproductive barriers ( $\chi^2 = 24.7$ ,  $N = 25$ ,  $P < 0.001$ ; Fig. 2). Pairwise comparisons among mechanisms showed that ecological isolation (EI) was greater than isolation by maternal investment

(EI versus MI:  $Z = -4.07$ ,  $P < 0.01$ ) and sexual selection (EI versus SI:  $Z = -3.54$ ,  $P < 0.01$ ). There was no difference between MI and SI ( $Z = -1.68$ ,  $P = 0.18$ ). These results are consistent with a coupling of species diversification and ecological divergence as expected during ecological speciation, and work in several poeciliid systems has highlighted the importance of ecological factors for species divergence on microevolutionary scales<sup>34–36</sup>. Despite strong sexual selection and divergence in female reproductive life history in many groups of poeciliids, it seems that natural selection has played a prominent role in poeciliid diversification.

## Discussion

Overall, our results provide several new insights about phenotypic diversification and speciation on macroevolutionary scales. Phylogenetic comparative studies often infer causation of evolutionary radiations based on patterns or rates of phenotypic evolution<sup>37,38</sup>,

but do not evaluate the relative contributions of specific selective mechanisms that give rise to these patterns (but see ref. <sup>39</sup>). However, mechanisms of species diversification may not be predictable from mechanisms driving phenotypic diversification. In poeciliids, strong evidence for sexual selection contributing to phenotypic diversification might suggest that it has also played an important role in divergence between species. But this was not the case, and ecological divergence was stronger between closely related species. While inferences of the forces that drive species diversification are often made in macroevolutionary studies<sup>37,40,41</sup>, our results highlight that ignoring alternative mechanisms and failing to test the relative importance of those mechanisms may misrepresent the importance of a particular source of selection in shaping macroevolutionary patterns. Hence, evidence for strong selection on specific traits that have been linked to the emergence of reproductive barriers (speciation phenotypes) on microevolutionary scales may not accurately predict macroevolutionary dynamics<sup>42</sup>. While our study is limited to a family of fishes, our results should have implications on broader taxonomic scales. For example, microevolutionary processes that contribute to evolution of reproductive isolation are not correlated with rates of speciation on macroevolutionary scales across drosophilid flies and birds<sup>42</sup>. More rigorous comparison between microevolutionary processes and macroevolutionary patterns with an emphasis on contrasting the relative influence of natural and sexual selection<sup>13</sup> promises to generate new insights into broad-scale evolutionary phenomena.

Our work found no evidence that the extent of maternal provisioning has played a major role in phenotypic or species diversification on a macroevolutionary scale, even though there is evidence of morphological variation and hybrid inviability associated with reproductive traits in intra- and inter-specific studies<sup>27,30,43</sup>. Viviparity has evolved numerous times in vertebrates, and squamate reptiles exhibit phenotypic constraints<sup>44</sup> and species diversification<sup>45</sup> associated with parity mode. Similarly, the evolution of viviparity in poeciliids and other cyprinodontiform fishes has been correlated with increased species diversification rates<sup>46</sup>. However, the evolution of placenta-like structures and variation in maternal provisioning within poeciliids do not seem to have played a direct role in macroevolutionary diversification. It is important to note, however, that variation in matrotrophy probably had important indirect effects on phenotypic diversification by driving shifts in the extent of sexual selection<sup>21</sup>, which was associated with male body shape diversification in our study.

Finally, our results clearly demonstrate the importance of sex-specific evolution on macroevolutionary scales. This is the first study to explicitly demonstrate the biases generated by using sex-averaged phenotypes, which misrepresented the adaptive landscape and extent of convergent evolution, and did not accurately reflect rates of phenotypic evolution. Averaging morphological data for males and females effectively generates nonexistent phenotypes that produce inaccurate results, with potentially important implications for studies in evolutionary biology. For example, sex-specific evolution may influence macroevolutionary inferences of brain size evolution<sup>47</sup>, the relationship between morphological and species diversification<sup>16,48</sup>, and even physiological tolerances<sup>18</sup>. Furthermore, phylogenetic comparative methods have become increasingly popular as a means to detect patterns of divergent and convergent evolution in ecological niches, and both naturally and sexually selected morphological traits across diverse taxa<sup>47–49</sup>. Considering the ubiquity of sexual dimorphism in functional traits throughout the animal kingdom<sup>13</sup> and some model adaptive radiations<sup>16</sup>, explicitly accounting for sex-specific patterns and rates of evolution will be critical to accurately reconstruct the macroevolutionary dynamics that gave rise to biodiversity. To evaluate the robustness of inferences drawn based on phylogenetic comparative methods, it will be important also to expand current frameworks that implement Ornstein–Uhlenbeck

models on adaptive landscapes to incorporate intraspecific variation rather than just species means, which will shed light into how measurement error affects the estimation of these adaptive landscapes and macroevolutionary dynamics.

## Conclusions

Some of our most basic tenets and enduring theories of evolution have come from systems in which phenotypes or species have diversified in response to putatively clear and strong sources of selection. However, even in those systems, evolutionary dynamics are more complex than previously assumed<sup>16,39</sup>. Compartmentalizing our understanding of diversification into male or female (or, worse yet, sex averages), natural or sexual selection, phenotypic or species patterns, and micro- or macroevolution provides an incomplete assessment of evolutionary patterns and processes. Only integrative analysis of evolutionary dynamics across these areas will allow us to develop robust understanding of the origins of biodiversity.

## Methods

**Geometric morphometrics.** Body shape variation was quantified for males and females of 112 species of New World poeciliids using a geometric morphometric approach. Sample sizes averaged 14 specimens per species (see Supplementary Table 4 for sample sizes by sex and species), consistent with previous studies of sex-specific evolution of functional morphology<sup>16</sup>. Specimens were obtained through museum visits, specimen loans and photographs shared by other researchers, as well as from the authors' own collections (Supplementary Table 4). To quantify variation in body shape, lateral digital photographs were taken of the left side of adult individuals with a Nikon (Tokyo, Japan) D90 camera. We digitized 17 homologous and seven semi-landmark points (Supplementary Table 5 and Supplementary Fig. 3) for each image using the software tpsDig2 v2.16<sup>50</sup>. A geometric morphometric analysis<sup>51</sup> was conducted on the basis of the 17 homologous landmarks. Landmark coordinates were aligned using least-squares superimposition as implemented in the program tpsRelw<sup>52</sup> to remove effects of translation, rotation and scale. Based on the aligned coordinates, we calculated centroid size and partial warp scores with uniform components for each individual (weight matrix). The weight matrix was subjected to a phylogenetic principal component analysis (PCA) based on a covariance matrix using *phyl.pca* in the R package PHYTOOLS<sup>53</sup> to reduce data dimensionality. We retained the first two axes (pPC1 and pPC2) as shape variables for subsequent analyses. We aligned male and female data in the same morphospace for analyses in which we made direct comparisons between the sexes (adaptive landscapes and evolutionary rates), and in separate morphospaces for detecting factors that have influenced sex-specific body shape evolution. Analyses of sex-averaged morphology were conducted by aligning males and females in the same morphospace and generating a single sex-averaged weight matrix prior to phylogenetic PCA. Unless otherwise stated, statistical analyses were performed using SPSS 17 (SPSS, 2008).

**Phylogenetic framework.** Sequences for six mitochondrial (*12S*, *COI*, *CytB*, *ND2*, *tRNA<sup>Valu</sup>* and *tRNA<sup>Leu</sup>*) and 11 nuclear genes (*Beta Actin*, *CCND1*, *ENC1*, *GLYT*, *MYH6*, *RAG1*, *Rhodopsin*, *RPS7*, *SH3PX3*, *T36* and *XSRC*) were obtained for all species of the family Poeciliidae for which we had digital images, plus the outgroup *Fundulus cingulatus* (Family: Cyprinodontidae), from GenBank (Supplementary Table 6). Sequences for each gene were aligned with eight iterations using the software Muscle<sup>54</sup>, subsequently trimmed and manually aligned in MEGA6<sup>55</sup>, with a final alignment using default parameters in ClustalX v2.1.16<sup>56</sup>. Sequences for all 17 genes were then concatenated within species using the program Sequence Matrix v1.8<sup>57</sup>. A maximum likelihood phylogenetic analysis was conducted using RAxML-HPC version 8<sup>58</sup> on the CIPRES computer cluster (San Diego State University, San Diego, CA, USA). A partitioned analysis was used in which the mitochondrial genes were grouped into a single partition and each nuclear gene had its own partition for a total of 18,809 characters (post-alignment) across 12 partitions. As RAxML does not allow different substitution models for different partitions, a GTR+G model of substitution was used across all partitions. The search for the best scoring maximum-likelihood tree was conducted simultaneously with 750 bootstrap replicates with a GTRGAMMA model using the rapid bootstrapping approach as implemented in RAxML-HPC version 8. The best scoring tree was highly similar to previously published trees<sup>21,59</sup> (Supplementary Fig. 2). We calibrated the phylogeny using multiple calibration points with 'chronos' in the package APE<sup>60</sup>. Cross-validation was first conducted with 'chronopl' to choose the best value of the smoothing parameter lambda. Owing to a poor fossil record, time calibration has been a historically difficult task in poeciliids. Some have recently argued for the use of secondary fossil calibrations, highlighting that previous estimates of divergence dates based on geological events may be prone to error due to uncertainties in the dating of those events<sup>61</sup>. However, all divergence estimates including fossil calibrations are subject to uncertainty, because primary

fossil calibration provides only a lower bound for node age, and secondary fossil calibrations are subject to the uncertainties of the original calibration. We used three calibration points spanning the depth of our phylogeny. First, we used a primary fossil calibration separating the outgroup *Fundulus* from all poeciliids (55–99 Myr ago<sup>62</sup>; Ma). Second, we used the same secondary fossil calibration as ref. <sup>61</sup> separating *Heterandria formosa* from the genus *Poecilia* (9.3–19 Ma). Finally, while vicariant geological events are subject to uncertainties, endemism based on distinct geological formations such as volcanic lakes are more robust. Although it is possible that lake endemics diverged before a lake formed, subsequently colonized the lake and then went extinct outside the lake, the more parsimonious explanation is that endemics diverged in the lake. We therefore used the formation of the Laguna de Catemaco<sup>63</sup> as a constraint on the age of *Poeciliopsis catemaco*, one of several poeciliids endemic to the lake. The maximum age of *P. catemaco* was set at 2 Ma based on the date of the lake's formation<sup>63</sup>, and the minimum was set at 0.5 Ma based on previous estimation of the divergence time between *P. catemaco* and its sister species *P. gracilis* (~0.75–1.5 Ma<sup>64</sup>).

**Evolutionary rate comparisons.** Rates of evolution along major morphological axes were compared in a phylogenetic comparative manner using maximum likelihood<sup>65</sup>. We used the first two pPC scores for shape variation from the male, female and sex-averaged data, and compared rates in a pairwise manner. Rate comparisons were performed with the best scoring tree and differences were assessed with LRT, comparing the observed rate variation to a scenario where both groups are constrained to the same rate.

**Predictors of diversification.** We used the same data to characterize natural selection, sexual selection and reproductive constraint for analyses of diversification in the context of functional morphology and speciation.

**Environmental data.** To generate a metric for variation in natural selection, we quantified species-specific environmental niches. We obtained 73,398 geo-referenced locality points from multiple sources (<http://fishnet2.net/>, <http://gbif.org/>, primary literature), representing the known distributions for all 112 species included in our study. We first removed duplicate points. As some species were vastly overrepresented compared with others, we then randomly sampled locality points for each species, retaining a maximum of 100 localities. This number is sufficient to capture environmental variation even in wide-ranging species<sup>66</sup>. All data points were verified against distribution maps of the native range of each species<sup>67,68</sup> (<http://nas.er.usgs.gov/>; <http://iucnredlist.org/>). We further verified that all data points for a given species were at least 1 km apart to match the spatial resolution of environmental data. Any locality that did not meet this criterion was either removed for species with <100 localities or replaced by another randomly drawn locality for species with >100 localities. For all locality records, we then extracted values for 19 climatic (Worldclim: <http://worldclim.org/>) and three hydrological (elevation, slope and compound topographic index; Hydro1k: <https://lta.cr.usgs.gov/HYDRO1K/>) variables at a spatial resolution of ~1 km<sup>2</sup> (30 arcsec) in ArcMap version 10.2.2 (ESRI Inc, Redlands, CA, USA). For each species, we calculated the median value for all 22 variables. To reduce multicollinearity, we removed Worldclim variables that were strongly correlated ( $r > 0.8$ ) with other variables, preferentially retaining variables that reflect extremes and heterogeneity<sup>69</sup>, and then summarized ecological niche space with a phylogenetic PCA on the remaining ten Bioclim and three Hydro1k variables (Supplementary Table 7). The first four principal components explaining 77% of the variance were retained for analyses of phenotypic diversification based on the broken-stick criterion<sup>70</sup>.

**Sexual selection.** The extent of pre-copulatory sexual selection within poeciliid species was quantified using an SSI based on the degree of sexual dimorphism in body shape and multiple fin traits (see semi-landmarks in Supplementary Table 5) that reflect variation in female mating preferences and male mating strategy<sup>24,71,72</sup>. Specifically, we calculated the absolute difference in length of the anal fin, the lower margin of the caudal fin and the height of the dorsal fin between males and females within species. We also calculated the degree of overall body shape dimorphism between males and females based on the weight matrices from the geometric morphometric analysis. A PCA was conducted on the dimorphism in the three fin lengths and body shape, and the first principal component was retained as the SSI. This index of sexual selection reflected a continuum from species characterized by long gonopodia and coercive mating tactics (negative PC1 scores) to species with short gonopodia and courtship (positive PC1 scores). This index prevented the exclusion of species for which behavioural data previously used to characterize sexual selection were lacking ( $N = 15$  species from our dataset). Should we change the two occurrences of PC1 in this sentence to pPC1, as used throughout the rest of the paper? No, this is a principal component analysis, not a phylogenetic principal component analysis. No change is required.

**Reproductive constraint.** The extent of post-fertilization maternal provisioning of embryos was quantified using ln-transformed values of the matrotrophy index, which is determined by the ratio of offspring mass at birth compared with mass at fertilization. Species in which mothers exhibit higher levels of provisioning

have higher values of the dimensionless matrotrophy index. Matrotrophy values were gathered from the literature<sup>31,73</sup> and we were able to obtain data for 94 of the 112 species.

**Analyses of body shape diversification.** *Adaptive landscapes.* The macroevolutionary adaptive landscape was characterized using the SURFACE method<sup>74</sup> as implemented in the I1ou package in R<sup>75</sup>. This approach uses stepwise model fitting during a forward phase, in which new adaptive regimes are added along the phylogeny, and a backward phase in which regimes are collapsed to identify convergent peaks. We used the first two pPC scores for males, females and sex-averaged morphology. To control for phylogenetic uncertainty and variation that can occur between individual runs of SURFACE, we ran analyses on male and female data across 100 trees and conducted 100 bootstrap replicates within each tree (Supplementary Table 1). To better summarize the extent to which male and female adaptive landscapes differed, we calculated a 'shift concordance fraction'. The shift concordance fraction is simply the number of adaptive shifts that occurred in the same location for both males and females on a given phylogenetic tree divided by the total number of shifts. A value of 0 indicates that no shifts occurred in the locations, and a value of 1 indicates that all observed shifts occurred in the same location for males and females.

*Predictors of body shape variation.* PGLS was used to evaluate relationships between sex-specific patterns of body shape and predictor variables. For males, separate PGLS were used to evaluate the first two axes of body shape variation with the SSI and axes of the ecological niche as predictors. For females, the same procedure was conducted, but using the ln-transformed matrotrophy index as a predictor rather than SSI. We used the corPagel structure and optimized lambda in each PGLS analysis as implemented in 'gls' of the R package nlme<sup>76</sup>. Although bootstrap support values on the best scoring tree were generally strong, we accounted for phylogenetic uncertainty by conducting these PGLS analyses across 250 trees drawn at random from the bootstrap replicates. The test statistics and *P* values reported in the text and supporting table were averaged across results from the 250 trees. As data on matrotrophy were available for only 94 species, analyses of female body shape using matrotrophy as a predictor were conducted on reduced trees generated by pruning tips for which matrotrophy data were missing.

**Analyses of species diversification.** We followed established procedures for quantifying the relative contribution of different mechanisms of divergence previously used to investigate patterns of species diversification in freshwater fishes<sup>77</sup>.

*Selection of species pairs.* Pairs of species representing most of the main genera were selected from the best scoring maximum-likelihood tree. As in ref. <sup>77</sup> and to facilitate comparisons among studies, we focused on more recently diverged pairs of species. Specifically, we selected only bifurcating tips of the phylogeny for which we had data on matrotrophy (Supplementary Fig. 2). The most recent common ancestor of the resulting 25 species pairs ranged from 0.3 to 10.2 Ma. There was no overlap in the branches connecting any of the species pairs, indicating no direct interrelationship among any pairs. While the pairs nonetheless share some evolutionary history, because they are members of the same family that eventually share common ancestors, we were not making comparisons among species pairs. Traditional statistical approaches were therefore appropriate for our analyses<sup>77</sup>. For all of the subsequently described metrics of isolation, we *z*-transformed values across species within each metric prior to the calculation of the Euclidean distance between the two species of each pair. This procedure yielded standardized metrics of isolation to enable comparisons among reproductive barriers within species pairs. We then calculated *z*-scores across metrics within species pairs to evaluate the relative contribution of each metric to divergence between species.

*Mechanisms of isolation.* Quantifying divergence in ecology. The same axes of the ecological niche described above were used to characterize the ecological distance between species. Distance between species was calculated across the first two major pPC axes of the niche (explaining 53% of variance). The results of the overall analysis were qualitatively identical regardless of whether additional axes were used to estimate ecological divergence.

Quantifying divergence in maternal investment. Evidence for the viviparity-driven conflict hypothesis was estimated by the extent of divergence in matrotrophy between species<sup>33</sup>. We used the ln-transformed matrotrophy index as the raw data to calculate distances between species.

Quantifying divergence in sexual selection. To assess the role of sexual selection in species divergence, we used the SSI (calculation described above), which quantifies the extent of sexual dimorphism in traits associated with pre-copulatory sexual selection. The SSI therefore summarized where species fall on the continuum of pre-copulatory sexual selection from coercive mating with little courtship to increased courtship and female mate choice. The distance between two species in the SSI therefore represents the extent of divergence in pre-copulatory sexual behaviour.

Statistical analyses. To evaluate whether any of the three mechanisms was consistently stronger across the 25 species pairs, a non-parametric Friedman's test was used to assess barrier strength by accounting for species pair identity (as in a repeated-measures design). Pairwise comparisons among the barriers were subsequently evaluated with post hoc Wilcoxon signed rank tests. All *P* values from post hoc tests were Bonferroni corrected to account for multiple comparisons. All statistical tests were conducted in SPSS v17.0 (IBM) and were two-tailed.

**Data availability.** All specimens, genetic data and occurrence records used in this study are publicly available from sources listed within the Methods or the Supplementary Information. In addition, the best scoring maximum-likelihood tree, the 250 random trees used to account for phylogenetic uncertainty, data from morphological analyses and the basic scripts for analyses have been deposited on GitHub ([https://github.com/michtobler/NEE\\_Sex-specific-evolution](https://github.com/michtobler/NEE_Sex-specific-evolution)).

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### Author contributions

Z.W.C. and M.T. conceived the project. Z.W.C. collected and analysed the data. Z.W.C. and M.T. wrote the manuscript.

### Competing interests

The authors declare no competing financial interests.

### Additional information

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