Mode of maternal provisioning in the fish genus *Phalloceros*: a variation on the theme of matrotrophy

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The placenta is a complex organ that shows high morphological diversity. Among fish, the first vertebrates that have evolved a placenta, the family Poeciliidae exhibits very diverse modes of maternal provisioning even among congeneric species. Here, we investigated the embryonic growth curve across seven recently-described species of the highly diverse genus *Phalloceros* (Eigenmann, 1907). We also investigated possible intraspecific differences and whether other female characteristics affected embryo mass. We found that embryo mass decreased until around stage 20 and then increased, resulting in a 1.5 to 3-fold mass gain from fertilization to birth. Embryo mass changed non-linearly with stage of development and was affected by species identity (or locality) and female somatic dry mass. This initial loss then gain of embryonic mass during development is unique among other Poeciliidae species and was conserved across populations and species, even though size at birth can vary. Other species instead either lose mass if they lack placentas or gain mass exponentially if they have placentas. The *Phalloceros* mode of maternal provisioning could thus represent a different form from that seen in other species of Poeciliidae.

ADDITIONAL KEYWORDS: embryonic growth curve – fish – matrotrophy index – offspring size – placentation – Poeciliidae

INTRODUCTION

Placental viviparity, defined as 'any intimate apposition or fusion of the fetal organs to the maternal . . . tissues for physiological exchange' (Mossman, 1991), is not an exclusive characteristic of mammals. It is found across a vast array of invertebrates [e.g. cockroaches, terrestrial and aquatic gastropods, clams, reviewed in Ostrovsky *et al.* (2016)] and all vertebrate taxa, except birds [fish (Wourms *et al.*, 1988); amphibians (Greven, 1998); reptiles (Blackburn, 1992)]. Placental or matrotrophic females have small-size eggs at fertilization that grow throughout gestation as the female provides the developing embryos with nutrients (Blackburn, 1992). There is no general consensus on why placentas evolve. Proposed hypotheses include diverse adaptive hypotheses (e.g. resource availability, locomotor costs, life-history facilitation) and parent-offspring conflict (summarized in Pollux *et al.*, 2009). A potential hallmark of conflict is the bewildering diversity in the structure of the mammalian placenta (Mossman, 1991; Crespi & Semeniuk, 2004).

Placentation as a life-history trait in organisms other than mammals has been poorly characterized. The characterization of physiological, anatomical and other aspects of maternal provisioning occurring throughout gestation can shed light into its evolution and adaptive value. Comparative studies of placentation may inform us of the ecological circumstances that are associated with placentation. If there is variation in the extent to which placentation is developed, especially among closely related species, then it may also provide the necessary raw material for characterizing how and why placentas evolve.

Fish are the first vertebrates to evolve a placenta (Long *et al.*, 2008) and fishes in the family Poeciliidae are of special

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interest because of their diversity in maternal provisioning (Pollux *et al.*, 2009, 2014). All species save one—*Tomeurus gracilis* Eigenmann (1909)—are livebearers (Pollux *et al.*, 2009). Most of the livebearing species are lecithotrophic, meaning that the mothers fully provision eggs before they are fertilized, then retain the embryos until development is complete (Pollux *et al.*, 2014). Around 20% of the species are instead placentotrophic, as indicated by their embryos dry mass change during development (Pollux *et al.*, 2014). The magnitude of post-fertilization provisioning varies both among and within species and ranges from just enough to sustain body mass during development to provisioning sufficient to cause a greater than 100-fold increase in dry mass between fertilization and birth. The placenta has evolved at least nine times in the family (Pollux *et al.*, 2014). Crespi & Semeniuk (2004) argue that the evolution of viviparity could set the stage for the subsequent evolution of the placenta because the prolonged close contact between mother and developing embryos gives the embryos the opportunity to gain additional resources from the mother. Once this threshold is crossed, Trivers (1974) predicts that the ideal quantity of resources that is in the best interests of the embryo to acquire from its mother will exceed what is in the best interests of the mother to provide. Placentas are a by-product of the resulting tug of war between maternal control of allocation and embryonic control of acquisition.

All livebearing Poeciliidae retain developing embryos within the maternal follicle until development is complete (Turner, 1940). Most species with extensive placentotrophy have follicles that become thickened and highly vascularized with dense microvilli on the inner surface, presumably to facilitate the transfer of nutrients from the mother to developing young (Turner, 1940; Panhuis *et al.*, 2017; Olivera-Tlahuel *et al.*, 2018). The South American genus *Phalloceros* (Eigenmann, 1907) is of special interest because it has an unusually large number of species and the few species studied thus far are placental. It was considered a single species for over a century, until Lucinda (2008) revisited it and concluded that it comprises 22 separate but closely related species. This makes *Phalloceros* the most species-rich South American genus in the family Poeciliidae. More recent molecular analysis confirmed the classification of the 22 morphologically-defined species by showing that they mostly correspond to monophyletic lineages, sometimes living in sympatry (Thomaz *et al.*, 2019). This work also suggested the existence of hidden diversity and undescribed species in the genus, as also shown by Souto-Santos *et al.* (2019). The large number of species with undescribed modes of maternal provisioning thus represent an important target to study.

Arias and Reznick (2000) reported on the life histories of what were considered four populations of *Phalloceros caudimaculatus* (Hensel, 1868) and found variation in the maternal provisioning to the embryos and an unusual pattern of embryo growth. The embryos tended to increase in dry mass during development, up to a factor of 2 to 3-fold in one population, but the pattern of growth appeared to differ from what was seen in other placental species, where it is best described by an exponential growth function. Embryos of some *Phalloceros* populations instead appeared to first lose, then gain mass. The difference in embryonic growth suggests the existence of a different mechanism of maternal provisioning. However, small sample sizes precluded an accurate description of the pattern of maternal provisioning in those populations. The four populations considered in Arias and Reznick (2000) are now tentatively classified as four distinct species, based on where they were collected.

Intraspecific variation in maternal provisioning has been shown in *Phalloceros harpagos* Lucinda, 2008, with predation regime being the driver of the degree of matrotrophy (Gorini-Pacheco *et al.*, 2018). Additionally, one population of *P. harpagos* has superfetation whereas all other populations and species studied thus far lack superfetation (Gorini-Pacheco *et al.*, 2018). 'Superfetation' means that females carry multiple broods of young in different stages of development (Thibault & Schultz, 1978). Such diversity in modes of reproduction within what appear to be rapidly diversifying lineages presents a great opportunity for evaluating the conditions that favour the evolution of placentation and superfetation and to test the generality of the results of earlier comparative analyses (Lucinda, 2008).

The objective of this study is to investigate the mode of maternal provisioning throughout gestation of seven recently described species of *Phalloceros* and to analyse interspecific and intraspecific variation with the benefit of larger sample sizes and multiple species and the goal of obtaining a better characterization of the patterns of embryonic growth. We evaluated the mode of maternal provisioning in the seven species from 12 localities in south-eastern Brazil. Specifically, we explored how mean embryo mass changes during ontogeny in different populations of these seven species. We investigated how maternal provisioning changed throughout embryonic development by calculating a matrotrophy index and by looking at embryos' growth patterns through developmental stages. Finally, we also asked if embryo mass was correlated to other characteristics of the females, such as size and reproductive allocation.

MATERIAL AND METHODS

COLLECTIONS AND SPECIMEN PREPARATION

We collected seven species of *Phalloceros*— *Phalloceros aspilos* Lucinda, 2008, *Phalloceros anisophallos* Lucinda, 2008, *Phalloceros enneaktinos* Lucinda, 2008, *Phalloceros leptokeras* Lucinda, 2008,

Phalloceros harpagos Lucinda, 2008, *Phalloceros tupinamba* Lucinda, 2008 and *Phalloceros uai* Lucinda, 2008—in the type localities described in Lucinda (2008) and other localities in the states of Rio de Janeiro, São Paulo and Minas Gerais in south-eastern Brazil. Some of the species occurred in sympatry and some were collected in multiple localities (localities summarized in Table 1; Supporting Information, Fig. S1; Table S4). The fish were collected between 2013 and 2015 using seine nets, preserved in 10% formalin, then transferred to 70% ethanol. Females of all sizes were collected to include the complete range of reproductive individuals. All fish were identified at the Museu Nacional, Universidade Federal do Rio de Janeiro (MNRJ), following the species classification proposed by Lucinda (2008). Voucher specimens were deposited at the Ichthyological Collection of the MNRJ. Fish were measured for standard length (mm) and weighed (g), and dissected to remove the ovaries with embryos, which were counted and classified according to developmental stage following Reznick (1981). For purposes of statistical analyses, developmental stages were given numerical values that ranged from 0 (unfertilized egg) to 50 (fully developed embryo), as described in Reznick (1981). Embryos and all reproductive tissues were dried in a drying oven at 50 °C and then weighed. The female body without the reproductive tissue was also dried and weighed. We thus obtained the following measurements: female standard length, female wet mass with ovary, female dry mass without ovary (somatic dry mass), reproductive tissues dry mass, number of embryos, embryo total and mean dry mass.

Table 1. Locality data of samples of *Phalloceros* collected for this study. Species that occurred in sympatry with other species of Poeciliidae are indicated by asterisks. State abbreviations correspond to the Brazilian states of Minas Gerais (MG), Rio de Janeiro (RJ) and São Paulo (SP). South-east refers to small coastal river drainage of the states of Rio de Janeiro and São Paulo

Species	Voucher (MNRJ)	State	Municipality	Drainage	Sub-drainage	Geographical coordinates
P. tupinamba	43236	SP	Ubatuba	South-east	Rio Grande de Ubatuba	S 23° 24' 46.0" W 45° 06' 50.2"
*P. tupinamba**	43544	SP	Ubatuba	South-east	Itamambuca	S 23° 23' 19.0" W 45° 01' 23.8"
P. enneaktinos	43460	SP	Ubatuba	South-east	Tavares	S 23° 26' 43.9" W 45° 05' 23.8"
P. enneaktinos	43245	RJ	Paraty	South-east	Toca do Boi	S 23° 19' 44.8" W 44° 40' 54.6"
*P. anisophallos**	43504	RJ	Paraty	South-east	Paraty Mirim	S 23° 19' 03.7" W 44° 43' 24.7"
*P. aspilos**	43505	RJ	Paraty	South-east	Paraty Mirim	S 23° 19' 03.7" W 44° 43' 24.7"
*P. anisophallos**	43507	RJ	Angra dos Reis	South-east	Caputera	S 22° 56' 29.0" W 44° 18' 41.1"
*P. harpagos**	43508	RJ	Angra dos Reis	South-east	Caputera	S 22° 56' 29.0" W 44° 18' 41.1"
*P. tupinamba**	43600	RJ	Cachoeira de Macacu	South-east	Macacu	S 22° 29' 12.2" W 42° 39' 42.8"
P. harpagos	50835	RJ	Cachoeira de Macacu	South-east	Guapiaçu (Itaperiti)	S 22° 28' 17.46" W 42° 41 '49.86"
P. harpagos	52716	RJ	Maricá	South-east	Ubatiba	S 22° 52' 17.10" W 42° 44' 14.04"
*P. leptokeras**	43596	RJ	Sapucaia	Paraiba do Sul	Paquequer Grande	S 22° 03' 46.0" W 42° 47' 44.7"
P. uai	43598	MG	Lagoa Santa	São Francisco	Rio das Velhas	S 19° 34' 28.5" W 43° 55' 27.3"
P. uai	43597	MG	Conceição do Mato Dentro	Doce	Santo Antônio	S 19° 04' 28.4" W 43° 26' 43.1"

STATISTICAL ANALYSIS

We performed the analyses on two levels: (a) interspecific and (b) intra-specific. For the intra-specific analyses, we included only those species for which we had data for more than one population (i.e. locality), namely: *P. anisophallos* (Caputera, Paraty Mirim), *P. harpagos* (Caputera, Ubatiba), *P. tupinamba* (Macacu, Rio Grande de Ubatuba, Itamambuca), *P. enneaktinos* (Toca do Boi, Tavares) and *P. uai* (Santo Antônio, Rio das Velhas). Only localities with data on more than four individuals were included.

We first performed an ANOVA with mean embryo mass as a response and developmental stage as a predictor (fixed factor) to assess the way embryo mass changes during development. We then ran additional ANOVAs considering only embryos from developmental stage 45 and looked at species (for the interspecific analysis) and locality (for the intraspecific analysis, performing separate ANOVAs for each species) effects. Lastly, we included somatic dry mass as a covariate to the analysis of embryo mass at birth (stage 45) to assess whether or not the size of the mother influenced the size of the offspring at birth.

We then modeled how embryo mass changed during development with generalized additive nonparametric models with mixed effects (a.k.a. GAMMs, Zuur *et al.*, 2009). GAMMs allow for non-linear relationships between the response variable and multiple explanatory variables, which have an important biological meaning in the case of mean embryo mass variation. They represent generalized linear models where the predictor includes a sum of the smooth functions of the covariates. The smooth functions may be cubic, natural splines, B-splines, thin-plates or smoothing splines, and are treated as random effects. The mean embryo mass change during development is a growth curve and we hypothesized that this growth is non-linear, as implied by the results of Arias and Reznick (2000), where embryos initially lose mass, then gain mass beginning mid-way through development (Arias & Reznick, 2000; trend also visible in fig. 1b). Because embryo mass at any stage depends on the mass at previous stages, it is meaningful to consider a non-linear smoother for the variables. Stage of development was added to the models as a fixed effect. The following models were compared to evaluate if embryonic growth was linear or non-linear: (a) a null model, (b) a model where mean embryo mass increases linearly with stage of development, (c) mean embryo mass increases non-linearly with stage of development (smoother type: cubic regression spline) and (d) mean embryo mass increases non-linearly with stage of development (smoother type: thin-plate spline). Within models (c) and (d), different numbers of knots (k) were tested, where $k = 3,...,10$.

Having selected the most plausible model to explain how mean embryo mass varies with stage of development, based on Akaike's Information Criterion (AIC; Akaike 1987), we then added the *species* variable (or in the case of intra-specific analyses, the *locality* variable). The *species/locality* variables were added either as a random intercept, a random intercept and slope, or a random smooth. In the following steps the remaining variables (female somatic dry mass and reproductive tissue dry mass) were added as a thin-plate spline smoother in a step-wise manner. In the last step of the analysis, the remaining variables were added as non-isotropic terms of interaction with stage of development (i.e. where the interacting variables are measured on different scales and with different units). Finally, to compare the effect between *locality* and *species* variables on mean embryo mass variation in the intraspecific analysis, we added species as a random intercept and slope because the model selection in the interspecific analysis indicated that this was the most plausible model. The most plausible model(s) was(were) chosen based on the Deviance information criterion (DIC; Spiegelhalter *et al.*, 2002), a generalized version of the AIC (Akaike, 1987), where the lowest value(s) indicated the most plausible model(s). The analyses were performed in RStudio [v.1.1.463 – 2009–2018 RStudio, Inc., under R v.3.5.2 (2018-12-20)] using the *rjags* (Plummer *et al.*, 2019) and *jagsUI* (Kellner, 2019) packages.

We also estimated a Matrotrophy Index (MI), which is the ratio of embryo mass at birth divided by the egg mass at fertilization (sensu Reznick *et al.*, 2002). We used stage 45 for the embryos' mass at birth and stage 0 for the egg mass at fertilization. MI characterizes the extent of post-fertilization maternal provisioning. In lecithotrophic species, which fully provision eggs before fertilization, MI is less than one because embryos lose dry mass during development. If the mother continues to provision embryos after fertilization, then MI can have values > 1. At the extreme, some species have values > 100.

RESULTS

INTER-SPECIFIC ANALYSIS

The values of mean embryo dry mass varied from 0.085 to 1.85 mg and showed an increasing trend when plotted against the developmental stages. The mass increment was non-linear, with a clear decline from fertilization through to stage 20–25 (stage 20 corresponds to the first appearance of eye pigment) (Fig. 1). The decline after stage 20–25 was common to all of the species and the curvature was very similar among species (Fig. 1, see modeling results below).
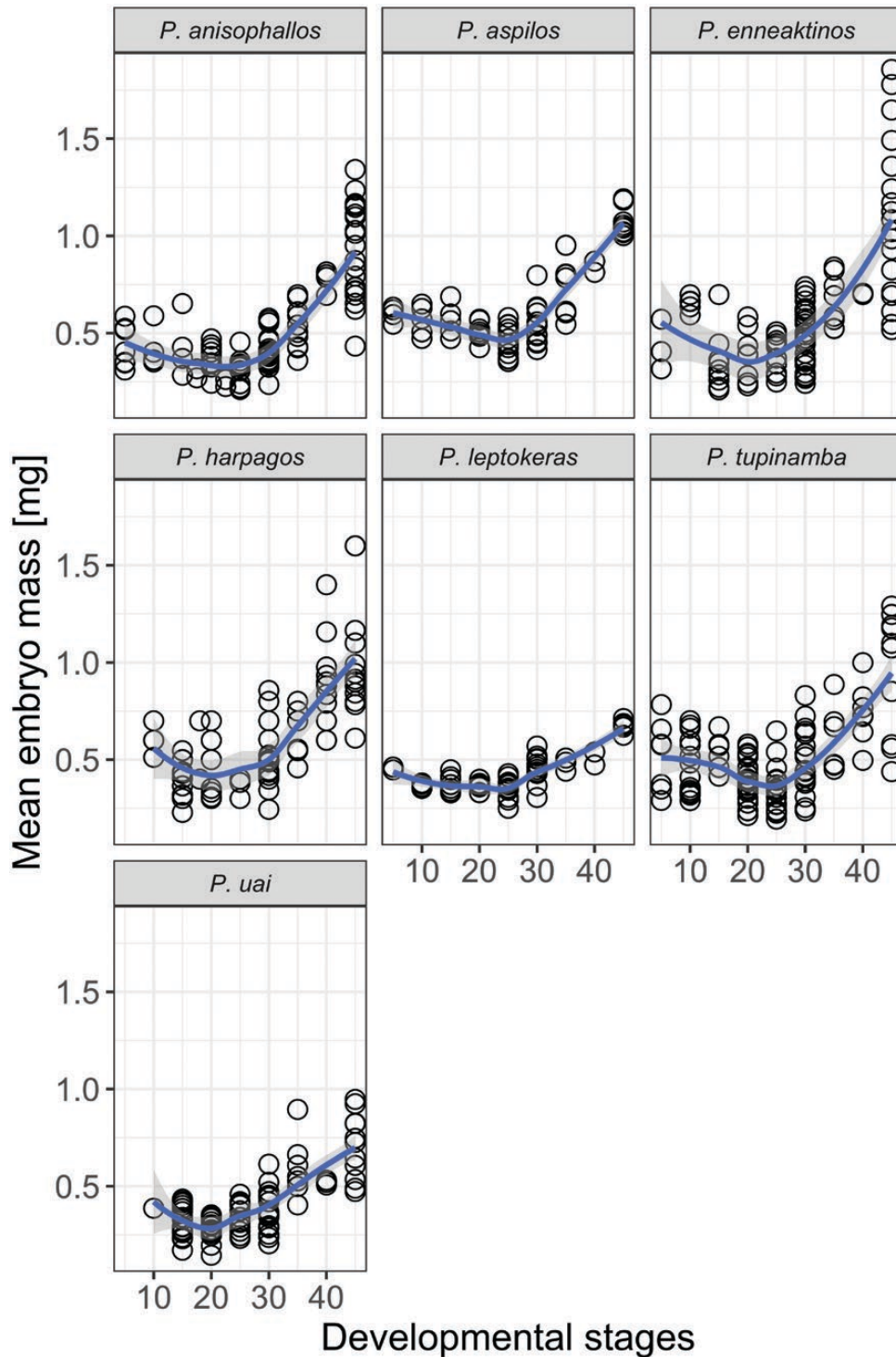
Figure 1. Mean embryo mass variation (mg) as a function of stage of development for the females of seven species of *Phalloceros* collected in the states of Rio de Janeiro, São Paulo and Minas Gerais, south-eastern Brazil. For details on definition of stages of development, see Reznick (1981) and Gorini-Pacheco *et al.* (2018). The curve represents a smoother on developmental stages, which differs among species. The grey shading represent 95% confidence intervals.
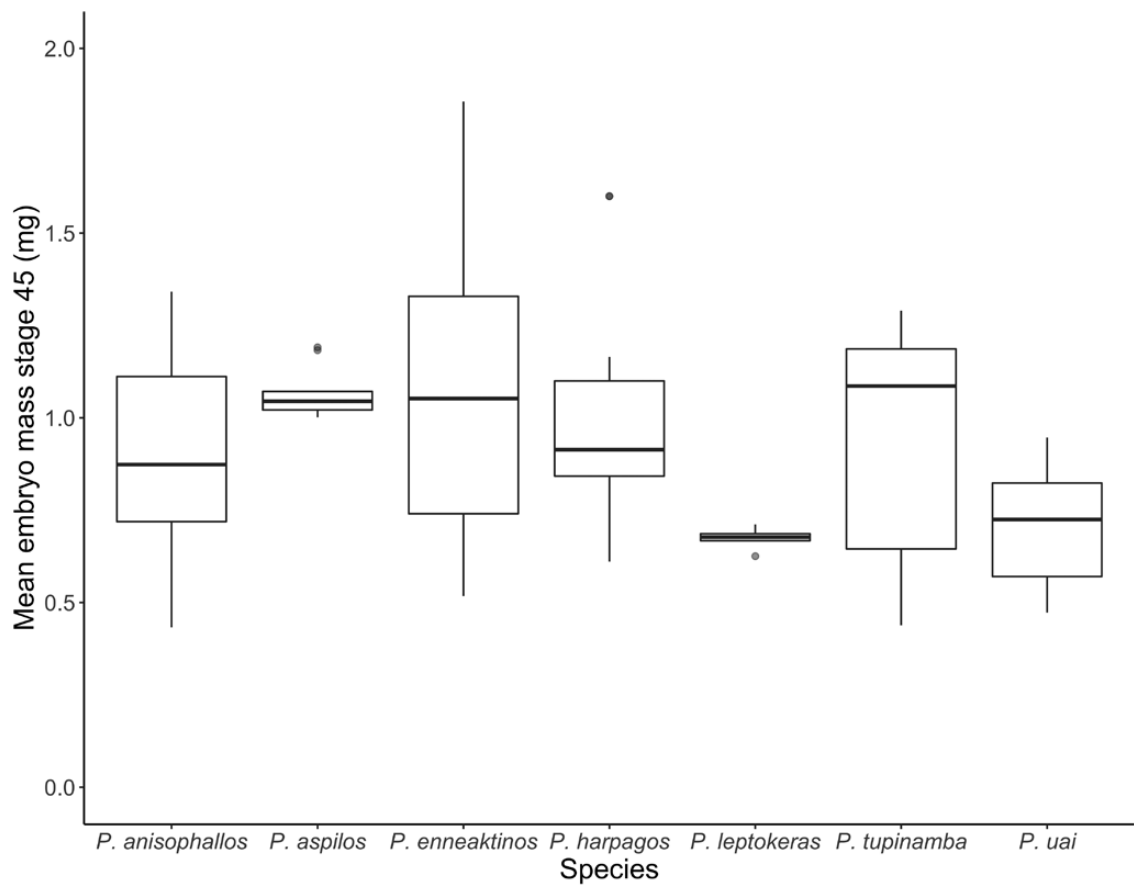
Figure 2. Mean embryo mass variation (mg) in seven species of *Phalloceros* collected in the states of Rio de Janeiro, São Paulo and Minas Gerais, south-eastern Brazil. Only values for developmental stage 45 are shown. The mid-line in the box represents the median, the outer margins represent the 25th and 75th percentiles while the whiskers are the 10th and 90th percentiles.

Table 2. Results of GAMM model selection procedure for inter-specific variation of mean embryo mass for seven species of *Phalloceros*: searching for the most plausible model to explain how mean embryo mass varied with the developmental stages. Bold indicates best models (with lowest AIC and highest R² values)

	Model selection results			
Stage of development	d.f.	AIC	GCV	R2
Null model	2	79.77	0.067	0
Linear	3	-168.67	0.044	0.346
Smooth (cubic regression spline)	**5**	**-417.83**	**0.029**	**0.574**
Smooth (thin-plate spline)	**5**	**-417.72**	**0.029**	**0.574**

(SD effect on mean embryo mass)

The difference in mean embryo mass among different stages of development was significant (ANOVA, F-value = 238.31, $P < 0.001$). The difference in embryo mass for developmental stage 45 among species was significant (ANOVA, F-value = 3.43, $P < 0.001$) which implies variation among species in the size at birth (Fig. 2). The post-hoc test indicated that the mean embryo mass for developmental stage 45 in *P. enneaktinos* was significantly larger than in *P. uai* and *P. leptokeras* (Fig. 2; Supporting Information, Table S1). The effect of added covariate (somatic dry mass, which represents the size of the mother) was not significant (ANCOVA, F-value = 1.0228, $P = 0.315$). The effect of species remained significant in this model (ANCOVA, F-value = 3.426, $P = 0.005$).

The model selection procedure indicated that mean embryo mass variation with stages of development was non-linear and could be best described using either a cubic regression spline or a thin-plate spline, since these two models revealed the lowest AIC value (Table 2,
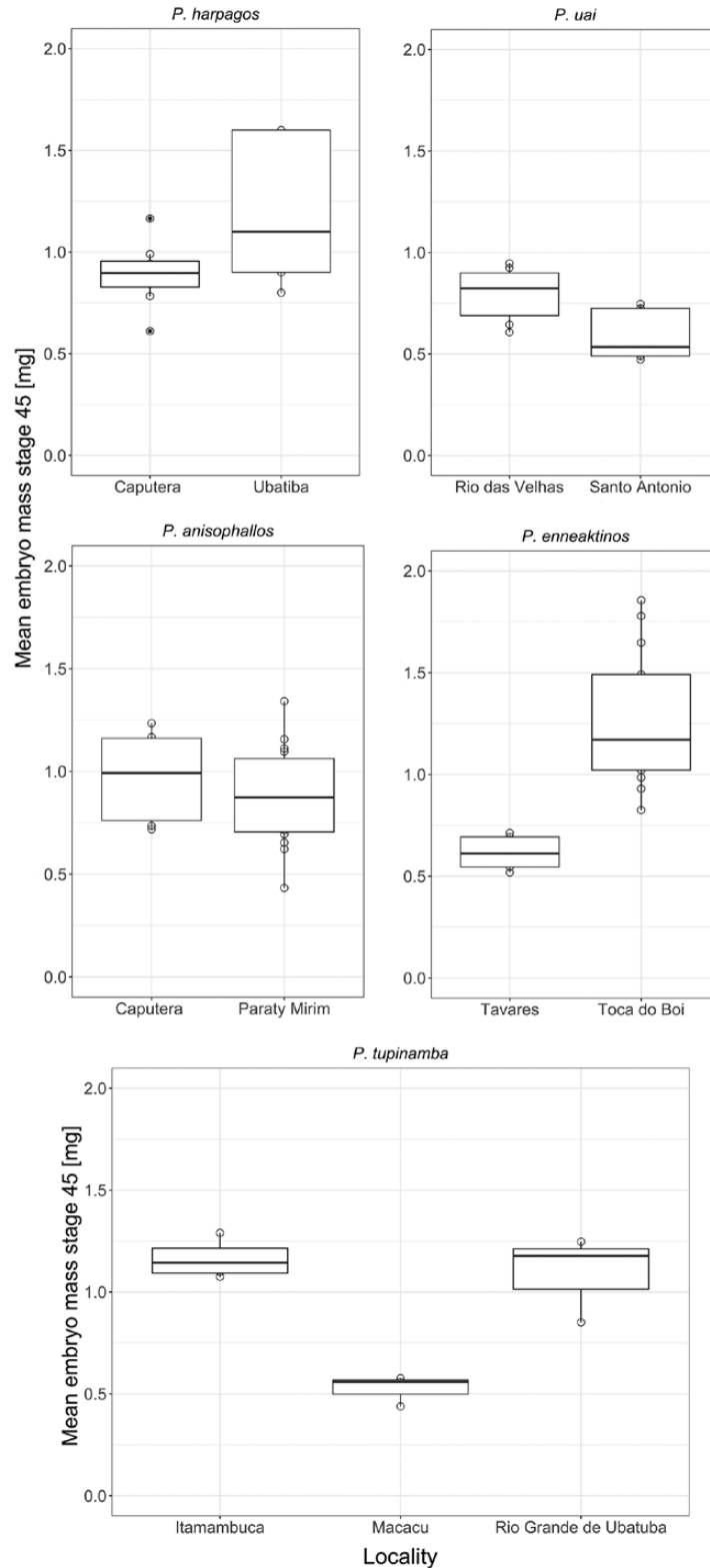
Figure 3. Intra-specific variation in mean embryo mass (mg) in different populations of five species of *Phalloceros* collected in the states of Rio de Janeiro, São Paulo and Minas Gerais, south-eastern Brazil. Only values for developmental stage 45 are shown. The mid-line in the box represents the median, the outer margins represent the 25th and 75th percentiles while the whiskers are the 10th and 90th percentiles.

models in bold, AIC = -417.83 for cubic regression spline and AIC = -417.72 for thin-plate spline model). Since the AIC values for the two models are so close, either model represents the data equally well. We chose the thin-plate spline model to be used in the subsequent steps of the analyses. The thin-plate spline model was preferred because it represents the nature of our data better, given its better depiction of smooth functions as compared to smoothers with a selected number of base knots (for details see Wood 2003).

We found that developmental stage, species and female body mass influenced the mean embryo mass (Fig. 1). The best-ranked model was one where mean embryo mass was expressed in terms of stage of development (as a thin-plate spline), species (as a random intercept and slope) and female somatic dry mass (as a thin-plate spine) (Supporting Information, Table S2, model in bold, DIC = -367.0). The second-best ranked model (containing somatic dry mass as a non-isotropic interaction with developmental stages) resulted in a DIC = -360.0, leaving the *delta*DIC high enough to distinguish the first ranked model as a single plausible model to explain the observed data. In conclusion, the way mean embryo mass changed during development, with a decline to a minimum value at stage 20–25 followed by an increase in mass, differed among species in the intercept as well as in the slope. Female somatic dry mass influenced the shape of the curvature in a non-linear way, but it did not affect embryo mass at birth, which was only affected by species identity (see ANCOVA results).

INTRA-SPECIFIC ANALYSIS

Mean embryo mass of stage 45 in five *Phalloceros* species that were collected on multiple locations varied considerably among locations for three of the five species, with the exception being *P. anisophallos* and *P. harpagos* (Fig. 3). *P. tupinamba* differed significantly in mean embryo

Table 3. Results of GAMM model selection procedure for intra-specific variation of mean embryo mass for four species of *Phalloceros*: searching for the most plausible model to explain how mean embryo mass varied with the stages of development. Bold indicates best models (with lowest AIC and highest R^2 values)

	Model selection results			
Stage of development	d.f.	AIC	GCV	R2
Null model	2	107.52	0.073	0
Linear	3	-93.54	0.048	0.352
Smooth (cubic regression spline)	**6**	**-284.95**	**0.032**	**0.573**
Smooth (thin-plate spline)	**6**	**-284.85**	**0.032**	**0.573**

SD effect on mean embryo mass

Table 4. Matrotrophy Index (MI) and average female somatic dry mass (g) for the seven species and the 11 populations studied. The MI was calculated as the average dry mass of stage 45 divided by the average dry mass of stage 0. *N* indicates sample size.

Species	Stage 0 (*N*)	Stage 45 (*N*)	MI	Average female somatic dry mass (N; max-min)
Phalloceros anisophallos	67	22	2.64	0.082 (95)
Caputeira	24	6	3.29	0.072 (36; 0.196-0.038)
Paraty Mirim	33	15	2.35	0.089 (59; 0.219–0.029)
Phalloceros enneaktinos	54	18	2.88	0.089 (91)
Tavares	28	5	2.28	0.113 (34; 0.217-0.015)
Toca do Boi	26	13	2.57	0.074 (57; 0.155-0.021)
Phalloceros harpagos	60	17	2.51	0.075 (77)
Caputeira	20	8	2.63	0.067 (46; 0.116-0.039)
Ubatiba	26	5	2.41	0.087 (31; 0.143-0.032)
Phalloceros tupinamba	69	10	2.36	0.071 (115)
Itamambuca	20	4	2.28	0.064 (39; 0.227-0.024)
Macacu	29	3	1.71	0.064 (45; 0.144-0.035)
Rio Grande—tributary	20	3	2.53	0.091 (31; 0.260-0.052)
Phalloceros uai	52	11	2.61	0.078 (85)
Rio das Velhas—tributary	28	6	2.50	0.103 (49; 0.214-0.025)
Santo Antônio—tributary	24	5	2.77	0.044 (36; 0.259-0.023)
Phalloceros aspilos	20	10	2.50	0.058 (66; 0.134-0.027)
Phalloceros leptokeras	28	6	1.52	0.093 (55; 0.179-0.041)

mass of stage 45 among the pairs of localities Macacu / Rio Grande de Ubatuba (P = 0.004) and Macacu / Itamambuca (P = 0.001, Fig. 3). *P. uai* differed significantly in mean embryo mass of stage 45 between the localities Rio das Velhas and Santo Antonio (F-value = 5.895, P = 0.038), similarly as in *P. enneaktinos* for localities Tavares and Toca do Boi (F-value = 18.247, P = 0.001, Fig. 3). For *P. harpagos* and *P. anisophallos*, the difference in mean embryo mass of stage 45 between localities was not significant (F = 4.245, P = 0.064 and F = 0.539, P = 0.472, respectively, Fig. 3). The effect of added covariate (female somatic dry mass) to the variable locality was not significant for any of the species, while the effect of locality remained significant after controlling for the female somatic dry mass covariate in the three species (ANCOVA, F-value for *P. tupinamba*: 18.493, P = 0.003; F-value for *P. uai*: 5.410, P = 0.048; F-value for *P. enneaktinos*: 18.147, P = 0.001).

The model selection procedure in the analysis that included all stages of development revealed that mean embryo mass in the five species analysed increased with increasing stage of development in a non-linear way, best explained by either a thin-plate spline or a cubic regression spline model (Table 3, models in bold, AIC = -284.95 for cubic regression spline and AIC = -284.85 for thin-plate spline model). We opted to use the thin-plate spline model for further analysis, because it represents the nature of our data better (for details see Wood, 2003). When further variables were added to the thin-plate spline model, the best-ranked model included a locality effect (as random intercept and slope), as well as a female somatic dry mass effect (as a thin-plate spline) (Supporting Information, Table S3, model in bold, DIC = -399.3). Thus, there was a difference in the embryonic growth function throughout development among localities and the curvature of this variation was affected by female somatic dry mass. However, female somatic dry mass did not affect differences between species in embryo size at birth (see ANCOVA results).

The results for all species and populations revealed that the mass of embryos at birth exceeds the mass of the eggs at fertilization, so the MIs always exceed 1. Among the 7 species, *P. leptokeras* showed the lowest MI value (1.52) and *P. enneaktinos* the highest (2.88) (Table 4; Fig. 1). Among the populations analysed, *P. anisophallos* from the Caputera locality showed the highest MI value recorded (3.29).

DISCUSSION

All 12 populations of *Phalloceros* showed a non-linear increase of embryo mass through gestation. In all species, embryo mass initially decreased until around stage 20–25 (Fig. 1) then steeply increased, ultimately resulting in offspring at birth that were approximately twice the mass of the egg at fertilization. This growth function is conserved across populations and species, even though size at birth can vary. Our results showed that the change in the mean embryo mass can be explained in terms of developmental stages, species and female body mass. The increase in dry mass after stage 20–25 observed in all species implies the presence of placentation, or the continuation of maternal provisioning after the egg is fertilized. Furthermore, the fitted curves show that there was a net gain of dry mass between fertilization and birth. Overall, the embryos increased in dry mass 1.5 to 3-fold from fertilization to birth, which is, however, relatively low in comparison to the range of values seen throughout the family (Pollux *et al.*, 2009).

The embryo mass at the end of gestation (stage 45) differed among species and populations, indicating that there is variation among populations and species in embryo size at birth. Size at birth is not influenced by female body mass. Other selective forces could be affecting local adaption within species, as observed in populations of *P. harpagos* and *P. anisophallos* under different predation pressure (Gorini-Pacheco *et al.*, 2018). In this species, as also observed in other poecilids [e.g. *Poecilia reticulata* Peters, 1859, Reznick & Endler, 1982; Zandonà *et al.*, 2011; *Brachyrhaphis rhabdophora* (Regan, 1908), Johnson & Belk, 2001; *Gambusia hubbsi* Breder, 1934 (= *Gambusia puncticulata* Poey, 1854); Riesch *et al.*, 2013], high predation pressure selects for smaller offspring size at birth. Other factors, such as density and resource availability, can also affect offspring size at birth (e.g. Jørgensen *et al.*, 2011). The twelve localities where we sampled our seven *Phalloceros* species differed in many biotic and abiotic characteristics, mostly in terms of fish community and *Phalloceros* density, all of which could have played some role in shaping the evolution of offspring size.

The most remarkable feature of these results is the nature of the function that describes the relationship between embryo mass and stage of development. In *Phalloceros*, embryos loose dry mass early in development, implying that they are sustained by nutrients stored in their relatively large eggs prior to fertilization. Post-fertilization provisioning, and increase in embryo dry mass, does not become evident until offspring development has progressed to the development of eye-pigment (stage 25). This growth function differs from all other placental species in this family. In all other published accounts to date, there is a monotonic increase in mass during development (Pollux *et al.*, 2009). The embryo growth curve in *Phalloceros* implies a mode of maternal provisioning that is different from all other placental Poeciliidae. In all well-studied poecilids, offspring develop within the maternal follicle (Turner, 1940). Those species that are placental tend to have follicles that are structurally distinct from

non-placental species because they are vascularized and lined with microvilli (Turner, 1940; Olivera-Tlahuel *et al.*, 2018). Although the mechanism of provisioning has not been well characterized in placental species, it presumably involves some form of direct absorption of maternal nutrients by the developing embryo, such as through the integument or the externalized pericardial membrane, portions of which have been described as microvillous and well adapted for absorption of nutrients (Grove & Wourms, 1991; Guernsey *et al.*, 2020). However, the limited comparative studies of the embryonic integument have yet to reveal consistent differences in the embryos of placental vs. non-placental species (Panhuis *et al.*, 2017).

The lag between fertilization and mass gain in *Phalloceros* species is reminiscent of what was found in some matrotrophic species in the family Zenarchopteridae (freshwater halfbeaks—genera *Nomorhamphus* Weber & de Beaufort, 1922 and *Dermogenys* Kuhl & van Hasselt, 1823), which shed the maternal follicle early in development, then complete development in the lumen of the ovary (Reznick *et al.*, 2007). They appear to acquire maternal nutrients by imbibing fluid in the ovary (Meisner & Burns, 1997), an alternative form of matrotrophy referred to as 'histophagy' (Crespi & Semeniuk, 2004). The embryos of these species gain little dry mass until they reach stages 20–30, then growth accelerates substantially, resulting in as much as a 200-fold increase in dry mass at birth. The timing of the acceleration appears to align with organogenesis and the development of the digestive tract (Meisner & Burns, 1997). A similar pattern of embryonic growth is seen in the one-sided livebearer *Jenynsia multidentata* (Jenyns, 1842) [currently a synonym of *Jenynsia lineata* (Jenyns, 1842), but more likely to represent *Jenynsia darwini* Amorim, 2018 based on geographic location], family Anablepidae. Development in this species is not intra-follicular and the embryos do not gain mass in the initial stage of development. There is a sharp increase in dry mass later in development, which leads to a greater than 60-fold mass gain of the clutch (López-Rodríguez *et al.*, 2017). The steep increase in dry mass corresponds to the intrusion of maternal tissues—highly vascularized ovarian folds—into the embryo's gills, providing nourishment to the developing offspring (López-Rodríguez *et al.*, 2017). Since the timing is the same in *Phalloceros*, it seems plausible that the unusual growth occurs via the mechanisms seen in the Zenarchopteridae or *Jenynsia* in spite of development being intra-follicular.

Anatomical, histological and functional studies are needed to shed light on the role of the maternal (follicles) and embryonic (e.g., pericardial membrane, yolk sacs, integument) components of the placenta in the transfer of nutrients throughout pregnancy in *Phalloceros*. These species are unique in that, in the initial stages of development, they behave like a lecithotrophic species, where embryos lose mass due to metabolic costs (Turner, 1940), then later they behave like a matrotrophic species and gain dry mass. The *Phalloceros* mode of maternal provisioning may reflect the diversity of maternal provisioning that is predicted by Crespi & Semeniuk (2004) if the placenta evolves as a by-product of mother-offspring conflict over resource provisioning.

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DATA AVAILABILITY

The data from this study are available to download from the Dryad digital repository (Zandonà *et al.*, 2021).

REFERENCES

Akaike H. 1987. Factor analysis and AIC. *Psychometrika* **52:** 317–332.

Arias AL, **Reznick D. 2000.** Life history of *Phalloceros caudiomaculatus*: a novel variation on the theme of livebearing in the family Poeciliidae. *Copeia* **2000:** 792–798.

Blackburn DG. 1992. Convergent evolution of viviparity, matrotrophy, and specializations for fetal nutrition in reptiles and other vertebrates. *American Zoologist* **32:** 313–321.

Crespi B, **Semeniuk C. 2004.** Parent-offspring conflict in the evolution of vertebrate reproductive mode. *The American Naturalist* **163:** 635–653.

Gorini-Pacheco B, **Zandonà E**, **Mazzoni R. 2018.** Predation effects on matrotrophy, superfetation and other life history traits in *Phalloceros harpagos*. *Ecology of Freshwater Fish* **27:** 442–452.

Greven H. 1998. Survey of the oviduct of salamandrids with special reference to the viviparous species. *Journal of Experimental Zoology* **282:** 507–525.

Grove BD, **Wourms JP. 1991.** The follicular placenta of the viviparous fish, *Heterandria formosa*. I. Ultrastructure and development of the embryonic absorptive surface. *Journal of Morphology* **209:** 265–284.

Guernsey MW, **Van Kruistum H**, **Reznick DN**, **Pollux BJA**, **Baker JC. 2020.** Molecular signatures of placentation and secretion uncovered in *Poeciliopsis* maternal follicles. *Molecular Biology and Evolution* **37:** 2679–2690.

Johnson JB, **Belk MC. 2001.** Predation environment predicts divergent life-history phenotypes among populations of the livebearing fish *Brachyrhaphis rhabdophora*. *Oecologia* **126:** 142–149.

Jørgensen C, **Auer SK**, **Reznick DN. 2011.** A model for optimal offspring size in fish, including live-bearing and parental effects. *The American Naturalist* **177:** E119–E135.

Kellner K. 2019. *jagsUI: a wrapper around 'rjags' to streamline 'JAGS' analyses.* R package version 1.5.1. https://CRAN.R-project.org/package=jagsUI.

Long JA, **Trinajstic K**, **Young GC**, **Senden T. 2008.** Live birth in the Devonian period. *Nature* **453:** 650–652.

López-Rodríguez NC, **De Barros CM**, **Petry AC. 2017.** A macroscopic classification of the embryonic development of the one-sided livebearer *Jenynsia multidentata* (Teleostei: Anablepidae). *Neotropical Ichthyology* **15:** 1–10.

Lucinda PHF. 2008. Systematics and biogeography of the genus *Phalloceros* Eigenmann, 1907 (Cyprinodontiformes: Poeciliidae: Poeciliinae), with the description of twenty-one new species. *Neotropical Ichthyology* **6:** 113–158.

Meisner AD, **Burns JR. 1997.** Viviparity in the halfbeak genera *Dermogenys* and *Nomorhamphus* (Teleostei: Hemiramphidae). *Journal of Morphology* **234:** 295–317.

Mossman HW. 1991. Comparative morphogenesis of the fetal membranes and accessory uterine structures. *Placenta* **12:** 1–5.

Olivera-Tlahuel C, **Moreno-Mendoza NA**, **Villagrán-Santa Cruz M**, **Zúñiga-Vega JJ. 2018.** Placental structures and their association with matrotrophy and superfetation in poeciliid fishes. *Acta Zoologica* **100:** 167–181.

Ostrovsky AN, **Lidgard S**, **Gordon DP**, **Schwaha T**, **Genikhovich G**, **Ereskovsky AV. 2016.** Matrotrophy and placentation in invertebrates: a new paradigm. *Biological Reviews* **91:** 673–711.

Panhuis TM, **Fris M**, **Tuhela L**, **Kwan L. 2017.** An examination of surface epithelium structures of the embryo across the genus *Poeciliopsis* (Poeciliidae). *Journal of Morphology* **278:** 1726–1738.

Plummer M. **2019.** *rjags: bayesian graphical models using MCMC*. R package version 4-10. https://CRAN.R-project.org/package=rjags.

Pollux BJA, **Meredith RW**, **Springer MS**, **Garland T**, **Reznick DN. 2014.** The evolution of the placenta drives a shift in sexual selection in livebearing fish. *Nature* **513:** 233–236.

Pollux BJA, **Pires MN**, **Banet AI**, **Reznick DN. 2009.** Evolution of placentas in the fish family Poeciliidae: an empirical study of macroevolution. *Annual Review of Ecology, Evolution, and Systematics* **40:** 271–289.

Reznick D. 1981. 'Grandfather effects': the genetics of interpopulation differences in offspring size in the mosquito fish. *Evolution* **35:** 941–953.

Reznick D, **Endler JA. 1982.** The impact of predation on life history evolution in Trinidadian guppies (*Poecilia reticulata*). *Evolution* **36:** 160–177.

Reznick D, **Meredith R**, **Collette BB. 2007.** Independent evolution of complex life history adaptations in two families of fishes, live-bearing halfbeaks (Zenarchopteridae, Beloniformes) and Poeciliidae (Cyprinodontiformes). *Evolution* **61:** 2570–2583.

Reznick DN, **Mateos M**, **Springer MS. 2002.** Independent origins and rapid evolution of the placenta in the fish genus *Poeciliopsis*. *Science* **298:** 1018–1020.

Riesch R, **Martin RA**, **Langerhans RB. 2013.** Predation's role in life-history evolution of a livebearing fish and a test of the Trexler–DeAngelis model of maternal provisioning. *The American Naturalist* **181:** 78–93.

Souto-Santos ICDA, **Ferraro GA**, **Jennings WB**, **Vergara GLDS**, **Buckup PA. 2019.** Geographic distribution of *Phalloceros* Eigenmann, 1907 (Cyprinodontiformes, Poeciliidae) in the Ilha Grande bay hydrographic region, Rio de Janeiro, Brazil. *Check List* **15:** 181–192.

Spiegelhalter DJ, **Best NG**, **Carlin BP**, **van der Linde A. 2002.** Bayesian measures of model complexity and fit. *Journal of the Royal Statistical Society: Series B (Statistical Methodology)* **64:** 583–639.

Thibault RE, **Schultz RJ. 1978.** Reproductive adaptations among viviparous fishes (Cyprinodontiformes: Poeciliidae). *Evolution* **32:** 320–333.

Thomaz AT, **Carvalho TP**, **Malabarba LR**, **Knowles LL. 2019.** Geographic distributions, phenotypes, and phylogenetic relationships of *Phalloceros* (Cyprinodontiformes: Poeciliidae): insights about diversification among sympatric species pools. *Molecular Phylogenetics and Evolution* **132:** 265–274.

Trivers RL. 1974. Parent-offspring conflict. *Integrative and Comparative Biology* **14:** 249–264.

Turner CL. 1940. Pseudoamnion, pseudochorion, and follicular pseudoplacenta in poeciliid fishes. *Journal of Morphology* **67:** 59–89.

Wood SN. 2003. Thin plate regression splines. *Journal of the Royal Statistical Society: Series B (Statistical Methodology)* **65:** 95–114.

Wourms JP, **Grove BD**, **Lombardi J. 1988.** The maternal-embryonic relationship in viviparous fishes. *Fish Physiology* **11:** 1–134.

Zandonà E, **Auer SK**, **Kilham SS**, **Howard JL**, **López-Sepulcre A**, **O'Connor MP**, **Bassar RD**, **Osorio A**, **Pringle CM**, **Reznick DN. 2011.** Diet quality and prey selectivity correlate with life histories and predation regime in Trinidadian guppies. *Functional Ecology* **25:** 964–973.

Zandonà E, **Kajin M**, **Buckup P**, **Amaral J**, **Souto-Santos I**, **Reznick D. 2021.** Mode of maternal provisioning in the fish genus *Phalloceros*: a variation on the theme of matrotrophy. *Dryad, Dataset.* https://doi.org/10.5061/dryad.t76hdr80z.

Zuur A, **Ieno EN**, **Walker N**, **Saveliev AA**, **Smith GM. 2009.** Mixed effects models and extensions in ecology with R. Springer Science & Business Media.

SUPPORTING INFORMATION

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

Figure S1. Map of the sampled localities for the seven species studied.

Table S1. Post-hoc test with Bonferroni adjustment indicating which *Phalloceros* species presented significantly different mean embryo weights in developmental stage 45.

Table S2. Results of GAMM model selection procedure for inter-specific variation of mean embryo weight for seven species of *Phalloceros*: searching for the most plausible model to explain how mean embryo weight varied with the remaining variables after the *mean embryo weight ~ developmental stages* relation had been fixed. The most plausible models are in bold. df - degrees of freedom; AIC - Akaike's Information Criterion; GCV - generalized cross-validation; R2 - model fit.

Table S3. Results of GAMM model selection procedure for intra-specific variation of mean embryo weight for four species of *Phalloceros*: searching for the most plausible model to explain how mean embryo weight varied with the remaining variables after the *mean embryo weight ~ developmental stages* relation had been fixed. The most plausible models are in bold. df - degrees of freedom; AIC - Akaike's Information Criterion; GCV - generalized cross-validation; R2 - model fit.

Table S4. Characteristics of the localities where *Phalloceros* samples were collected for this study.