

# Are superfetation and matrotrophy more prominent in small species?

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## ABSTRACT

**Background:** Superfetation is the ability of females to simultaneously bear multiple broods of embryos at different developmental stages. Matrotrophy is the post-fertilization transfer of nutrients to developing embryos by the mother. In viviparous fishes of the family Poeciliidae there is wide interspecific variation in the degree of these two reproductive modes. Given that superfetating and matrotrophic females increase less in mass and volume when producing a particular number of embryos compared with females of species that lack these reproductive modes, we propose the hypothesis that their joint presence allows females to fit a relatively large number of embryos in any given volume of the reproductive tract. Thus, matrotrophy and superfetation should be more advantageous for small species, because their presence could compensate the space restrictions of a small body size by maximizing their reproductive output.

**Question:** Do superfetation and matrotrophy compensate the reproductive constraints imposed by a small body size, and thus are more prominent in small species?

**Data description:** Previously published data on female size, degree of superfetation, matrotrophy index, and total fecundity of 91 poeciliid species.

**Search method:** We implemented phylogenetic generalized least squares regressions to examine potential effects of female size on superfetation and matrotrophy. In addition, we compared total fecundity among four categories of species that differ in the presence or absence of matrotrophy and superfetation, accounting for both phylogenetic relatedness and differences in female size among species.

**Conclusions:** We failed to find support for the hypothesis that superfetation and matrotrophy increase the total reproductive output of small species. Small species do not exhibit more pronounced superfetation and matrotrophy than large species. Therefore, these two reproductive strategies do not function as mechanisms to compensate the physical restrictions of a small body size.

*Keywords:* body size, comparative analysis, reproductive modes, Poeciliidae, viviparous fishes.

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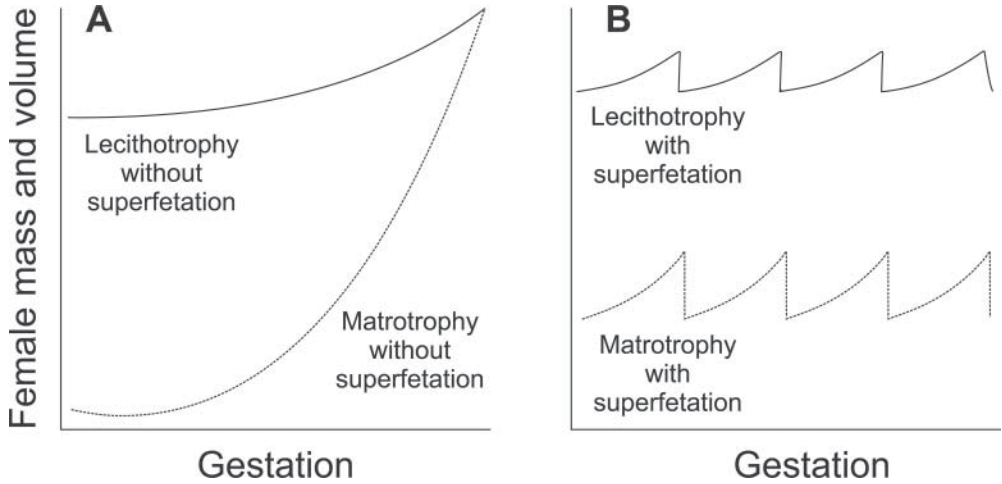
## INTRODUCTION

Superfetation is the ability of females to simultaneously bear two or more groups of embryos at different developmental stages (Turner, 1937; Scrimshaw, 1944a). This is a rare reproductive strategy that occurs in three families of viviparous fishes [Clinidae, Poeciliidae, and Zenarchopteridae (Gunn and Thresher, 1991; Reznick *et al.*, 2007; Pires *et al.*, 2011)] and in bivalves of the family Sphaeriidae (Cooley and Foighil, 2000). In mammals, superconception and superfertilization are similar phenomena that have been observed in a few species only [European brown hare, American mink, and European badger (Roellig *et al.*, 2011)]. Within the Poeciliidae, a Neotropical family of viviparous fishes, superfetation is relatively frequent and has evolved independently multiple times (Pollux *et al.*, 2009; Meredith *et al.*, 2011; Gorini-Pacheco *et al.*, 2018). In addition, there is wide variation among species as well as among populations within species in the degree of superfetation (i.e. in the number of simultaneous broods that females bear), from only two [for example in *Poeciliopsis catemaco* (Bassar *et al.*, 2014)] up to eight simultaneous broods [for example in *Heterandria formosa* (Scrimshaw, 1944b)].

Matrotrophy is the post-fertilization provisioning of nutrients to developing embryos by the mother (Wourms, 1981; Marsh-Matthews, 2011). In matrotrophic species, nutrient transfer occurs through placental structures (Kwan *et al.*, 2015; Olivera-Tlahuel *et al.*, 2019). Matrotrophy is the opposite of lecithotrophy, in which mothers deposit nutrients into the mature ova in the form of yolk before fertilization. In lecithotrophic species, developing embryos feed on the yolk with no additional source of nutrients from the mother (Wourms, 1981). Poeciliid fishes exhibit wide interspecific variation in the amount of nutrients that females transfer to their developing embryos after fertilization, from strictly lecithotrophic species to species that exhibit extensive matrotrophy, with numerous species in between these extremes that vary in the relative amounts of pre- and post-fertilization maternal provisioning (Reznick *et al.*, 2002; Pollux *et al.*, 2009, 2014). Extensive matrotrophy has evolved independently multiple times within the fish family Poeciliidae and some studies have demonstrated that the amount of matrotrophy may also vary among populations within species (Riesch *et al.*, 2010a; Zúñiga-Vega *et al.*, 2011; Gorini-Pacheco *et al.*, 2018).

Given the multiple independent evolutionary origins of both superfetation and matrotrophy and the wide variation among and within species in the degree of these two reproductive modes, some studies have attempted to show their potential benefits in terms of fitness (Travis *et al.*, 1987; Trexler and DeAngelis, 2003, 2010; Macías-García and González-Zuarth, 2005; Zúñiga-Vega *et al.*, 2007, 2010, 2017; Olivera-Tlahuel *et al.*, 2015). Most of these studies have focused separately on either superfetation or matrotrophy, thereby ignoring the fact that there is an evolutionary correlation between the two strategies (Meredith *et al.*, 2011; Pollux *et al.*, 2014). This means that species with the most pronounced matrotrophy also tend to overlap more broods and vice versa, although there are a few exceptions whereby superfetating species are lecithotrophic or matrotrophic species do not exhibit superfetation (Reznick and Miles, 1989; Arias and Reznick, 2000; Pires and Reznick, 2018). Therefore, it is necessary to analyse the two traits together when attempting to understand their adaptive value.

One hypothesis that has been proposed to explain the benefits of the joint presence of superfetation and matrotrophy suggests that, assuming an equal number of embryos produced at any particular point in time, the body mass and volume of pregnant females may be substantially lower in matrotrophic and superfetating species than in lecithotrophic and non-superfetating species (Reznick and Miles, 1989; Pollux *et al.*, 2009). In matrotrophic species, mature ova are quite small and, after fertilization, they gradually grow in mass and volume during development because females provide them with nutrients throughout gestation. Small initial embryo size and gradual increases in mass during development imply that the mass and volume of pregnant females increase little during a large proportion of pregnancy, at least before the late phases of embryo



**Fig. 1.** Predicted changes throughout gestation in the mass and volume of pregnant females for lecithotrophic (solid lines) and matrotrophic (dashed lines) species without (A) and with (B) superfetation. Modified from Pollux *et al.* (2009).

development (Fig. 1A) (Fleuren *et al.*, 2018). In contrast, in lecithotrophic species, mature ova are large and full of yolk before fertilization, and hence the mass and volume of reproductive females are considerably large even before fertilization and they may increase further throughout pregnancy due to water uptake by developing embryos (Fig. 1A).

With respect to superfetation, females that adopt this strategy appear to split a large reproductive bout into two or more smaller broods. Given that wet mass of embryos increases as development progresses, superfetating species overall increase less in mass and volume during pregnancy because embryos in early developmental stages are smaller than embryos in late stages (Zúñiga-Vega *et al.*, 2010). In contrast, non-superfetating species produce all their embryos in a single bout and all of them reach the latest stages (when they become largest) at the same time, which results in pregnant females with substantially larger body mass and volume. This expected difference between superfetating and non-superfetating species also assumes that the total number of embryos carried by females is the same.

Lower female mass and volume during pregnancy should be true for either superfetation or matrotrophy (i.e. considering each of these strategies separately), but this benefit is presumably greatest when the two strategies occur together in a single species (Pollux *et al.*, 2009). Matrotrophy allows the body mass and volume of pregnant females to remain relatively unchanged during the early phases of embryo development and, in concert with superfetation, body mass and volume increase only little when pregnancy progresses, because only a subset of the developing embryos reach the largest sizes (Fig. 1B).

Based on this hypothesis, we wish to go one step further and propose that, given that matrotrophic and superfetating females increase less in mass and volume when producing a particular number of embryos compared with lecithotrophic and non-superfetating females (i.e. a particular number of developing embryos weigh less and occupy a smaller volume of the reproductive tract in matrotrophic and superfetating females), matrotrophic and superfetating females would be able to fit a larger number of embryos in any given volume of the reproductive tract

than lecithotrophic and non-superfetating females. Therefore, matrotrophy and superfetation should be more advantageous for small species, because the presence of these two reproductive modes could compensate the space restrictions of a small body size by maximizing their fecundity. In many taxa, smaller species produce fewer offspring than larger species (Hines, 1991; Sokolovska *et al.*, 2000; Prado and Haddad, 2005). Hence, natural selection could promote the evolution of anatomical, behavioural, physiological, or life-history adaptations that allow small species to maximize their total reproductive output (Blanckenhorn, 2000; Bergström and Wiklund, 2002). Therefore, we predict that higher levels of matrotrophy and superfetation will be more frequent in small than larger taxa. In addition, given that the range of female sizes within poeciliid species may be quite wide (Johnson and Bagley, 2011), such as in other vertebrates with indeterminate growth, we also tested the prediction that, within populations of species that exhibit both matrotrophy and superfetation, smaller females should exhibit a higher degree of both reproductive modes than larger females.

According to our hypothesis, matrotrophy and superfetation may allow females from small species to reach total fecundities that are relatively similar to those of large species, because small species with matrotrophy and superfetation would be able to produce a relatively large number of newborns despite the space restrictions that are inherent to a small body size. To test this hypothesis, we compared fecundity among four categories of species that differ in the presence or absence of matrotrophy and superfetation (lecithotrophic species with and without superfetation and matrotrophic species with and without superfetation). In this comparison, we also accounted for differences in average female size among species and predicted that small species with matrotrophy and superfetation should have: (1) higher fecundities than similar-sized species that lack these two reproductive modes, and (2) similar fecundities to those of larger species.

## METHODS

### Study system and data collection

We focused on the fish family Poeciliidae because of the wide interspecific variation in matrotrophy, superfetation, and female size (Reznick and Miles, 1989; Pollux *et al.*, 2009, 2014). We conducted a literature search to gather data on average standard length (mm) of reproductive females, degree of superfetation (average number of simultaneous broods), and the amount of nutrients transferred to embryos during development (estimated by means of the matrotrophy index) for 91 poeciliid species (see [evolutionary-ecology.com/data/3178Appendix.pdf](http://evolutionary-ecology.com/data/3178Appendix.pdf), Table S1). The matrotrophy index (MI) is a standard measure of post-fertilization maternal provisioning and is calculated as the dry mass of the offspring at birth divided by the dry mass of the egg at fertilization (Reznick *et al.*, 2002; Marsh-Matthews, 2011). MI values  $<0.75$  indicate that all nutrients are provided before fertilization in the form of yolk (i.e. strict lecithotrophic species); MI values between 0.75 and 1 indicate that small amounts of nutrients are provided during embryo development (i.e. incipient matrotrophy); MI values  $>1$  indicate that substantial amounts of nutrients are provided after fertilization; and MI values  $>5$  indicate extensive matrotrophy (Reznick *et al.*, 2002; Pollux *et al.*, 2009; Marsh-Matthews, 2011; Pires *et al.*, 2011). Even though developing embryos of some species with lecithotrophy or incipient matrotrophy can absorb radiotracers shortly after injecting pregnant females, which has been interpreted as the presence of true matrotrophy in these species (Marsh-Matthews *et al.*, 2001, 2005), a recent study demonstrated that eggs from oviparous species can also absorb radiolabelled compounds from their surrounding environment (Morrison *et al.*, 2017). Thus, the presence of radioactivity in embryos of lecithotrophic species does not indicate active transfer of nutrients by the mother, but instead represents a general capacity of embryos of both oviparous and

viviparous species to absorb organic molecules from their surrounding environment. Hence, our classification of species as having lecithotrophy, incipient matrotrophy, or true matrotrophy based on the matrotrophy index is appropriate.

In addition, for all 91 species we also obtained data on total fecundity (i.e. total number of embryos carried by females at any particular point in time) (see Table S1). For non-superfeting species, this was equivalent to brood size. However, for superfeting species, we calculated total fecundity as brood size multiplied by the number of simultaneous broods (assuming that the number of embryos per brood is on average similar among all the broods within a female). We log-transformed all these variables prior to our comparative analyses.

### Morphological comparisons

Our hypothesis that matrotrophy and superfetation should be more prominent in small species – and in small females within species – is based on the assumption that, for any given body length, higher degrees of these two reproductive strategies should result in a lower body volume of reproductive females compared with species and females with less pronounced matrotrophy and superfetation (Fig. 1). We searched for evidence that would support this assumption by comparing the body profile of pregnant females from the following six species: *Poecilia mexicana*, *Xiphophorus hellerii* (both are lecithotrophic and lack superfetation), *Poecilia butleri* (matrotrophic without superfetation), *Poeciliopsis gracilis* (lecithotrophic with superfetation), *P. infans* (has incipient matrotrophy with superfetation), and *P. prolifica* (highly matrotrophic with superfetation). We obtained preserved females of *Poecilia mexicana*, *X. hellerii*, and *Poeciliopsis prolifica* from the National Collection of Fishes (Instituto de Biología, Universidad Nacional Autónoma de México) and digital photographs of pregnant females of *Poecilia butleri*, *Poeciliopsis gracilis*, and *P. infans* from the archives of Zúñiga-Vega *et al.* (2011) and Frías-Alvarez and Zúñiga-Vega (2016) (sample size per species varied from 16 to 123 females). We digitized 17 landmarks on the photographs of all these pregnant females (as per Zúñiga-Vega *et al.*, 2011) and applied geometric morphometric techniques to obtain shape variables using the programs tpsDig v.2.17 and tpsRelw v.1.69 (Zelditch *et al.*, 2004; Rohlf, 2013, 2017).

We statistically compared the first shape axis (i.e. the first relative warp) among species by comparing the fit of a linear model that considers differences among species against the fit of a model that does not include interspecific differences (an intercept-only model). For this purpose, we used the Akaike Information Criterion adjusted for small sample sizes [AICc (Burnham and Anderson, 2002)]. We visualized shape differences among species by means of deformation grids (Zelditch *et al.*, 2004) and, according to the main assumption underlying our hypothesis, we predicted that pregnant females would have narrower abdomens in *P. infans* and *P. prolifica* because these two species exhibit matrotrophy with superfetation.

In addition, we also analysed body shape within two species of the genus *Poeciliopsis*: *P. gracilis* and *P. infans*. We obtained reproductive data and digital photographs of pregnant females for two populations of each species from Frías-Alvarez and Zúñiga-Vega (2016). In both species, the two populations differed in their degree of matrotrophy (see Results) but showed little variation in the number of simultaneous broods. Thus, our comparisons between populations tested only the effect of matrotrophy on body shape. It should be noted here that *P. gracilis* has been considered as lecithotrophic, with an average matrotrophy index of 0.72 (Bassar *et al.*, 2014; Pollux *et al.*, 2014). However, data from Frías-Alvarez and Zúñiga-Vega (2016) indicate that some populations of this species exhibit incipient matrotrophy, which reveals intraspecific variation in the amount of post-fertilization maternal provisioning. Based on digital photographs of

pregnant females, we implemented the same protocol that we explained above to obtain shape variables. Then, we compared the first shape axis between populations (separately for each species) by comparing the fit of a model that included inter-population differences against the fit of an intercept-only model. Model fit was assessed using AICc. We expected narrower abdomens in the populations with higher MI values.

### Interspecific evaluation of the effect of size

We implemented a phylogenetic comparative method [phylogenetic generalized least squares, PGLS (Martins and Hansen, 1997)] to search for a statistical effect of average female size (independent variable) on both matrotrophy and superfetation (dependent variables) while accounting for phylogenetic relatedness. We based these analyses on a robust phylogeny of the family Poeciliidae that was assembled from 28 different genes (Pollux *et al.*, 2014). We trimmed this phylogeny to our 91 species of interest using the program Mesquite v.3.51 (Maddison and Maddison, 2018) and transformed it to an ultrametric tree using a semi-parametric smoothing method based on penalized likelihood (Sanderson, 2002) in the R package ‘ape’ (Paradis *et al.*, 2004; R Core Team, 2018) (Fig. 2). PGLS incorporates the phylogenetic information as part of the error term of the regression model (Martins and Hansen, 1997).

We compared the fit of four different evolutionary assumptions when analysing the effect of female size on superfetation and matrotrophy by means of PGLS implemented in the R package ‘ape’. First, we assumed that traits accumulate changes steadily through time in a way that is well-described by a Brownian motion model (PGLS-BM), which results in substantial phylogenetic signal in the residuals of the regression model. Second, we assumed stabilizing selection towards a fixed optimum, which corresponds to an Ornstein-Uhlenbeck model of evolution (PGLS-OU). This model estimates  $\alpha$  as a measure of the strength of stabilizing selection. Hence, when  $\alpha$  is close to zero, selection is weak and phenotypes retain substantial phylogenetic signal. As  $\alpha$  increases, selection is strong and phenotypes adapt quickly to the local environment, leaving behind no phylogenetic signal. Third, we directly quantified the amount of phylogenetic signal in the residuals of the relationships between female size and superfetation and matrotrophy by estimating  $\lambda$  (Pagel, 1999), a scaling parameter that weights the influence of the phylogeny in explaining the observed data (PGLS- $\lambda$ ). A  $\lambda$ -value close to zero indicates independence from the phylogeny, whereas a  $\lambda$ -value close to one indicates strong similarity between related species. Fourth, we assumed that the phylogenetic relationships have not influenced the correlated evolution between female size, superfetation, and matrotrophy by fitting an ordinary least squares regression (OLS). We used AICc to compare the fit of these four evolutionary assumptions.

We conducted a phylogenetic principal components analysis [PPCA (Revell, 2009)] on both degree of superfetation and the matrotrophy index to create a combined measure of these two reproductive strategies. For this purpose, we used the R package ‘phytools’ (Revell, 2012). PPCA incorporates the expected covariance among phenotypic traits that would result from phylogenetic relatedness into the principal components analysis. This phylogenetic PCA resulted in a single factor with an eigenvalue  $>1$ , which means that the information contained in both variables can be summarized in a single principal component. The largest values of this first principal component (PC1) corresponded to species with the highest degrees of both matrotrophy and superfetation, whereas the lowest values corresponded to lecithotrophic and non-superfeting species.

We implemented the three PGLS models and OLS regression to examine the potential effect of female size on (1) degree of superfetation, (2) matrotrophy index, and (3) the joint presence

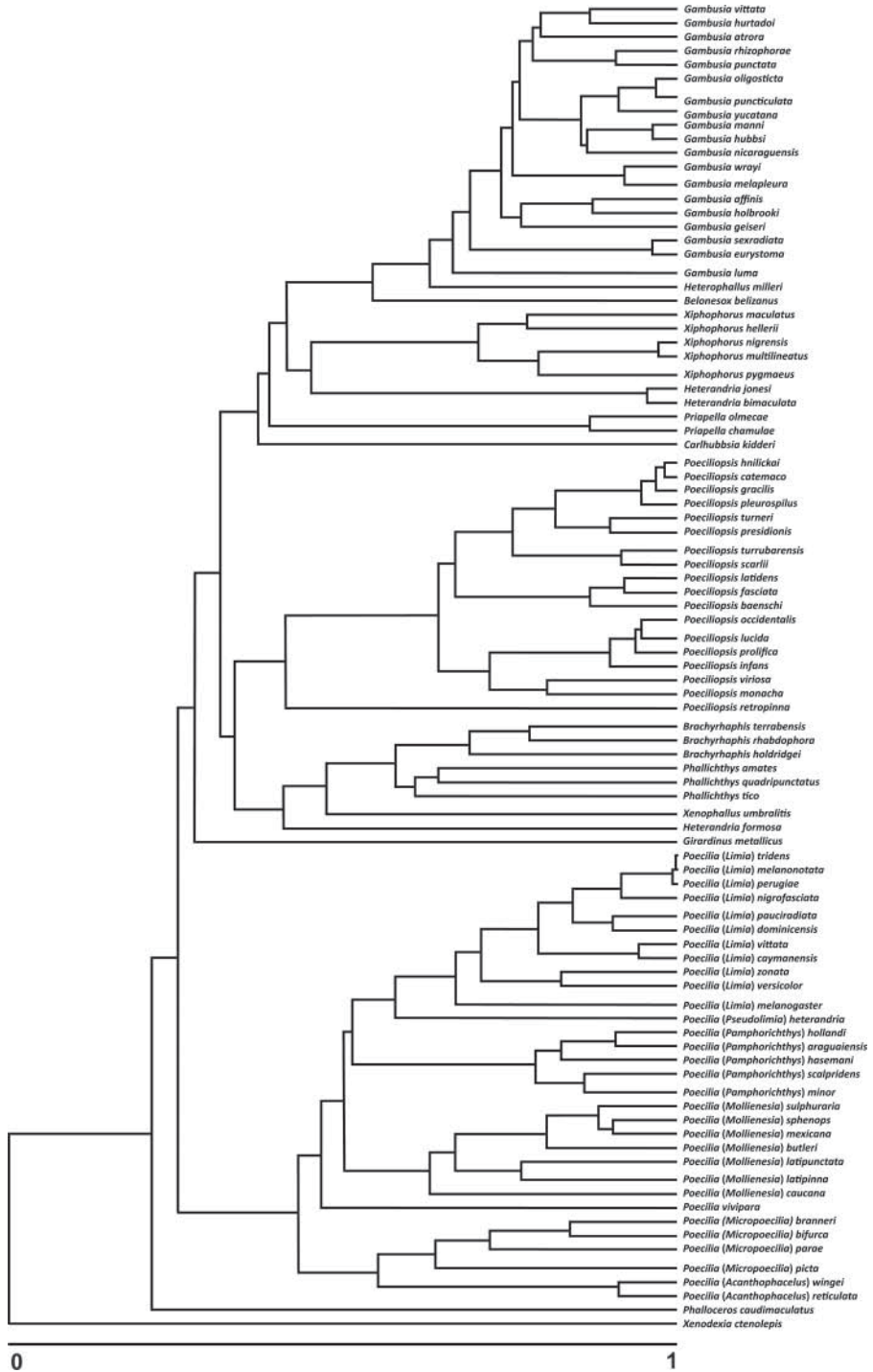


Fig. 2. Phylogenetic relationships among 91 species of fishes from the family Poeciliidae according to Pollux *et al.* (2014). Branch lengths represent relative time.

of superfetation and matrotrophy (PC1). Before running these models, we log-transformed all variables, except PC1 (which followed a normal distribution). These models test our hypothesis that a small body size promotes the evolution of a higher degree of both superfetation and matrotrophy.

### Intraspecific evaluation of the effect of size

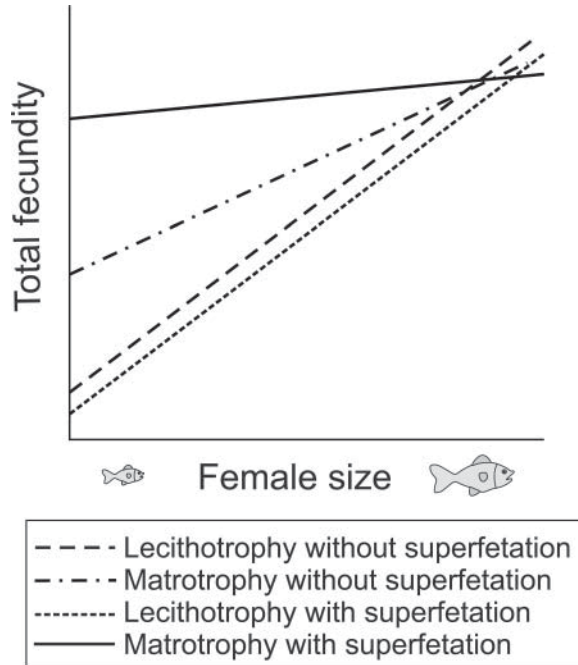
We examined the influence of female size on superfetation and matrotrophy at the intraspecific level by comparing the average degree of these two reproductive modes among three size categories: small, medium-sized, and large females. We used data from two populations of *P. gracilis* and two populations of *P. infans* (Frias-Alvarez and Zúñiga-Vega, 2016). The small size category included all females that measured less than  $-0.5$  standard deviation (SD) of the mean standard length, the medium size category included females that measured between  $-0.5$  and  $+0.5$  SD, and the large size category included females that measured more than  $+0.5$  SD. In these intraspecific analyses, we treated size as a categorical variable because the matrotrophy index cannot be calculated for each individual, but instead is calculated for a group of individuals by regressing log-transformed embryo mass on developmental stage and then dividing the predicted embryo mass at stage 11, which is the estimated size of full-term embryos according to Haynes (1995), by the predicted mass at stage 4, which is the estimated size at fertilization (Riesch *et al.*, 2013). In this regression, each data point corresponds to an individual female.

We statistically compared degree of superfetation (number of simultaneous broods per female) among size categories by means of a generalized linear model with log link function and Poisson distribution of the error term. We used AICc to compare the fit of this model against the fit of an intercept-only model. Given that the matrotrophy index is calculated for a group of individuals and not for each individual female, we conducted randomization tests (Manly, 2007) to compare the index among size categories. These randomization tests consisted of the following steps. First, we calculated an MI value for each of the three size categories. Second, we calculated the standard deviation (SD) among these three MI values. Third, we randomized all data across all size categories and drew three random samples from this pooled dataset. Fourth, we calculated MI values for each of these three random samples as well as the SD among these new MI values. We repeated the last two steps 1000 times and compared the originally observed SD with the distribution of 1000 SD values that resulted from randomizing the data. Evidence of differences among categories in the matrotrophy index would be provided by our observed SD being higher than the 950<sup>th</sup> sorted value of the resulting distribution (this would be equivalent to  $P < 0.05$  in a one-tailed test). We also calculated 95% bootstrap confidence intervals for the MI values (as per Zúñiga-Vega *et al.*, 2011). These analyses were conducted separately for each population.

### Fecundity comparisons

We classified our 91 species into one of the following four categories: lecithotrophy without superfetation, lecithotrophy with superfetation, matrotrophy without superfetation, and matrotrophy with superfetation, and then compared total fecundity among these four categories. For this purpose, we also implemented the PGLS and OLS regression models that we described above in which fecundity was the response variable and reproductive mode (our four categories) served as the main factor. We included average female size as a covariate in these models and predicted that, for small species, total fecundity should be greater in species that exhibit both matrotrophy and superfetation than in the other three categories (Fig. 3). This difference should





**Fig. 3.** Predicted relationships between average female size and total fecundity for four distinct reproductive modes. Small species that exhibit both matrotrophy and superfetation should have (1) higher fecundity than small species that exhibit the other three reproductive modes, and (2) similar fecundities to those of larger species.

be more pronounced with respect to lecithotrophic species (both with and without superfetation) (Fig. 1). We also predicted that small species with matrotrophy and superfetation should have similar fecundities to those of larger species (regardless of the particular reproductive mode of such larger taxa; Fig. 3). Evidence supporting these predictions would be provided by a regression line (between fecundity and average size) with higher intercept and less steep slope for matrotrophic and superfetating species compared with all other reproductive modes, such as we depict in Fig. 3. This means that we expected a significant interaction between reproductive mode and female size as well as a significantly different intercept for matrotrophic and superfetating species with respect to the intercepts for all other reproductive modes.

### Phylogenetic signal

From PGLS- $\lambda$ , we obtained estimates of phylogenetic signal in the residuals of the regressions between female size and our reproductive variables (superfetation, matrotrophy index, PC1, and total fecundity). These estimates of lambda indicate how important shared ancestry has been in the correlated evolution between female size and these reproductive traits. However, these estimates of lambda derived from PGLS do not quantify the amount of phylogenetic signal in the traits themselves (Symonds and Blomberg, 2014). Thus, we used the R package ‘phytools’ to estimate lambda for all traits and to evaluate whether lambda was significantly different than zero (i.e. significant phylogenetic signal) using likelihood ratio tests.

## RESULTS

### Superfetation and matrotrophy promote slender bodies

We found differences in female morphology among species and these differences were consistent with the hypothesis that pregnant females that exhibit superfetation and matrotrophy have less distended abdomens than females that lack these reproductive strategies. The intercept-only model had substantially weaker support than the model that included differences among species ( $\Delta\text{AICc} = 638.28$ ). *Poeciliopsis prolifica* (which exhibits substantial matrotrophy and superfetation) had the lowest scores in the first shape axis, indicating the thinnest morphologies (Fig. 4A). *Poeciliopsis infans* (incipient matrotrophy and superfetation) and *P. gracilis* (lecithotrophic with superfetation) had relatively low values in the first shape axis, also indicating slender female bodies. In contrast, *Poecilia mexicana*, *Xiphophorus hellerii* (both are lecithotrophic and lack superfetation), and *Poecilia butleri* (matrotrophic without superfetation) had higher scores in the first shape axis, indicating robust females (Fig. 4A).

In both *P. gracilis* (Fig. 4B) and *P. infans* (Fig. 4C), females from the population with higher MI values exhibited thinner bodies (lower scores in the first shape axis) than females from the population with lower MI values, which had more distended abdomens (higher scores in the first shape axis). In both species, the intercept-only model had weaker support than the model that included a difference between populations (*P. gracilis*:  $\Delta\text{AICc} = 36.68$ ; *P. infans*:  $\Delta\text{AICc} = 18.79$ ).

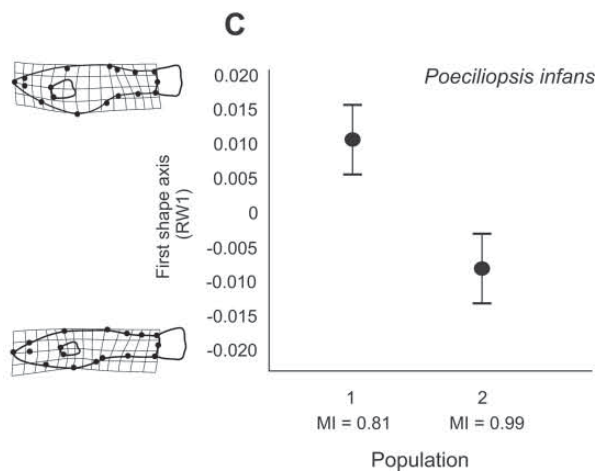
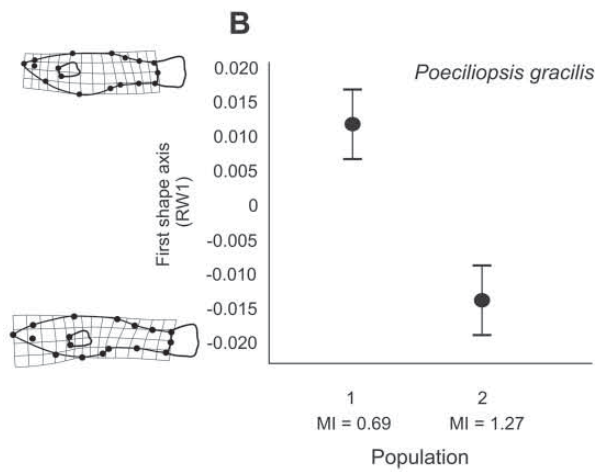
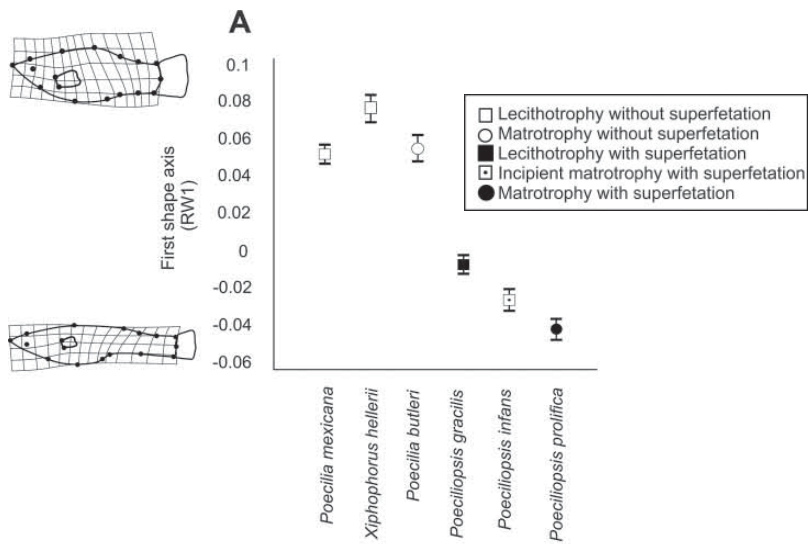
### Superfetation and matrotrophy are not more prominent in small species

We found no significant effects of female size on either superfetation, matrotrophy index, or PC1 (Table 1). The slope that represents the effect of female size on these traits was not statistically different than zero in almost all the evolutionary models that we tested (Fig. 5). The only exception was the effect of female size on the matrotrophy index assuming that phylogeny is unimportant (OLS regression). In this case, the slope indicated a negative relationship between these two traits (Table 1). However, the relative support for this model was extremely weak ( $\Delta\text{AICc} = 57.9$  with respect to the best-fitting model).

In all cases, the models with strongest support indicated that the potential effect of female size on these reproductive strategies evolved in a way that is consistent with a Brownian motion model of evolution. PGLS-BM provided the best fit for both MI and PC1 (Table 1). In the case of the potential effect of female size on superfetation, PGLS- $\lambda$  provided the best fit, with an estimate of  $\lambda$  equal to 1.03 (Table 1), which is also indicative of high phylogenetic signal,

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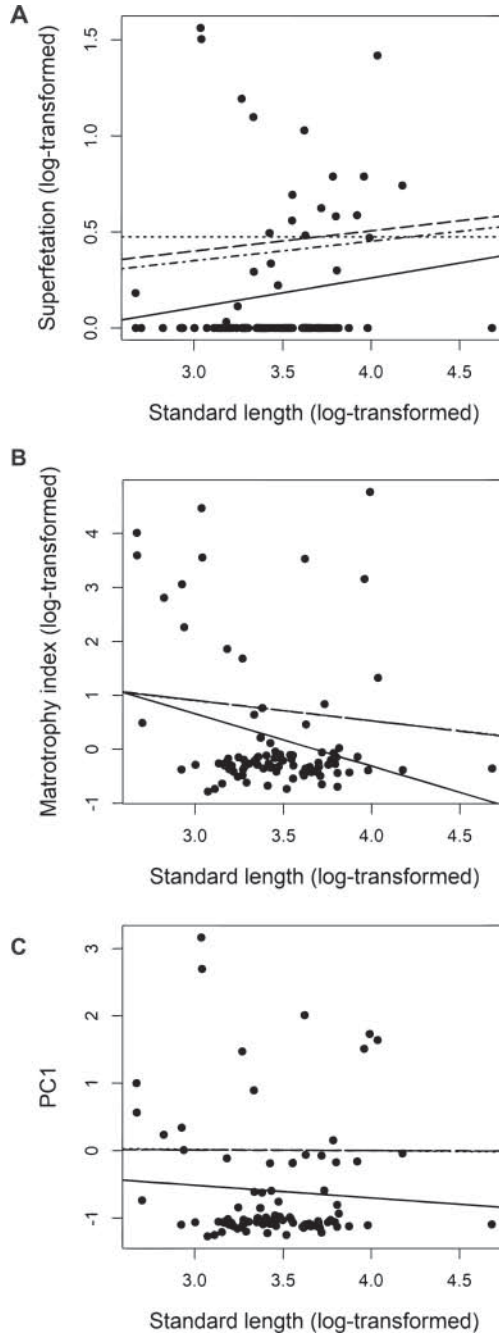
**Fig. 4.** Morphological comparisons among six species with different reproductive modes (A) and between two populations of *Poeciliopsis gracilis* (B) and *P. infans* (C). The two populations of *P. gracilis* and *P. infans* differed in the matrotrophy index (MI). In all cases, we compared the first shape axis (first relative warp, RW1) derived from geometric morphometric analyses. Deformation grids represent deviations from the consensus shape for females with positive and negative scores in the RW1. Error bars represent 95% confidence intervals.



**Table 1.** Results from phylogenetic generalized least squares (PGLS-BM, PGLS-OU, and PGLS- $\lambda$ ) and ordinary least squares (OLS) regression models fitted to test the effect of female size on superfetation and matrotrophy (MI) for 91 fish species from the family Poeciliidae

Regression model	Evolutionary assumption	AICc	$\Delta$ AICc	Regression slope	$P$	$\alpha$	$\lambda$
<b>Superfetation – female size</b>							
	PGLS- $\lambda$	18.56	0	-0.00001 (0.00002)	0.97		1.03
	PGLS-BM	29.41	10.85	0.11 (0.08)	0.21		
	PGLS-OU	31.01	12.45	0.10 (0.09)	0.24	0.41	
	OLS	87.78	69.22	0.16 (0.11)	0.18		
<b>MI – female size</b>							
	PGLS-BM	244.86	0	-0.38 (0.28)	0.18		
	PGLS- $\lambda$	247.03	2.17	-0.37 (0.28)	0.19		0.99
	PGLS-OU	247.05	2.19	-0.38 (0.28)	0.18	$2 \times 10^{-7}$	
	OLS	302.76	57.90	-0.97 (0.38)	0.013		
<b>PC1 – female size</b>							
	PGLS-BM	190.78	0	-0.01 (0.21)	0.97		
	PGLS- $\lambda$	192.90	2.12	-0.02 (0.20)	0.92		1.001
	PGLS-OU	192.97	2.19	-0.01 (0.21)	0.97	$4 \times 10^{-7}$	
	OLS	251.49	60.71	-0.19 (0.29)	0.52		

*Note:* PGLS was implemented using three evolutionary models: Brownian motion (BM), Ornstein-Uhlenbeck (OU), and phylogenetic signal ( $\lambda$ ). The fit of each model to the data was evaluated using the Akaike Information Criterion (AICc), with the smallest value indicating the best-fitting model. We also show differences in AICc values between each model and the best-fitting model ( $\Delta$ AICc). Models are listed according to their AICc values. Standard errors of the regression slopes are shown within parentheses. PGLS-OU estimates an additional parameter,  $\alpha$ , which measures the strength of stabilizing selection.  $\lambda$  is a scaling parameter that weights the influence of the phylogeny in explaining the observed data. PC1 represents a combined measure of superfetation and matrotrophy.



**Fig. 5.** Potential effect of female size (standard length) on superfetation (A), matrotrophy index (B), and a combined measure of superfetation and matrotrophy (PC1) (C) for 91 species of fishes from the family Poeciliidae. In all panels, the solid line corresponds to an ordinary (non-phylogenetic) least squares regression. The other lines represent phylogenetic generalized least squares regressions (PGLS) assuming a Brownian motion model of evolution (dashed), an Ornstein-Uhlenbeck model of evolution (dot-dashed), or estimating the amount of phylogenetic signal ( $\lambda$ ) in the residuals (dotted).

a pattern that is consistent with the Brownian motion model. However, as we emphasized above, the estimated effects of female size derived from these models were not statistically significant (Fig. 5).

### Within species, superfetation – but not matrotrophy – may increase in larger females

Contrary to our prediction that superfetation and matrotrophy would be more prominent in small females, medium-sized and large females exhibited higher degrees of superfetation than small females in the two populations of *P. gracilis* (Fig. 6A, B). In both cases, the model indicating differences among size categories had strong support ( $\Delta\text{AICc} = 0$  and 1.33) and the 95% confidence interval for small females did not overlap with those for medium-sized and large females. In one population of *P. infans*, the degree of superfetation was similar among size categories as indicated by weak support for the model that included differences among categories ( $\Delta\text{AICc} = 4.52$ ; Fig. 6C). In the other population of *P. infans*, the model indicating differences among size categories provided the best fit, whereas the intercept-only model had less support ( $\Delta\text{AICc} = 2.79$ ). In this population, large females had more pronounced superfetation than small and medium-sized females (Fig. 6D).

Regarding matrotrophy, the matrotrophy index was similar among size categories in all four populations (Fig. 6E–H). In all cases, the 95% bootstrap confidence intervals for the three size categories overlapped each other and the standard deviation of the actual MI values was similar to the SD values that we obtained after randomizing the data ( $P > 0.15$  in all four populations).

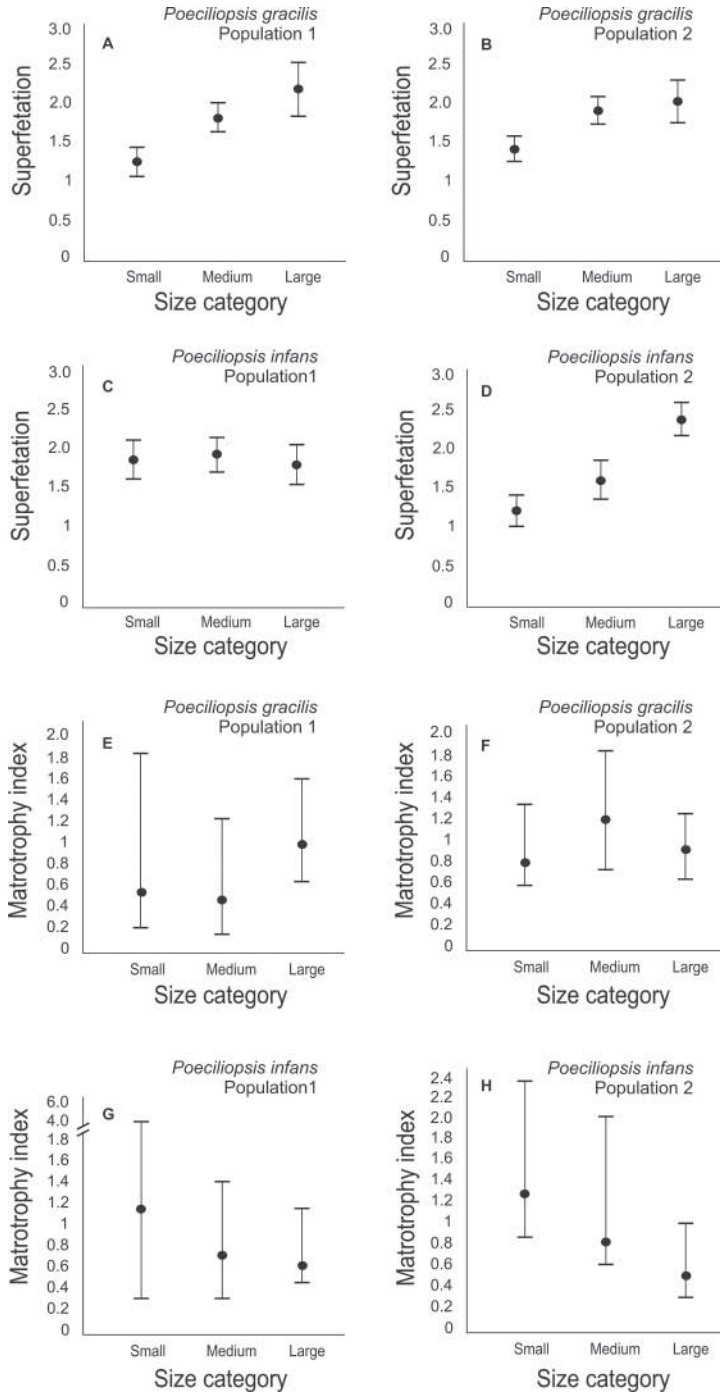
### Superfetation and matrotrophy do not maximize fecundity

The model that assumed independence from the phylogeny (OLS regression) provided the best fit to the fecundity data (Table 2). PGLS-OU and PGLS- $\lambda$  also had considerable support ( $\Delta\text{AICc} < 2$  in both cases), with a large estimate of  $\alpha$  (35.37) and a low estimate of  $\lambda$  (0.18), both of which also indicate independence from the phylogeny. PGLS-BM had little support (Table 2). For simplicity, we present and interpret results from the OLS regression (all other models yielded similar results). The main effect of female size was positive and significant ( $F_{1,83} = 12.25$ ,  $P = 0.001$ ). Thus, in general, females of larger species carry more embryos than females of smaller species (Fig. 7). In contrast, the main effect of reproductive mode and the interaction

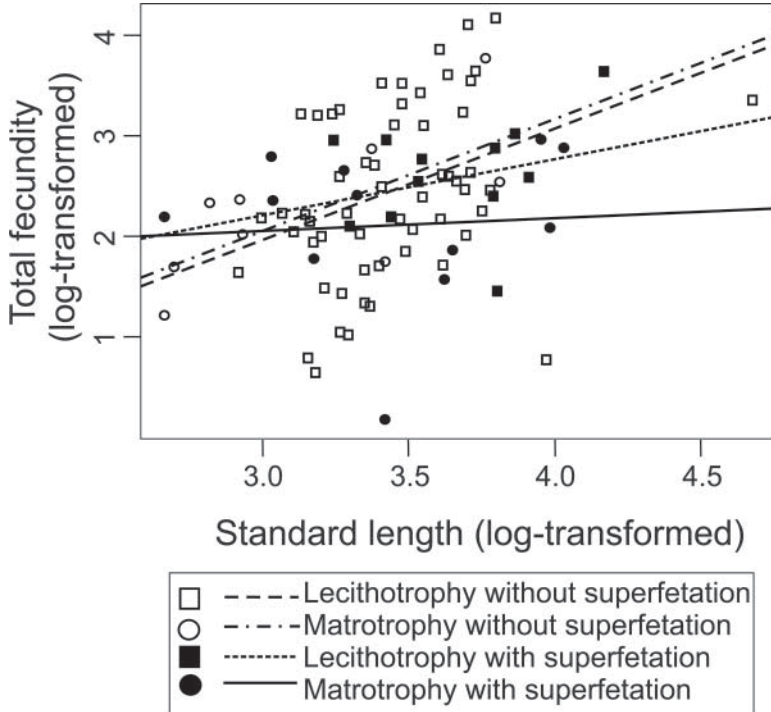
**Table 2.** Results from phylogenetic generalized least squares (PGLS-BM, PGLS-OU, and PGLS- $\lambda$ ) and ordinary least squares (OLS) regression models fitted to test the effects of female size and reproductive mode on total fecundity for 91 fish species from the family Poeciliidae

Evolutionary assumption	AICc	$\Delta\text{AICc}$	$\alpha$	$\lambda$
OLS	225.25	0		
PGLS-OU	225.91	0.66	35.37	
PGLS- $\lambda$	226.60	1.35		0.18
PGLS-BM	277.45	52.20		

*Note:* We classified our 91 species into one of four distinct reproductive modes: lecithotrophy without superfetation, lecithotrophy with superfetation, matrotrophy without superfetation, and matrotrophy with superfetation. All abbreviations and symbols are as defined in the footnote to Table 1.



**Fig. 6.** Average number of simultaneous broods (i.e. degree of superfetation) (A–D) and matrotrophy index (E–H) for two populations of *Poeciliopsis gracilis* and two populations of *P. infans*. Females were classified into three different size categories. Error bars represent 95% parametric (A–D) and bootstrap (E–H) confidence intervals.



**Fig. 7.** Relationships between average female size and total fecundity for 91 fish species from the family Poeciliidae that we classified into four distinct reproductive modes. For species with superfetation, total fecundity was calculated as brood size multiplied by the average number of simultaneous broods. We show the fitted lines for all four reproductive modes according to an ordinary (non-phylogenetic) least squares regression.

between reproductive mode and female size were not significant ( $F_{3,83} = 0.60$ ,  $P = 0.61$  and  $F_{3,83} = 0.88$ ,  $P = 0.46$ , respectively) (Fig. 7).

Table 3 shows the estimated regression coefficients from the OLS model. We used the category of matrotrophy with superfetation as the reference level. In this way, regression coefficients indicate if the intercepts and slopes of the other three categories differ with respect to those of matrotrophic and superfetating species. Neither the intercepts nor the slopes of the other three categories differed significantly from the intercept and slope estimated for matrotrophic and superfetating species (Table 3). Therefore, small species that exhibit both matrotrophy and superfetation do not produce more embryos than small species that exhibit the other three reproductive modes, and do not have similar fecundities to those of larger species (Fig. 7).

### Phylogenetic signal

Our estimates of  $\lambda$  for individual traits revealed strong phylogenetic signal in female size ( $\lambda = 0.83$ ), superfetation ( $\lambda = 0.93$ ), MI ( $\lambda = 0.99$ ), and PC1 ( $\lambda = 1.00$ ;  $P < 0.001$  in all cases). In contrast, we found no phylogenetic signal in total fecundity ( $\lambda = 6.6 \times 10^{-5}$ ,  $P = 1$ ).



**Table 3.** Estimated regression coefficients and standard errors (SE) from the ordinary least squares (OLS) regression that tested the effects of female size and reproductive mode on total fecundity for 91 fish species from the family Poeciliidae

Model terms	Regression coefficient	SE	P
Intercept (matrotrophy with superfetation)	1.68	1.87	0.37
Female size	0.13	0.54	0.81
Lecithotrophy with superfetation	-1.16	3.69	0.75
Matrotrophy without superfetation	-2.96	2.72	0.28
Lecithotrophy without superfetation	-3.07	2.23	0.17
Female size (lecithotrophy with superfetation)	0.43	1.02	0.67
Female size (matrotrophy without superfetation)	0.98	0.82	0.24
Female size (lecithotrophy without superfetation)	0.99	0.65	0.13

*Note:* We classified our 91 species into one of four distinct reproductive modes: lecithotrophy without superfetation, lecithotrophy with superfetation, matrotrophy without superfetation, and matrotrophy with superfetation. We used matrotrophy with superfetation as the reference level.

## DISCUSSION

We found no patterns in support of the hypothesis that superfetation and matrotrophy are more common in smaller species or individuals. Based on the theoretical consideration that a particular number of embryos occupy a smaller volume of the reproductive tract in females of species that exhibit both reproductive modes than in females of species that lack these reproductive modes (Thibault and Schultz, 1978; Reznick and Miles, 1989; Pollux *et al.*, 2009), we proposed that matrotrophic and superfetating females would be able to fit more embryos in any given volume of the reproductive tract than lecithotrophic and non-superfetating females. This would be a major benefit for small species or individuals, given the space limitations associated with a small body size. However, we found that: (1) small species do not have more pronounced superfetation and/or matrotrophy, and (2) their joint presence does not increase the total reproductive output of small species, and hence these reproductive strategies do not function as mechanisms to compensate the physical restrictions of a small body size. Therefore, in poeciliid fishes, the evolution of small body size has not promoted the evolution of more prominent superfetation and/or matrotrophy.

The reverse direction of evolutionary causality would be that the evolution of these two reproductive modes facilitates the evolution of a small body size, because small females with more pronounced matrotrophy and superfetation would be able to produce as many embryos as large females, which relaxes the selective pressure towards attaining a large body size. However, the lack of statistical relationships between female size and these two reproductive modes does not support this reverse direction of causation either.

Our results also revealed that, at the intraspecific level, large females may overlap more broods than small females, a pattern that is opposite to what we predicted. Thus, at least within two species (*P. gracilis* and *P. infans*), superfetation may be more prominent in larger females than in smaller ones. In all poeciliid species, females keep growing after attaining sexual maturity, which means that the range of female sizes within populations may be quite wide (Turner and Snelson, 1984; Abney *et al.*, 2005; Riesch *et al.*, 2012). For this reason and based on our hypothesis, we predicted more pronounced superfetation and/or matrotrophy in small females. Contrary to this prediction, we found that the amount of post-fertilization provisioning is not affected by female

size and, more interestingly, that larger females exhibit more prominent superfetation. These results not only provide further evidence against our hypothesis, but also imply that any interspecific comparison may be problematic if the average values of the reproductive traits cannot reflect the wide variation that occurs within species. In our case, at the interspecific level, we found no statistical relationship between average female size and superfetation, but this lack of association could have arisen erroneously if, for example, the average values of superfetation for some of the large species came from biased samples that contained only a few of the largest reproductive females, which may exhibit the greatest degree of superfetation, but which do not provide a representative value for the majority of the females from such species. Thus, the interpretation of our interspecific patterns must be treated with caution.

Two other hypotheses have been proposed to explain the potential benefits of the joint presence of both superfetation and matrotrophy. First, these two reproductive modes may enhance locomotor (swimming) performance because relatively low body mass and volume during pregnancy should allow females to: (1) exhibit less-impaired escape responses, (2) experience less drag forces during swimming, and (3) maintain a steady velocity (Thibault and Schultz, 1978; Pollux *et al.*, 2009; Zúñiga-Vega *et al.*, 2010). Therefore, these two reproductive modes should be advantageous in environments where fish must swim fast to escape from abundant predators or where there are strong water currents. This hypothesis has not been formally tested by considering the two reproductive strategies together. The only existing evidence that partially supports this hypothesis comes from a recent study by Gorini-Pacheco and colleagues (2018), who studied *Phalloceros harpagos* in three populations that differ in predation pressure and found the most pronounced matrotrophy and superfetation in the population with the highest predation risk. However, these authors did not quantify body shape or swimming performance.

Here, we examined if the presence of both superfetation and matrotrophy would result in pregnant females having less distended abdomens. Consistent with this expectation, the thinnest females were those of *P. prolifica*, a species that exhibits a high degree of both superfetation and matrotrophy [up to four simultaneous broods and MI = 5.4 (Pires *et al.*, 2007; Pollux *et al.*, 2014)]. In addition, *P. gracilis* and *P. infans*, both of which display superfetation and limited post-fertilization maternal provisioning to developing embryos, also exhibited slender bodies. However, we recognize here that this morphological comparison among few species provides only preliminary evidence in favour of superfetation and matrotrophy promoting streamlined phenotypes, because thin bodies may simply be a shared feature of the entire genus *Poeciliopsis*. Using a substantially larger number of species and accounting for phylogenetic relatedness, further comparative studies are required to examine, at the interspecific level, whether the joint presence of matrotrophy and superfetation is associated with more streamlined phenotypes and enhanced locomotor performance.

Few other studies have examined the potential benefits of either superfetation or matrotrophy (not considering them together) in terms of promoting more streamlined phenotypes. Zúñiga-Vega *et al.* (2007) found that females of *Poeciliopsis turrubarensis* with the most pronounced superfetation are thinner, more elongated, and inhabit environments with stronger water currents than females that overlap fewer broods, which are more robust and inhabit environments with less strong currents. However, studies on other congeneric species (*P. gracilis*, *P. infans*, and *P. baenschi*) did not observe this same pattern (Frias-Alvarez and Zúñiga-Vega, 2016; Zúñiga-Vega *et al.*, 2017). Fleuren *et al.* (2018) examined morphological differences between a matrotrophic species (*P. turneri*) and a lecithotrophic species (*P. gracilis*) during pregnancy and found that females of *P. turneri* are more slender than those of *P. gracilis* during early pregnancy. They inferred

that pregnant females of *P. turneri* would experience less drag forces and better swimming performance than pregnant females of *P. gracilis*. Here, we also provided evidence, in two different species (*P. gracilis* and *P. infans*), that greater post-fertilization maternal provisioning (higher MI) may result in thinner females.

Second, if indeed a particular number of embryos weigh less and occupy a smaller volume of the reproductive tract in females that exhibit superfetation and matrotrophy, then such females may have some additional space to produce larger newborns (Olivera-Tlahuel *et al.*, 2015). Under particular ecological circumstances, larger offspring have higher fitness than smaller offspring (Bashey, 2008; Gordon *et al.*, 2009; Riesch *et al.*, 2010b). However, phylogenetically corrected evidence from 44 poeciliid species did not support this hypothesis: species that exhibit these two reproductive strategies do not produce larger newborns than lecithotrophic and non-superfeting species (Olivera-Tlahuel *et al.*, 2015). This hypothesis has yet to be tested at the intraspecific level.

Here, we failed to find support for the hypothesis that superfetation and matrotrophy are mechanisms that maximize total fecundity of small species. Small species that exhibit superfetation and matrotrophy have neither higher fecundities than similar-sized species that lack these two reproductive modes, nor similar fecundities to those of larger species. Hence, we now should ask if small poeciliids have some other way to compensate the space restrictions imposed by their small body on their reproductive output. We hypothesize that smaller poeciliid species may have faster developmental rates (i.e. shorter gestation periods) or shorter non-breeding intervals. Quantification of gestation length and yearly reproductive cycles in natural conditions remains limited (Turner, 1937; Greven, 2011).

Finally, our estimates of phylogenetic signal from both residuals and traits indicate that body size as well as the degree of both superfetation and matrotrophy have evolved by gradually accumulating changes throughout time, which resulted in substantial similarity among closely related species. This is an interesting pattern given the multiple independent times that the capacity of females to superfetate and the extensive post-fertilization maternal provisioning of nutrients to developing embryos have evolved in these livebearing fishes (Pollux *et al.*, 2009, 2014; Meredith *et al.*, 2010, 2011). Thus, once these two reproductive strategies arise, the amounts of superfetation and matrotrophy steadily accumulate small changes with little influence of the local environment. This high phylogenetic signal in both matrotrophy and superfetation has already been documented, although with fewer ( $n = 44$ ) species (Olivera-Tlahuel *et al.*, 2015). Regarding female size, our study is the first to document that in poeciliid fishes this trait has also evolved in a way that is consistent with a Brownian motion model of evolution. In contrast, we found no phylogenetic signal in total fecundity, which indicates that this life-history trait adapts quickly to the local environment. This result is consistent with the well-documented selective effects of particular environmental factors on offspring size and number – predation, salinity, population density, food availability (Reznick *et al.*, 1990; Johnson and Belk, 2001; Bashey, 2008; Johnson and Bagley, 2011) – as well as with the low phylogenetic signal observed in the size of each individual newborn in poeciliid fishes (Olivera-Tlahuel *et al.*, 2015).

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