INDEPENDENT EVOLUTION OF COMPLEX LIFE HISTORY ADAPTATIONS IN TWO FAMILIES OF FISHES, LIVE-BEARING HALFBEAKS (ZENARCHOPTERIDAE, BELONIFORMES) AND POECILIIDAE (CYPRINODONTIFORMES)

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We have previously documented multiple, independent origins of placentas in the fish family Poeciliidae. Here we summarize similar analyses of fishes in the family Zenarchopteridae. This family includes three live-bearing genera. Earlier studies documented the presence of superfetation, or the ability to carry multiple litters of young in different stages of development in the same ovary, in some species in all three genera. There is also one earlier report of matrotrophy, or extensive postfertilization maternal provisioning, in two of these genera. We present detailed life-history data for approximately half of the species in all three genera and combine them with the best available phylogeny to make inferences about the pattern of life-history evolution within this family. Three species of *Hemirhamphodon* have superfetation but lack matrotrophy. Most species in *Nomorhamphus* and *Dermogenys* either lack superfetation and matrotrophy or have both superfetation and matrotrophy. Our phylogenetic analysis shows that matrotrophy may have evolved independently in each genus. In *Dermogenys*, matrotrophic species produce fewer, larger offspring than nonmatrotrophic species. In *Nomorhamphus*; matrotrophic species instead produce more and smaller offspring than lecithotrophic species. However, the matrotrophic species in both genera have significantly smaller masses of reproductive tissue relative to their body sizes. All aspects of these results are duplicated in the fish family Poeciliidae. We discuss the possible adaptive significance of matrotrophy in the light of these new results. The two families together present a remarkable opportunity to study the evolution of a complex trait because they contain multiple, independent origins of the trait that often include close relatives that vary in either the presence or absence of the matrotrophy or in the degree to which matrotrophy is developed. These are the raw materials that are required for either an analysis of the adaptive significance of the trait or for studies of the genetic mechanisms that underlie the evolution of the trait.

KEY WORDS: convergence, convergent evolution, evolution of complex traits, matrotrophy, placenta, Poeciliidae, superfetation, Zenarchopteridae.

The evolution of the same traits in different lineages offers an enhanced opportunity to study adaptation because it provides replicates in a natural experiment. These replicates in turn provide the raw material for comparative analyses that can implicate the agents of selection, lead to more refined tests of hypotheses and, in some cases, facilitate the analysis of the genetics of adaptation. The collected works on the evolution and developmental genetics of sticklebacks that have invaded freshwater environments serve

well as an example of the creative exploitation of such independent evolutionary events (Bell et al. 1993; Schluter and McPhail 1993; Bourgeois et al. 1994; Schluter 1994, 1995; Shapiro et al. 2004; Colosimo et al. 2005). Here we discuss the evolution of similar complex life-history adaptations between two families of atherinomorph fishes from different orders found on opposite sides of the earth. Fishes in the family Zenarchopteridae have evolved derived life-history traits multiple times and in similar ecological circumstances to the evolution of the same traits in the family Poeciliidae. The Zenarchopteridae are in the order Beloniformes and are found in southeast Asia and the Malay Archipelago whereas the Poeciliidae are in the order Cyprinodontiformes and are found primarily in the Neotropics. These derived traits include multiple origins of matrotrophy (postfertilization maternal provisioning, as in placental mammals) and the ability to carry multiple litters of young in different stages of development in the same ovary (superfetation). Such repeated evolution, with close relatives in all cases that lack such traits, affords an expanded opportunity to study how and why such complex traits evolved. The diversity of modes of reproduction in the Poeciliidae has been described earlier (Reznick and Miles 1989; Reznick et al. 2002); here we detail the Zenarchopteridae.

The evolution of the placenta is of particular interest because it represents a complex of maternal adaptations to provide nutrients to the developing young and embryonic adaptations to absorb them. It has been argued in the case of humans and other placental mammals that it is a frontier for intergenomic conflict (Haig 1993) between the mother and developing young, as evidenced by phenomena including the imprinting of genes associated with resource allocation and acquisition or the diverse mechanisms that resolve immunological incompatibility. Placentas have evolved in four classes of vertebrates in addition to the mammals (Reptilia, Amphibia, Osteichthys, and Chondrichthys) (Wourms et al. 1988; Wake and Dickie 1998; Blackburn 1999; Hamlett 1999) and a diversity of invertebrates (Campiglia and Walker 1995; Hart et al. 1997; Korniushin and Glaubrecht 2003; Korneva 2005) if we accept Mossman's (1937) generic definition of the placenta as an intimate apposition of maternal and embryonic tissues that sustains embryos through development.

Because placentas are so widespread, one might expect there to be considerable theory and speculation about the adaptive significance of placentas, yet there has been little consideration of the circumstances that might favor their evolution. One index of the lack of such a consideration is that they are barely mentioned in the three book-length reviews of life-history evolution (Roff 1992, 2002; Stearns 1992). It has also been suggested that placentas are not ecological adaptations and are instead a byproduct of the intergenomic conflict that arises after the evolution of viviparity (Crespi and Semeniuk 2004). Having multiple, independent origins of placentas and close relatives that lack them can provide

the raw material that is needed not only to address how and why placentas evolved, but also to provide a paradigm for addressing a more general issue, which is how what Darwin (1859) referred to as "organs of extreme perfection" evolve.

STUDY SPECIES

We report on life-history analyses of approximately half of the species in three genera that are a monophyletic subset of the family Zenarchopteridae (*Hemirhamphodon*, *Dermogenys*, and *Nomorhamphus*) (Jamieson and Grier 1993; Collette 2004; Lovejoy et al. 2004; Aschliman et al. 2005). All but one species in these three genera bear live young. The one egg layer (Bork and Mayland 1998) is *Hemirhamphodon tengah*. This family includes a fourth, egg-laying genus, *Zenarchopterus* and a fifth genus, *Tondanichthys*, which is known only from immature individuals, so its mode of reproduction is unknown (Collette 1995). The three live-bearing genera are found in brackish and freshwater environments in southeast Asia and the Malay Archipelago, whereas most species of *Zenarchopterus* are found in marine environments. Morphological traits that unite the four genera of Zenarchopteridae that are known from adults include the anal fin of males being modified as an "andropodium" and sperm that are packaged into a bundle known as spermatozeugmata (Grier and Collette 1987; Jamieson and Grier 1993). The andropodium may facilitate internal fertilization in the three live-bearing genera and appears to serve as a clasper during fertilization in some species of *Zenarchopterus* (Kottelat and Lim 1999); internal fertilization is a necessary precursor to the evolution of viviparity. All species in the sister clades to the Zenarchopteridae are egg layers and most occupy marine environments, which suggests that live bearing is a derived trait of these three genera that evolved when their common ancestor invaded a freshwater environment.

Meisner and Burns (1997), as part of their revision of the genera *Nomorhamphus* and *Dermogenys*, presented evidence that species vary in their patterns of maternal provisioning based on the ratio of the dry mass of late-term embryos divided by dry mass of early-term embryos. Some species registered weight losses during development, so that this ratio was less than one, whereas others had weight gains, which suggests that mothers continued to provision young after fertilization. Species that fully provision eggs prior to fertilization are referred to as "lecithotrophic" (yolk feeding) and typically lose 30–40% of their dry mass during development, which corresponds to the amount of weight loss seen between fertilization and birth in egg-laying species (Wourms et al. 1988). Species that continue to provision young after fertilization, resulting in weight gains during development, are referred to as "matrotrophic" (mother feeding). Meisner and Burns (1997) found that both genera had species with and without matrotrophy.

A second variation on the theme of live bearing seen in these fish is "superfetation," or the ability to carry multiple

litters of young in discretely different stages of development in the same ovary (Wourms 1981). In the Poeciliidae, there is a strong but not strict association between the presence of superfetation and matrotrophy, so much so that it was formerly suggested that they were two manifestations of the same trait (Constanz 1989). Trexler and DeAngelis (unpubl. ms.) have extended their earlier theory for the evolution of matrotrophy (Trexler and DeAngelis 2003) by showing that the prior existence of superfetation can facilitate the subsequent evolution of matrotrophy. It is thus of interest to know whether there is a similar association between these traits in the Zenarchopteridae. An unusual form of super-fetation was originally described by Soong (1968) and further detailed by Roberts (1989) and Brembach (1991) for *Hemirham-phodon pogonognathus*. The developing ova and embryos are ar-rayed single-file in long, narrow ovaries. Yolking ova are found at the anterior end of the ovaries, followed by embryos that are early in development, then by embryos that are progressively more ad-vanced in development toward the posterior of the ovary. There are generally only one or two embryos in the same stage of devel-opment. Brembach observed that these fish gave birth to one to four offspring at a time with only a few days between successive litters, so the ovary is like a linear assembly line and new ova are fertilized in a nearly continuous fashion, presumably with stored sperm. The more usual form of superfetation is for there to be a small number of litters that occupy discrete portions of an ovoid ovary. Meisner and Burns (1997) found species with and without superfetation in both *Dermogenys* and *Nomorhamphus*. The trend was for superfetation to be absent in lecithotrophic species and present in matrotrophic species.

The three genera of livebearers thus exhibit two additional derived life-history traits-–superfetation and matrotrophy. In this regard, they display a diversity of modes of reproduction that is similar to that seen in the Poeciliidae, which also appear to share live bearing, superfetation, and matrotrophy as derived life-history traits relative to their closest relatives in their order (Turner 1937, 1940b; Scrimshaw 1944, 1945; Reznick and Miles 1989). The two families also share similar ecologies, because both are rela-tively small fish that are found in brackish water, lakes, streams, and rivers.

Here we present an analysis of the patterns of mass change of embryos during development and the association between ma-trotrophy and superfetation. We also characterize other features of the life history, including offspring size, fecundity, and repro-ductive allocation, which is a measure of the total quantity of reproductive tissues relative to body size. We then evaluate the association between matrotrophy, superfetation, and these other components of the life history to determine whether their evolu-tion is associated with the evolution of traits such as offspring size or fecundity; such associations potentially yield clues to why these derived features of the life history evolve. Finally, we present a formal phylogenetic analysis of the distribution of these derived features of the life history that can reveal when and how often they evolved.

Methods
STUDY MATERIALS
We follow the classification of Meisner (2001) as our source of species designations. We were able to get sufficient data from five of the ten species of *Dermogenys*, seven of the 13 species of *Nomorhamphus* and three of the six species of *Hemirhamphodon* to characterize their patterns of maternal provisioning, the pres-ence or absence of superfetation, and other features of the life his-tory (see Table 1 and online Supplementary materials for details on museum collection numbers and locality information). We also report on data from four populations of *Dermogenys orientalis*. We dissected between three and 35 females from each collection and found that between three and 26 of these individuals were gravid. In one case, (*H. pogonognathus*) we only had access to a single gravid female but, because of the unusual form of super-fetation in this species, were able to make inferences about the mode of reproduction from a single dissection. We also obtained dissection data on small numbers of individuals from four addi-tional species of *Nomorhamphus*; although the sample sizes were insufficient to fully characterize their life histories, we include whatever summary data we were able to obtain for them.

DISSECTIONS
All methods follow those of Reznick (1981). Our measurements included the standard length of the female, measured from the tip of the upper jaw to the outer margin of the hypural plate, then the number, stage of development, and dry mass of any offspring that were present. Staging followed the developmental series summa-rized in Reznick (1981) and Haynes (1995). We also dried and weighed the remaining reproductive tissues, which consist pri-marily of the ovary and yolking ova. Stage of development was converted into a numerical score that ranged from 0 (yolked egg, no development) to 45 (fully developed embryo, ready to be born). Stage 50 represents a newborn embryo. These same numerical scores were used previously and generated a straight line with a negative slope in lecithotrophic species and a positive, exponen-tial growth curve in matrotrophic species in the family Poeciliidae (Reznick 1981; Reznick et al. 2002), so they serve well as a basis for making statistical comparisons among species in the patterns of mass change during development.

STATISTICS
Patterns of maternal provisioning were inferred from the relation-ship between the mean dry mass of developing embryos and their stage of development. Models were fit with the GLM procedure

Table 1. Summary of collection and life-history data for all samples included in our analyses. Variable definitions: Species, with N. = *Nomorhamphus*, H. = *Hemirhamphodon* and D. = *Dermogenys*. Cat. # = Catalog number of each collection. Collection acronyms: ZMH = Zoological Museum, Universitat Hamburg, CMK = C.M Kottelat private collection, SMF = Natur-Museum und /Forschungs-Institut, Senckenberg, USNM = National Museum of Natural History, SU = Stanford University, CAS = California Academy of Sciences. #Dissected = total number of dissected females, #Gravid = subset of those dissected that were carrying developing embryos. Min. Size Gravid = minimum size gravid female (mm). Mean Size = mean size of gravid females (mm). Ovary(mg) = total reproductive dry mass, which is the sum of the dry mass of all developing litters of young and all other reproductive tissues. MI = the "Matrotrophy Index," which equals the estimated mass of young at stage 45, which just precedes birth, divided by the estimated mass of the egg at fertilization. #Litters = the average number of developing litters of young in gravid females. #Embs/Litter = the average number of embryos per litter. Mass St.0(mg) = the estimated mass of an egg at fertilization. Mass St. 45 = the estimated mass of an advanced embryo that is approaching birth.

Zenarchopteridae data

Species	Cat. #	#Dissected	#Gravid	Min. size Gravid	Mean size	Ovary (mg)	MI	#Litters	#Embs/ Litter	Mass St.0(mg)	Mass St.45
N. liemi	ZMH 7157	5	2	.	62.2	.	.	1	.	.	.
N. kolonodalensis	CMK 7576	8	6	.	65.4	58.8	0.66	1	7.2	8.93	5.88
N. celebensis	CMK 8013	6	2	57.1	68.4	.	.	1	9.5	.	.
N. towoetii	CMK6187	4	2	53.2	56.7	.	.	1	10.5	.	.
N. hageni	SMF 6522	8	3	64.5	73.1	.	.	1	11.3	.	.
N. brembachi	ZMH 7616	7	5	56.2	63.9	8.5	11.4	1.8	8.2	0.14	1.6
N. rossi	USNM 363187	21	13	59.7	71.2	48.9	22	1.8	18.2	0.2	4.4
N. manifesta	SU29706	15	11	42.3	50.8	8.9	15.8	1.7	7.1	0.12	1.9
N. ebrardtii	ZMH7150	3	1	.	51.2	.	.	1	5	.	.
N. weberi	CMK6196A	21	15	55.6	60.7	31.8	0.77	1	2.1	14.92	11.56
N. megarrhamphus	CMK6234	9	6	73.2	79.4	107.4	0.84	1	6.5	12.89	10.78
N. bakeri	USNM 138667	17	5	44.7	51.2	7.9	3.44	1	15.6	0.3	1.03
N. vivipara	SU37829	30	18	37	42.6	23.2	0.79	1	12.7	1.47	1.16
H. pogonognathus	USNM 313957	1	1	.	44.3	.	0.64	6	2.7	0.9	0.57
H. kuekenthali	USNM 330839	35	26	38	46.9	.	0.58	5.5	3.5	1.14	0.66
H. kapuasensis	USNM 313653	3	3	43.8	45.8	.	0.61	6.3	3.2	1.22	0.75
D. sumatrana	USNM 306569	5	5	40.3	48	6.4	198.5	4	2.7	0.02	3.97
D. orientalis	CMK6147	8	8	37.2	43.3	12.42	16.46	2.4	14.2	0.07	1.1
D. orientalis	ZMH 7147	4	4	51	56.1	15.32	22.98	2.3	10.2	0.07	1.49
D. orientalis	CMK6102	4	3	36.5	42.3	7.1	16	2	10.4	0.05	0.81
D. orientalis	CMK6109	9	8	41.7	50.4	10.36	17.97	2.5	5.9	0.09	1.6
D. siamensis	USNM 119517	17	11	30.6	39.3	21.5	0.64	1	36.6	0.61	0.39
D. burmanica	CAS 134945	23	15	38.9	46.9	16.7	0.67	1	19	1.05	0.67
D. bispina	USNM 345500	9	6	36.1	41.2	2.7	152	2.5	3.4	0.014	2.13

(SAS Institute 1999), then the quality of the fit of a given model was based upon an evaluation of the residuals, using the Univariate Procedure. The fit was evaluated with a combination of tests for the normality of the distribution of residuals, the appearance of the plot of residual versus predicted values, and the normal probability plot. These combined evaluations reveal whether the model represents a good fit to the relationship between the mean dry mass of embryos and their stage of development. In species that are lecithotrophic or that have small amounts of maternal provisioning, this relationship is best described with a straight line that has a slope that ranges from negative (lecithotrophic) to positive (low levels of matrotrophy). In species in the family Poeciliidae that have more extensive matrotrophy, this relationship can be best described with either a quadratic or exponential model. In many species in the current study, the best description turned out to be a broken regression, with one straight line fitted to early developmental stages and a second to late developmental stages. In this case, the regressions were fit with the regression procedure available in SYSTAT (SPSS 1998).

The pattern of maternal provisioning was represented with the "Matrotrophy Index" (MI), which is the ratio of the estimated dry mass of offspring at birth divided by the estimated dry mass of eggs at fertilization, as estimated from the regression models for the relationship between stage of development and mean embryo dry weight. We used the criteria of Reznick et al. (2002), who employed a *t*-statistic to assess whether the observed MI value

was significantly greater than 0.7. The choice of 0.7 is arbitrary because we cannot assume that an absence of post-fertilization maternal provisioning will always be associated with a 30% loss in dry mass between fertilization and birth, but it provides a frame of reference for making comparisons among species. In practice, all values of MI in the current dataset that were less than 1 were also not significantly different from 0.7. These species were classified as "lecithotrophic" in all analyses whereas those with MI values greater than 1 were classified as matrotrophic, although the extent of post-fertilization maternal provisioning was highly variable among species in the latter category.

All subsequent univariate analyses were based on mean values per species or collection. We included only one, randomly selected, population of *D. orientalis* in these analyses because all four populations had very similar life-history traits and are not independent of one another. We evaluated the relative allocation of resources to reproduction (sum of dry mass of developing embryos and the dry mass of other reproductive tissues), the number of offspring per litter and the estimated size of offspring at birth (stage 45 in Table 1) as a two-way analyses with genus and mode of maternal provisioning as fixed effects. The mean length of females in a collection was evaluated as a covariate (GLM Procedure; [SAS Institute 1999]) and included if the assumptions of the analysis of covariance were met and if it then accounted for significant variation. In all cases we evaluated the assumptions of normality and heteroscedasticity of the residuals. We log transformed the values of some dependent variables to meet these assumptions. The means that are reported for these analyses are the least square means derived from these analyses. If the analyses were performed on log-transformed data, then the least square means were derived from the equivalent analysis on untransformed data. The tests of significance are always derived from the Type III sums of squares, which compensate for the inequality of observations in the four cells. Only the genera *Nomorhamphus* and *Dermogenys* were included in these analyses because they both had species with and without matrotrophy whereas all three species of *Hemirhamphodon* lacked matrotrophy.

We recognize that species are not necessarily independent of one another and that an analysis that incorporates phylogeny would be more appropriate than a simple two-way (genus × mode) analysis; the available phylogeny lacks sufficient resolution to justify such an approach (see Results). Because of the potential lack of independence among species, the probabilities associated with these statistics cannot be taken literally; the statistics serve instead as heuristic tool to illustrate trends in the data. Analyzing our results as two-way analysis of variances (ANOVAs) and analysis of covariances (ANCOVAs) facilitates comparisons because we are able to include size as a covariate and generate least square means that incorporate the effects of the covariate and compensate for differences among cells in sample sizes.

PHYLOGENETIC ANALYSES

We used the morphological data matrix in Meisner (2001) to construct a phylogeny and hence infer the possible patterns of evolution of derived life-history states (viviparity, matrotrophy, superfetation) in the family. We deleted Meisner's characters 40–44 from her 45 character dataset because they are highly correlated with the presence or absence of matrotrophy and superfetation. Because our goal is to evaluate the evolution of these traits, it is necessary that the phylogeny be independent of them. Furthermore, in the Poeciliidae, matrotrophy and superfetation are known to have evolved independently among closely related species (Reznick and Miles 1989; Reznick et al. 2002) so their utility in phylogenetic reconstruction is questionable. The new dataset consisted of the remaining 40 characters comprising 28 taxa (10 *Dermogenys* species, 13 *Nomorhamphus* species, and five outgroups). All characters were treated as equally weighted, unordered, and analyzed using the branch and bound algorithm as implemented in PAUP (version 4.0b10) (Swofford 2002). Matrotrophy indices were plotted onto the branch and bound strict consensus tree to look at their distribution within the family. *Nomorhamphus bakeri* was not included in the phylogenetic analysis because of the large number of uncoded characters in Meisner (2001). We include it in Figure 4 as an unresolved lineage within the *Nomorhampus* clade.

We were able to enhance the sample size in these analyses by including species from Meisner and Burns (1997), which were not included in our study. For a subset of their species, they evaluated the ratio of the mass of embryos that were advanced in development divided by the mass of embryos that were early in development. These ratios will tend to underestimate the value of the matrotrophy index in matrotrophic species and overestimate the index for lecithotrophic species because they will not necessarily capture the beginning and end of the process of development and hence will not capture the full extent of weight gain or weight loss during development. These results are also reported without sample sizes, so we cannot know how representative they are of a population as a whole, as opposed to being influenced by variation among individual mothers in offspring size. Nevertheless, their data enabled us to add four species to the analysis, because they either registered substantial losses in dry mass (lecithotrophy) or increases in dry mass (matrotrophy) during development. We report on the details of these added species in our online Supplementary materials.

Results

PATTERNS OF MATERNAL PROVISIONING

All three species of *Hemirhamphodon* and some species of *Dermogenys* and *Nomorhamphus* had embryos that progressively lost dry mass during development and hence had matrotrophy indices that were less than 1 (examples in Figs. 1, 2). In all cases, the
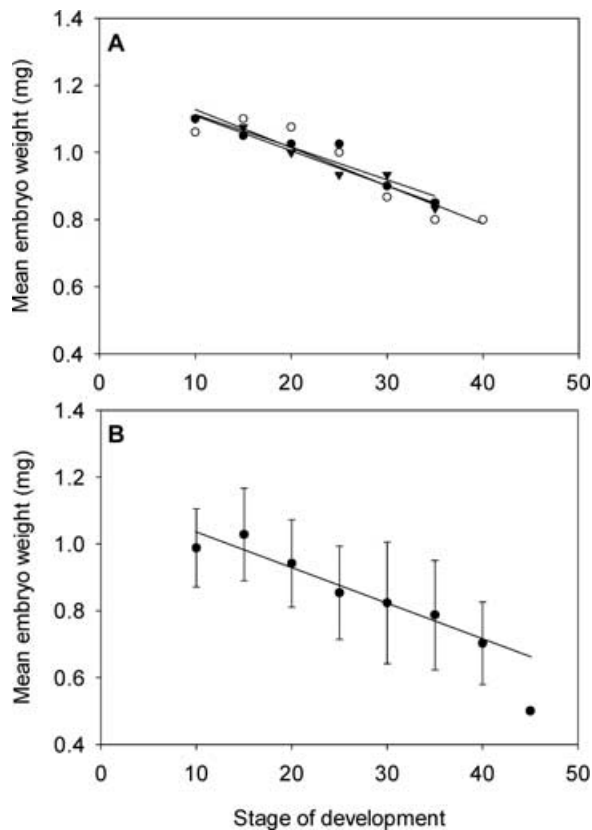
Figure 1. Patterns of change in mass of developing embryos as a function of their stage in development. The x-axis is the stage of development, with "0" representing a mature, unfertilized ovum and "45" representing an advanced embryo, soon to be born. The y-axis is the dry mass of developing embryos (in mg). (A) *Hemirhamphodon kapuasensis*, with a separate line fitted to the data for each of three individuals. (B) *H. kuekenthali*, showing the mean (+1 SE) for each stage of development, based on the dissection of 26 gravid females.

patterns of weight change during development were best described by a straight line with a negative slope. Some species of *Dermogenys* and *Nomorhamphus* gained significant amounts of dry mass during development, which is evidence of continued maternal provisioning after fertilization. Two of these (*N. brembachi, N. bakeri*) were well described by an exponential and a linear curve fit, respectively. The remainder had patterns of mass change that were not well described by either a linear, quadratic, or exponential curve. We found instead that the data could be described by a broken regression that fit one straight line of near-zero slope to early stages of development and a second straight line with a positive slope beginning with embryos that were sufficiently advanced to have begun to develop eye pigmentation (Fig. 2). We refer to all species with MI values greater than 1 as matrotrophic, but it is important to bear in mind that the degree of matrotrophy is highly variable.

## GENERAL DESCRIPTION OF GENERA
Hemirhamphodon

The three species of *Hemirhamphodon* all had very similar life histories. *Hemirhamphodon kuekenthali* and *Hemirhamphodon kapuasensis* and our single *H. pogonognathus* had ovaries that were similar in structure to the earlier descriptions of *H. pogonognathus* (Roberts 1989), with a single-file array of developing young; yolking ova were found at the anterior end of the ovary and progressively more advanced embryos are found toward the posterior end of the ovary. Females had from four to six litters of developing young according to our standards for scoring development, but perhaps more because there was a fairly continuous progression in the developmental stage of the embryos. All three species produced young that were similar in size (0.58 to 0.75 mg at birth), had similar litter sizes (2.7–3.5 offspring), and similar matrotrophy indices (0.58–0.64) (Fig. 1). Their indices indicate that all three species are lecithotrophic, which had been inferred from earlier observations of their large eggs (Roberts 1989) but had never been formally evaluated.

Nomorhamphus

Four species are lecithotrophic, with MI values that range from 0.66 to 0.84 (Table 1, Fig. 2). All females in all four species had only one litter of developing young, so it appears that they also lacked superfetation. These species produced the largest offspring of any in this study, with mean masses at birth ranging from 5.9 to 11.6 mg. They also had some of the smallest litters, with from two to seven offspring per litter. The four additional species for which we had too few datapoints to evaluate maternal provisioning (one to three gravid females per species) also had only one litter of developing young per female. In one of these (*N. towoetii*), the single litter that was at an intermediate stage of development had a mean embryo mass of 7.7 mg whereas the single litter that was at an advanced stage of development had an embryo mass of 3.8 mg; these results suggest that this species is lecithotrophic, which is also what Meisner and Burns (1997) report for this species. There were four matrotrophic species in this genus, with MI values ranging from 3.4 to 21. Females in three of the four species were almost all carrying two litters of developing young in discretely different stages of development and hence had superfetation. The fourth species, *N. bakeri*, had the lowest MI value and each female had only one litter of developing young. These data suggest that this species lacked superfetation, but it is possible for species that are capable of superfetation to not be carrying multiple litters, such as in response to low levels of food availability, so our conclusion concerning the absence of superfetation must be verified.

Dermogenys

Two species are lecithotrophic, with MI values of 0.64 and 0.67 (Table 1). All females in both collections were carrying only a
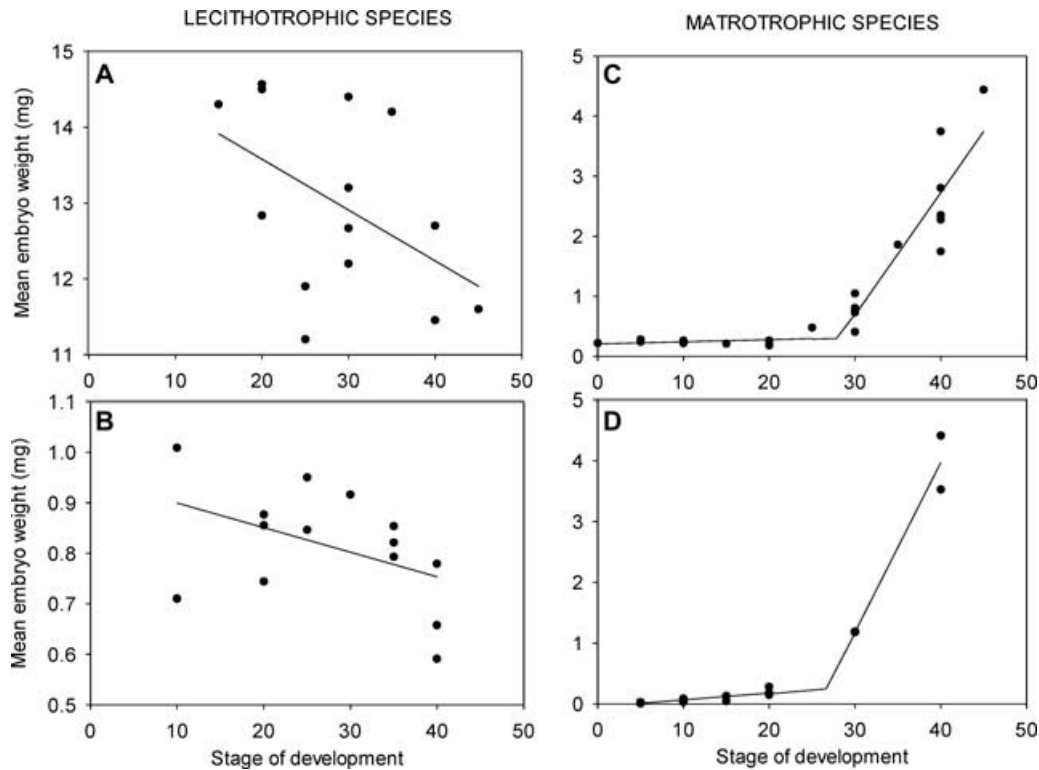
Figure 2. Patterns of change in mass in developing embryos as a function of stage of development. The x-axis and y-axis are as in Figure 1. There is one representative lecithotrophic and matrotrophic species from each genus presented here. (A) *Nomorhamphus weberi*, a representative lecithotrophic species. (B) *D. burmanica*, a representative lecithotrophic species. (C) *N. rossi*, a representative matrotrophic species. (D) *D. sumatrana*, a representative matrotrophic species.

single litter of developing young, so they also appear to lack superfetation. These species produced the smallest offspring (0.34 and 0.67 mg at birth) and had the highest mean number of offspring per litter (37 and 19, respectively) in the genus. There are three matrotrophic species in the genus, one of which is represented here by four populations, with MI values ranging from 16 to nearly 200. A 200-fold increase in dry mass during development represents one of the largest levels of post-fertilization provisioning for any nonmammalian, placental vertebrate. The females of two of these species (*D. orientalis, D. bispina*) were each carrying two or three litters of developing young, whereas females of the fourth species (*D. sumatrana*) were all carrying four litters of developing young. The two species with the highest MI values (*D. sumatrana*, and *D. bispina*) produced the largest offspring and also had the smallest number of offspring per litter. The four populations of *D. orientalis* were intermediate between these two species and the two lecithotrophic species for both offspring size and the number of offspring per litter.

COMPARISONS AMONG GENERA AND MODES OF REPRODUCTION

Because we found species with and without matrotrophy in the genera *Nomorhamphus* and *Dermogenys*, we performed a series of two-way ANOVA to extract independent estimates of the differences among genera, modes of reproduction, and the possible interaction between these two independent variables. Our primary goal was to see if the mode of reproduction is associated with differences in other features of the life history, in this case offspring number per litter, offspring size and reproductive allocation. For these analyses, all species with MI values > 1 were simply classified as matrotrophic. An analysis that explicitly incorporates phylogeny was not possible because of the lack of resolution in our tree (see below).

We first compared the mean standard lengths of the two genera and two modes of reproduction because length often figures as a covariate in the analyses of other dependent variables. *Nomorhamphus* were, on average, significantly longer than *Dermogenys* ($F_{1,13} = 8.81, P = 0.01$; *Nomorhamphus* lsmean $= 60.1$ mm, *Dermogenys* lsmean $= 45.0$ mm) and there was very little overlap in the ranges of lengths for the two genera. The means for the two modes of reproduction were nearly equal ($F_{1,13} = 0.04, P = 0.84$; lecithotrophic $= 52.0$ mm, matrotrophic $= 53.1$ mm) and there was no interaction between the two independent variables ($F_{1,13} = 0.29, P = 0.60$). The large differences in mean length among the genera mean that, if length is included as a covariate, then length will be confounded with genus and hence

Table 2. Two-way analyses of variance of the effects of genus (*Nomorhamphus* vs. *Dermogenys*) and mode of maternal provisioning (lecithotrophy vs. matrotrophy), with the natural log of mean female length as a covariate, where appropriate. The analysis of total reproductive dry mass and mass of offspring at birth was performed on natural log-transformed data. Dependent variables = number of offspring per litter, estimated dry mass of offspring at birth (mg), and total reproductive dry mass, which equals the dry mass of all developing young plus all yolking ova plus other reproductive tissues (mg). The degrees of freedom associated with the error mean square were equal to 11 if length was included as a covariate and 12 if it was not included.

Independent variable	Degrees of freedom	Number of offspring/litter	Mass of offspring at birth (mg)	*Total reproductive dry mass (mg)
log (length) (covariate)	1	–	9.12*	12.20**
Genus	1	6.69*	0.26 ns	0.09 ns
Maternal Provisioning	1	5.62*	0.15 ns	18.23**
G × M	1	16.12**	5.11*	0.02 ns
Error mean square	11(12)	33.63	0.4013	0.2443
R^2		0.63	0.67	0.80

*$P < 0.05$, **$P < 0.01$, ns = not significant.

will reduce the power of the genus comparison; however, length serves well as a covariate in the assessment of the effect of mode of reproduction on the remaining dependent variables.

Length proved to be nonsignificant as a covariate in the analysis of the number of embryos per litter ($F_{1,11} = 0.53$, $P = 0.48$) and was not included in that analysis. We found that there was a significant effect of genus and mode of reproduction, but a much larger interaction between the two (Table 2). The reason for the interaction was that the effect of the mode of reproduction was the opposite in each genus. In *Nomorhamphus*, the matrotrophic species produced substantially more offspring per litter whereas in *Dermogenys* they produced substantially fewer offspring (Fig. 3). We repeated the analysis for the total number of developing embryos, which is equal to the average number of embryos per litter times the average number of litters for those species that have superfetation. The results were qualitatively the same as those for just the number of young per litter, except that the mode of reproduction is no longer significant ($F_{1,12} = 0.08$, $P = 0.79$), and the effects of genus and the interaction were marginal (Genus: $F_{1,12} = 4.73$, $P = 0.0504$; Interaction: $F_{1,12} = 4.79$, $P = 0.0492$).

Length was a significant covariate in the analysis of the size of fully developed offspring (log transformed) and hence was included in the analysis. In this case, the effects of genus and mode of reproduction were not significant but the interaction between the two was significant (Table 2, Fig. 3). The cause of the interaction was again attributable to the opposite association between mode of reproduction and genus; in *Nomorhamphus* the matrotrophic species produced smaller offspring whereas in *Dermogenys* they produced larger offspring than their lecithotrophic congeners.

Length was again a significant covariate in our analysis of total reproductive mass, which was the sum of the total dry mass of developing embryos (multiple litters for species with superfetation) and the dry mass of other reproductive tissues, which includes the ovary and yolking ova. This variable is an

index of the relative quantity of resources devoted to reproduction, scaled on female size (the log transformation of female length). There was not a significant effect of genus or a significant interaction, but there was a highly significant effect of mode of reproduction; matrotrophic species have substantially smaller masses of developing young than do lecithotrophic species (Table 2, Fig. 3). This, then, is the only significant statistical association between matrotrophy and some other component of the life history.

PHYLOGENETIC DISTRIBUTION OF LIFE HISTORIES

The branch and bound analysis (Swofford 2002) resulted in 12 equally most parsimonious trees 51 steps long with consistency and retention indices of 0.980 and 0.995, respectively. The strict consensus tree (Fig. 4) is almost identical to the strict consensus branch and bound tree recovered by Meisner (2001) with the life-history traits included. The only difference is that the clade consisting of *N. pectoralis* + *N. manifesta* + *N. rossi* was not recovered by the new analysis. The presence of polytomies in all of the most parsimonious trees prevented us from being able to reconstruct ancestral states accurately using MacClade 4.08 (Maddison and Maddison 2000). We have plotted the life-history traits onto the strict consensus tree to illustrate their distribution within the family. The presence of viviparity throughout all three genera and its absence in the most closely related genus *Zenarchopterus* and in the most closely related families, the flying fish (Exocoetidae) and sauries (Scomberesocidae) (Lovejoy et al. 2004—not shown in Fig. 4) suggest that there is one common origin of viviparity for all three live bearing genera. This distribution leaves unanswered whether egg laying in *H. tengah* is a retained primitive trait or is a reversal from live bearing because this species is not included in any of our datasets. The distribution of superfetation (not shown) does not suggest any pattern of evolution. This lack of resolution is again a result of the highly unresolved tree. It thus remains
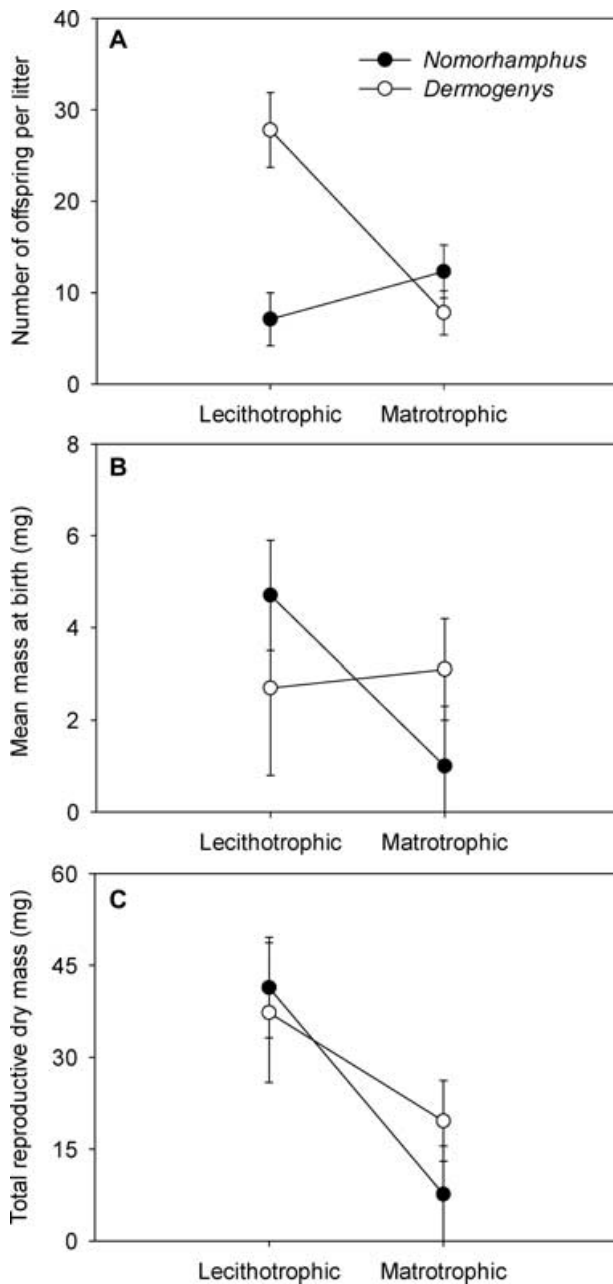
Figure 3. Results of the two-way analyses of variance summarized in Table 2. The two independent variables are lecithotrophy versus matrotrophy and *Dermogenys* versus *Nomorhamphus*. The plotted values are least square means from the analyses described in the text. (A) Number of offspring per litter. (B) Mean dry mass of offspring at birth, adjusted for mean female length. (C) Mean total dry mass of all developing offspring, adjusted for female mean length.

to be determined whether superfetation was ancestral to all three genera and was later lost in some species of *Nomorhamphus* and *Dermogenys* or if it instead evolved independently in all three genera. The distribution of lecithotrophy/matrotrophy (Fig. 4) suggests that there could be two or more independent ori-
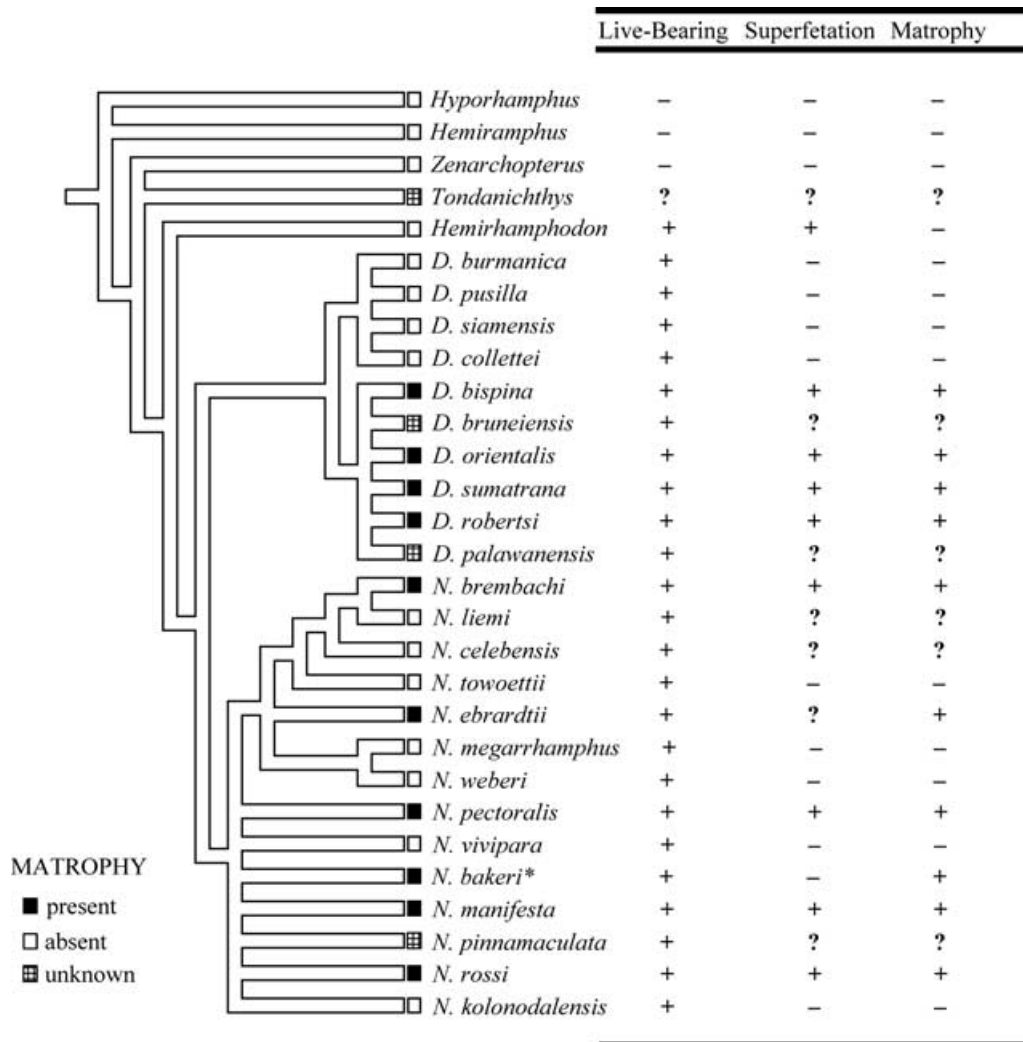
gins of matrotrophy—once in the *Dermogenys* clade and once in the *Nomorhamphus* clade. A more fully resolved tree is required to reveal the true number of independent origins of matrotrophy in *Nomorhamphus* but it is clear that all of these life-history traits are labile within the Zenarchopteridae.

Discussion

In summary, matrotrophy evolved at least once within the live-bearing genera of the Zenarchopteridae. The exact number events could be larger, but we cannot be certain until we have a more highly resolved phylogeny for the family. We cannot tell how many independent origins of superfetation there have been, again because of a lack of resolution in the phylogeny. There could have been a single origin of superfetation in the common ancestor of the three live-bearing genera followed by losses of superfetation in *Dermogenys* and *Nomorhamphus*. Alternatively, there could have been three or more independent origins, with at least one in each genus. There is also not a strict association between superfetation and matrotrophy because *Hemirhamphodon* have superfetation without matrotrophy. In *Dermogenys* and *Nomorhamphus*, most species are either lecithotrophic and lack superfetation or are matrotrophic and have superfetation, but one (*N. bakeri*) appears to be matrotrophic without superfetation. It thus appears that each of these traits can exist without the other, so they are not simply two manifestations of the same adaptation, as had once been suggested for the Poeciliidae (Constanz 1989). However, it is also clear that there is a strong association between the presence of both matrotrophy and superfetation in *Dermogenys* and *Nomorhamphus*.

Matrotrophy is highly variable in degree. Within the genus *Nomorhamphus*, we observed from a threefold to 20-fold increase in dry mass between fertilization and birth, whereas in *Dermogenys* the increase can be nearly 200 fold. For the Poeciliidae, Turner (1940a) reported that the elaborateness of the anatomical adaptations associated with matrotrophy seemed to increase with the extent of post-fertilization maternal provisioning, but there has yet to be a critical pairing of formal estimates of the matrotrophy index and anatomical studies in either the Zenarchopteridae or Poeciliidae. There have been such investigations in lizards in the family Scincidae, especially in the genus *Mabuya,* which have revealed a close association between the matrotrophy index and the anatomical complexity of the interface between the mother and developing young (Flemming and Blackburn 2003).

We also found that there is not a regular association between the presence of matrotrophy and either offspring number or offspring size, so it does not appear that matrotrophy evolves as a correlated response to the evolution of these two traits. This result is distinct from a similar analysis of the evolution of live bearing in fishes, because the transition from egg laying to live

	Live-Bearing	Superfetation	Matrophy
Hyporhamphus	−	−	−
Hemiramphus	−	−	−
Zenarchopterus	−	−	−
Tondanichthys	?	?	?
Hemirhamphodon	+	+	−
D. burmanica	+	−	−
D. pusilla	+	−	−
D. siamensis	+	−	−
D. collettei	+	−	−
D. bispina	+	+	+
D. bruneiensis	+	?	?
D. orientalis	+	+	+
D. sumatrana	+	+	+
D. robertsi	+	+	+
D. palawanensis	+	?	?
N. brembachi	+	+	+
N. liemi	+	?	?
N. celebensis	+	?	?
N. towoettii	+	−	−
N. ebrardtii	+	?	+
N. megarrhamphus	+	−	−
N. weberi	+	−	−
N. pectoralis	+	+	+
N. vivipara	+	−	−
N. bakeri*	+	−	+
N. manifesta	+	+	+
N. pinnamaculata	+	?	?
N. rossi	+	+	+
N. kolonodalensis	+	−	−

MATROPHY

■ present
□ absent
⊞ unknown

Figure 4. The strict consensus tree showing the distribution of maternal provisioning in the Zenarchopteridae. The tree is based on the character matrix in Meisner (2001), minus the four characters that are redundant with the mode of reproduction. The character states for the modes of reproduction are based on the results of the current study and augmented by the results of Meisner and Burns (1997). Meisner's character matrix, minus the four life-history characters (her #'s 41–44) and a table that reconciles the species names used by Meisner and Burns (1997) and the reclassifications by Meisner (2001) can be found in our online Supplementary Appendices.

bearing is generally associated with an increase in offspring size (Goodwin et al. 2002). There is, however, a common trend in *Dermogenys* and *Nomorhamphus* for species with matrotrophy to devote fewer resources to reproduction, when scaled by body size, than do species that are lecithotrophic.

These results are similar to our findings for the family Poeciliidae in all regards. The latter family has far greater taxonomic diversity (approximately 200 species and 20 genera), which means that there is a larger sample size for any type of analysis. On a family-wide basis, we again find that the presence or absence of superfetation and matrotrophy can be independent of one another, so that all four possible combinations of life-history traits (lecithotrophy with or without superfetation, matrotrophy with or without superfetation) can be found (Reznick and Miles 1989; Lorier and Berois 1995; Arias and Reznick 2000), yet most species

appear to be either lecithotrophic without superfetation or matrotrophic with superfetation. Seeing this pattern in both families is compelling in the light of Trexler and DeAngelis' (unpubl. ms.) theoretical result that the presence of superfetation can facilitate the evolution of matrotrophy.

Our ongoing work on the poeciliid genus *Poeciliopsis* shows that there is no regular association between the presence of matrotrophy and other features of the life history, including offspring number and offspring size. In the "northern clade" of the genus (Mateos et al. 2002) we find that matrotrophic species tend to have more and smaller offspring whereas in the "southern clade" of the genus they tend to have fewer and larger offspring (D. Reznick et al., unpubl. ms.). Reznick et al. (2002) found that matrotrophy evolved independently in each of these clades. However, we have also found (M. Pires et al., unpubl. ms.) that species that

are matrotrophic have relatively smaller reproductive allocations on average than those that are lecithotrophic. The reason for this difference is that the quantity of resources that are devoted to reproduction is very small at the time that eggs are fertilized, then gradually increases as the embryos grow throughout development. This quantity can be further reduced, without a reduction in the rate at which young are produced, when it is combined with superfetation. Species with superfetation not only reduce the number of young per litter but also reduce the time interval between successive litters, so the reproductive burden is divided among very small young that are early in development and progressively larger young that are later in development.

One feature of matrotrophy in *Dermogenys* and *Nomorhamphus* that is not in common with the Poeciliidae is the "broken-line" regression that describes the pattern of increase in dry mass during development in most matrotrophic species. In the Poeciliidae, the pattern of increase in dry mass during development is well described by either a quadratic or an exponential curve. The quadratic curve implies that development is initially fueled by yolk stored in the egg prior to fertilization and that active maternal provisioning is initiated some time later. Embryos thus first lose some dry mass early in development, then later increase in dry mass, presumably as the placenta becomes functional. The broken line regression and the details of the relationship between dry mass and stage of development (Fig. 2) imply instead that there is little maternal provisioning until an embryo has developed eye and body pigment, then there is the advent of a large additional source of maternal provisioning. One aspect of the anatomical differences in development between the two families is that larval *Dermogenys* and *Nomorhamphus* appear to have functional intestinal tracks and to be ingesting maternal secretions from the lumen of the ovary or follicle (Meisner and Burns 1997). The one species of poeciliid that has been studied in such detail does not have an active intestinal tract during development and instead absorbs all nutrients via structures on the surface of the body (Grove and Wourms 1991). The break in weight gain seen in the matrotrophic zenarchopterids may thus correspond to the maturation of the intestinal tract and the advent of such intraluminal feeding.

Adaptive significance of matrotrophy

Although our analysis focuses on the presence or absence of postfertilization maternal provisioning as inferred from changes in the dry mass of developing young (Fig. 1), anatomical studies of the Poeciliidae, Zenarchopteridae and other families (Turner 1940b; Wourms et al. 1988; Meisner and Burns 1997) show that the presence of matrotrophy is associated with the presence of maternal and embryonic structures that appear to be specialized for the transfer and absorption of resources during development. For example, Meisner and Burns (1997) found that matrotrophy was associated with embryos that had an expanded and highly vascu-

larized pericardial membrane and yolk sac, similar to those seen in matrotrophic Poeciliidae. The follicle and ovary were more highly vascularized than in the nonmatrotrophic species plus were lined on the inner surface with cells that appeared to have a secretory function, presumably to secrete nutrients into the lumen. It is the presence of these specialized structures that qualifies these organisms as having placentas, (Mossman 1937). Some authors have distinguished between "placentotrophy," or the tissue-to-tissue transfer of nutrients and matrotrophy, or the secretion of nutrients into the lumen, which are then actively ingested by the embryos (Wourms 1981; Blackburn 2000). *Dermogenys* and *Nomorhamphus* appear to have both mechanisms.

In both the Zenarchopteridae and Poeciliidae, species that are matrotrophic devote a smaller proportion of their body mass to reproduction (reproductive allocation) than do those that are lecithotrophic. This difference in reproductive allocation is independent of other life-history traits, such as fecundity or offspring size. Miller (1975) and Thibault and Schultz (1978) proposed that placentation can be an adaptation that reduces the average locomotory cost of reproduction because it reduces reproductive allocation. Plaut (2002) and Ghalambor et al. (2004) showed that there is such a locomotory cost of reproduction; Ghalambor et al. (2004) further showed that the cost increases as the reproductive burden increases. Both species in these studies (*Gambusia affinis* and *Poecilia reticulata*, respectively) are in the family Poeciliidae and are lecithotrophic. Ghalambor et al. (2004) showed that this cost was associated with a progressive increase in the wet mass of young during development because, even though their dry mass was declining, their wet mass increased between twofold and threefold between fertilization and birth. They also worked with four populations that differed in reproductive allocation and showed that those with higher reproductive allocations also had a higher locomotory cost of reproduction. If the locomotory cost is strictly a function of the mass or volume of developing young, then the results for *G. affinis* and *P. reticulata* suggest that the poeciliids and zenarchopterids with placentas will also have lower locomotory costs of reproduction than those species that lack placentas because they have lower reproductive allocations.

In the poeciliid genus *Poeciliopsis*, the lower values for reproductive allocation in association with matrotrophy are also associated with species that tend to live in larger rivers with higher currents (M. Pires, unpubl. ms.), so it is plausible that matrotrophy may serve as an adaptation to such habitats because they enable fish to reduce the size of their reproductive burden that results in a slimmer profile and a smaller reproductive cost of locomotion. There has been no attempt to evaluate such an association for the Zenarchopteridae.

If this reproductive allocation hypothesis proves to be true, then it can only represent one of a larger number of factors that select for the evolution of placentas, rather than being a general

argument for their evolution. Some of the other groups of fish that have evolved placentas share similar freshwater environments or live in the surf zone of marine environments in which current could play a similar role (e.g., the families Anablepidae, Embiotocidae or Goodeidae). However, there have been multiple origins of placentas in the Chondrichthys (Dulvy and Reynolds 1997) but without any similar habitat association. Furthermore, the equivalent of placentation has evolved in bivalves that have invaded freshwater streams and rivers (Korniushin and Glaubrecht 2003), but this trait cannot benefit such organisms on the basis of reduced locomotory costs of reproduction. It is also difficult to imagine the evolution of a mammalian placenta being associated with a reduced locomotory cost of reproduction given the impact of pregnancy on so many extant mammals, but in this case we are considering an event that occurred over 100 million years ago and for which we have no history. Inferences based on living mammals confuse animals as they are today with what their placental and nonplacental ancestors may have been like as the placenta was evolving.

Trexler and DeAngelis (2003) presented the first general, theoretical argument for the evolution of postfertilization maternal provisioning; they argue that matrotrophy can result in the production of offspring at a higher rate if food availability is high and relatively constant. This is true because species with maternal provisioning require fewer resources to initiate a litter but demand a constant supply of resources during development. Thibault and Schultz (1978) suggested that matrotrophy would be maladaptive in an environment with fluctuating resources because the development of embryos relies on the constant maternal input.

Crespi and Semeniuk (2004) proposed a stark alternative, which is that the placenta does not represent an adaptation to any external feature of the environment. It instead evolves as a consequence of the intergenomic conflict caused by the prolonged contact between the mother and developing young that follows the evolution of live bearing. In this case, we would not expect to see any association between the evolution of the placenta and any feature of either the environment or the life history beyond what would be caused by the prior evolution of live bearing. We can affirm that we are not finding a regular association between the placenta and most other aspects of the life history, but otherwise cannot find any support for or against their hypothesis. We note that it had already been argued that the presence of placentation will be associated with intergenomic conflict (Haig 1993), so that there will be continuous conflict-fueled evolution of the placenta once the trait has become established. Haig cites conflict as a potential cause of the remarkable variation that we see in the anatomy of the mammalian placenta. The added feature of the Crespi–Semeniuk hypothesis is that the conflict associated with the prior evolution of viviparity is what causes an organism to cross the threshold that divides lecithotrophy from matrotrophy.

Finally, Mank et al. (2005) offer additional insights into the evolution of matrotrophy, based on a phylogenetic analysis of a "supertree" of the ray-finned fish. Their analysis revealed at least 13 origins of internal fertilization, which is a necessary precursor to viviparity, followed by at least eight origins of viviparity. At least four of these eight lineages then evolved postpartum maternal care. Matrotrophy thus appears as a derived trait from lecithotrophy and is interpreted as an alternative route to increased maternal care. The origin of internal fertilization is apparently preceded by sexual dichromatism, so the sequence of events that lead to the evolution of viviparity then matrotrophy began with a sexual dimorphism associated with sexual selection. Mank and Avise (2006) performed a similar analysis for the Atherinomorpha, a superorder that forms a subset of the ray-finned fish and includes the two families considered in the current article. They confirmed at least four independent origins of live bearing in this group. In addition, they found that the live-bearing lineages tended to contain more extant species than the egg-laying lineages, which implies that the evolution of live bearing has enhanced the rate of speciation. Their analysis did not take into account the recent separation of the Zenarchopteridae from the Hemiramphidae. If this separation is made, then their results would still reach the same conclusion because the number of live-bearing species within the Zenarchopteridae (35) outnumbers the egg-laying species (21). Other authors (e.g., Rice and Holland 1997) have postulated that placentation can cause an increase in the rate of evolution of reproductive isolation, which can in turn increase the probability of speciation. Because all four lineages of the Atherinomorpha that evolved viviparity also evolved matrotrophy, an association between placentation and the rate of speciation may account for Mank and Avise' observation.

The value of our results is that they represent yet more raw materials for the study of the evolution of the placenta and the more general study of the evolution of complexity. This opportunity is not available in placental mammals because they share a placenta that evolved once over 100 million years ago; whatever conditions selected for the origin of this trait and whatever transitions were made from egg laying to this particular form of live bearing have been lost to history. The special feature of the Zenarchopteridae and the Poeciliidae is that we see, instead, multiple origins of the trait, variation in its presence or absence among close relatives, and dramatic variation in its degree of development among those species that have placentas. The multiple origins of derived, complex traits in combination with diversity among close relatives offer an opportunity for comparative studies that is unparalleled for the large majority of similar complex adaptations.

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