Title: Recent evolution of large offspring size and post-fertilization nutrient provisioning in swordtails

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Abstract

How organisms adapt to diverse environments is a foundational question in evolutionary biology. In addition to directly adapting to ecological conditions, organisms have evolved diverse reproductive strategies that impact the probability that their offspring survive to adulthood. Here, we describe divergence in reproductive strategy between two closely related species of swordtail fish (*Xiphophorus*). Swordtail fish and their relatives have evolved viviparity: they have internal fertilization and give birth to fully developed fry. We find that one species, *X. malinche*, which lives in high-elevation environments, has evolved larger offspring than its closest relative *X. birchmanni* and dwarfs the offspring size of other species in the genus. The larger fry of *X. malinche* are more resilient to starvation than their *X. birchmanni* relatives, hinting that the evolution of large offspring size may be an adaptation to the particularly challenging environments in which *X. malinche* are born. We find that *X. malinche* achieves larger offspring size in part by continuing to provision their offspring over the course of embryonic development after fertilization, the first time this process has been documented in the *Xiphophorus* genus. Moreover, we observe differential regulation in the ovary of genes associated with maternal nutrient provisioning in other species that use this reproductive strategy. Intriguingly, these reproductive differences may drive an asymmetric hybrid incompatibility, since *X. birchmanni* mothers pregnant with F_1 embryos give birth to premature and stillborn fry at an exceptionally high rate.

Introduction

Sexually reproducing organisms vary vastly in their investment in their offspring. In some species, investment stops prior to fertilization, whereas in others investment and parental care continue into adulthood (Gross 2005; Klug and Bonsall 2014; Furness and Capellini 2019). In vertebrates, for example, reproductive strategies range from broadcast-spawning millions of eggs (e.g. Atlantic cod; Roney et al. 2018) to a parent raising a single offspring over decades (e.g. orca whales; Weiss et al. 2023). Though the degree of parental investment is generally positively correlated with the probability of offspring survival (Brockelman 1975; Einum and Fleming 2000), there are well-documented tradeoffs between reproductive investment and life history traits like parent survivorship, frequency of reproduction, the number of offspring per reproductive cycle, and the total number over the parent's lifetime (Smith and Fretwell 1974; Brockelman 1975; Stearns 1989; Einum and Fleming 2000; Jørgensen et al. 2011; Roney et al. 2018).

One measure of reproductive investment that has been studied in diverse organisms across the tree of life is offspring size. Within species, size at birth or weaning is strongly correlated with the probability of offspring survival to reproductive maturity (although there are some exceptions; see (Kaplan 1992). The mechanisms through which larger offspring achieve better outcomes are incompletely understood but appear to be diverse and vary between species (such as by avoiding size-dependent mortality, starvation, disease, and conspecific competition (Rollinson and Hutchings 2013; Pettersen et al. 2022). Researchers have speculated that these factors could explain the evolution of differences in offspring size across species.

63 Another mechanism for increasing offspring survival that has recurrently evolved is
64 viviparity, or internal development of embryos. Viviparity reduces size-dependent mortality of
65 eggs and embryos (Jørgensen et al. 2011) and can offer improved environmental conditions for
66 developing offspring. For some species, it provides an opportunity to directly provision nutrients
67 to developing offspring (Griffith and Wagner 2017). While viviparous species typically provide
68 some level of nutrition to their offspring in the form of yolk in the egg, a strategy known as
69 lecithotrophy, some provision nutrients both before and after fertilization, a strategy known as
70 matrotrophy (Wourms et al. 1988). This post-fertilization provisioning is mediated through a
71 physical interface between parent and offspring tissue, called the placenta (Griffith and Wagner
72 2017; Whittington et al. 2022). Placentas have independently arisen over 130 times across
73 vertebrates (Blackburn 2015; Whittington et al. 2022), and complex placentas that exchange
74 large quantities of nutrients have arisen multiple times in squamates and ray-finned fishes
75 (Griffith and Wagner 2017; Whittington et al. 2022). While the complex mammalian placenta
76 evolved once over 100 million years ago (Meredith et al. 2011), lineages in which nutrient
77 provisioning evolved recurrently and recently can allow us to understand the ecological and
78 evolutionary pressures that drive post-fertilization nutrient provisioning and ask whether shared
79 genetic and regulatory mechanisms are responsible for its convergent evolution across
80 vertebrates.
81 Poeciliid fishes offer an opportunity to study the evolutionary drivers of viviparity, nutrient
82 provisioning strategies, and variation in offspring size. The common ancestor of poeciliids
83 evolved internal fertilization and live birth of juvenile fish, or fry (Pollux et al. 2009). Complex
84 placental structures have evolved at least nine times in this group of ~300 species and in some
85 species embryos grow to 100-120x their initial size over development (Reznick et al. 2002; Pires

86 et al. 2010). Notably, the molecular mechanisms associated with the independent evolution of
87 nutrient provisioning in the poeciliid genus *Poeciliopsis* is at least partially shared with mammals
88 (Guernsey et al. 2020).

89 Counterintuitively, the evolution of offspring size and matrotrophy are often decoupled in
90 poeciliids. While it might be expected that provisioning more nutrients during development
91 would lead to larger offspring at birth, empirical data have yielded no consistent association
92 across taxa (Olivera-Tlahuel et al. 2015; Meiri et al. 2020; Furness et al. 2021), although large-
93 bodied matrotrophic species seem to produce larger offspring relative to their lecithotrophic
94 counterparts (Furness et al., 2021). The link between offspring size and matrotrophy has been
95 difficult to pinpoint because offspring size is strongly impacted by maternal traits like age, size,
96 and condition (Berkeley et al. 2004; Marshall and Keough 2007; Hagmayer et al. 2018); but see
97 Marshall et al. 2010), as well as ecological factors that interact with maternal traits (Reznick et
98 al. 1996*a*; Jennions et al. 2006; Riesch et al. 2010; Pollux and Reznick 2011; Schrader and
99 Travis 2012; Leips et al. 2013). For example, even with no post-fertilization provisioning,
100 offspring of lecithotrophic *Poecilia* from environments with low predation and high competition
101 are 50% larger at birth than those from high predation, low competition environments (Reznick
102 1982*a*; Reznick and Endler 1982; Reznick and Bryga 1987; Reznick et al. 1996*c*; Bashey 2006*a*,
103 2008; Jørgensen et al. 2011), presumably as a result of mothers investing in larger eggs. Similar
104 environmental conditions appear to promote larger offspring size in some matrotrophic species
105 (Schrader and Travis 2012; Leips et al. 2013; but see Reznick et al. 1996*b*) but not in others
106 (Pollux and Reznick 2011). Overall, offspring size appears to be specific to species and
107 ecological-context.

108 Here, we address questions about the evolution of offspring size and matrotrophy in a
109 group of livebearing fish that are an important evolutionary and behavioral model system.
110 *Xiphophorus* species inhabit dramatically different ecological environments, from valley rivers
111 nearly at sea level to mountain streams in the Sierra Madre Oriental in central México. Past work
112 on a handful of species has suggested that although all *Xiphophorus* species are livebearing, they
113 do not invest in their offspring post-fertilization (i.e. they are lecithotrophic; Constantz et al.
114 1989). However, this hypothesis has not been evaluated on a genus-wide scale, and data is
115 particularly lacking in species living in extreme environments for the group (Morris and Ryan
116 1992; Pollux et al. 2014). There is no data available on variation in offspring size within or
117 between species.
118 We find that *X. malinche* have evolved exceptionally large offspring and explore the
119 mechanisms and pressures driving the evolution of this trait. By characterizing the change in
120 embryo size over development in the lab and wild for the closely related northern swordtail
121 species *X. malinche* and *X. birchmanni*, we find that *X. malinche* achieves larger offspring size
122 through post-fertilization maternal nutrient provisioning. Using a combination of approaches, we
123 investigate the potential mechanisms of post-fertilization nutrient in *X. malinche*. Finally, we
124 explore the possibility that a conflict between maternal nutrient provisioning and offspring
125 nutrient demands over development underlies asymmetric hybrid inviability between *X.*
126 *malinche* and *X. birchmanni*.
127
128

Methods

Measuring newborn fry size across Xiphophorus species

To compare the size of newborn fry in several *Xiphophorus* species, broods were collected in the laboratory on the day they were born. All tanks with gravid females were inspected multiple times a day for fry. Upon collection, all fry from a brood were photographed in shallow water from overhead for a dorsal view of each fry. Photos were imported to ImageJ2 and two measurements, standard length and head width, were made using the line measurement tool (Supp. Fig. 1). We measured both traits for fry from five *Xiphophorus* species – *X. birchmanni, X. malinche, X. cortezi, X. pygmaeus*, and *X. variatus*, and from *X. malinche x X. birchmanni* F_1, F_2, and lab-reared natural hybrids descended from individuals collected from the Calnali Low wild population. Note that we report newborn fry size for the *X. malinche x X. birchmanni* F_1 cross direction only, as F_1s from the reciprocal *X. birchmanni x X. malinche* are rarely carried to term (see below). Given that fry were collected from tanks with multiple gravid females, we were not able to collect covariates such as mother standard length. However, because average mother size is similar between species (see Supplementary Information 1), we expect that our large sample sizes (*X. malinche* broods N=35 and *X. birchmanni* broods N=48) will not be dominated by effects of individual mother size. Since broods varied in size, we averaged the standard length and head width for each brood and compared means with a Wilcoxon rank sum exact test. See Table S1 for raw fry standard length and head width and Table S2 for statistics. Using lab-reared species and hybrid fry, we also obtained an estimate of broad-sense heritability (H^2) of offspring standard length at birth (see Supplementary Information 2 for methods).

Measuring embryo weight across developmental stages

To compare embryo size throughout development in each species, we measured the dry weights of embryos across developmental stages from wild-collected females. We collected 49 pregnant and nonpregnant females (with fully-yolked eggs) from the *X. malinche* source population Chicayotla on the Río Xontla (1,003 m elevation; 20°55′27.24″N 98°34′34.50″W) across two seasons (45 in May 2022 and 4 in August 2020), 79 pregnant females from the *X. birchmanni* source population Coacuilco on the Río Coacuilco (320 m elevation; 21°5'50.85 N, 98°35'19.46 W) across three seasons (22 in February 2023, 30 in September 2022, and 27 in August 2020), and 19 pregnant females from the *X. cortezi* source population Puente de Huichihuayán on the Río Huichihuayán (89 m elevation; 21°26'9.95 N 98°56'0.00 W) in one season (February 2023). In practice, the sample of 19 females from *X. cortezi* was insufficient to capture developmental profiles because we had ≤1 brood for many stages, so we limit our discussion of these results to the supplement (Supplementary Information 3). Each mother was euthanized with MS-222, cut from the anal pore to the gills so as to expose the brood for fixation, and stored in 95% ethanol. Consistent with previous reports for *Xiphophorus* (Kindsvater et al. 2012; Furness et al. 2019), we found that all embryos in a brood were at roughly the same developmental stage.

Embryos were staged following the numerical scoring guidelines described by (Reznick 1981). Briefly, stages range from 0-50 (in intervals of 5), where stage 0 describes yolking or fully yolked unfertilized eggs and stage 50 describes fully developed fry with a closed pericardial cavity (see Table S3). Importantly, these stages are categorical based on key morphological features of developing embryos and do not reflect constant time intervals throughout development, as the temporal interval varies. Embryos were separated from the ovarian follicle,

175 staged under a microscope, and then dried following methods described in D. Reznick (1981).
176 The ovarian tissue and each staged embryo were placed in individual microcentrifuge tubes that
177 were dried in a laboratory oven overnight at 65°C. Each embryo was dry weighed using a
178 standard scale sensitive to 10^{-4} grams (Table S4).

179 It is difficult to discern whether stage 0 eggs are fully yolked or are still yolking.
180 Therefore, we noted whether unfertilized eggs had an even lipid distribution and were evenly
181 sized within their brood, which are the best visual indicators of completed yolking. We used the
182 subset of unfertilized eggs that met these criteria to compare pre-fertilized egg weight between
183 species but note that the distribution of these dry weights likely skews lower than the true pre-
184 fertilized egg weights for both species (see Table S5). We fit a linear model of egg dry weight by
185 species with brood size and mother standard length as selected covariates, performed pairwise
186 comparisons with an ANOVA and Tukey post-hoc test, and plotted partial residuals of egg dry
187 weights with R visreg::visreg (as above).

188

189 *Comparison of embryo weight over development between species*

190 Maternal size has previously been shown to strongly correlate with offspring size in
191 poeciliids, including in *X. birchmanni* (Jørgensen et al. 2011; Kindsvater et al. 2012; Hagmayer
192 et al. 2018), as have brood size and environmental variables like collection season (Kindsvater
193 et al. 2012). Therefore, we evaluated which covariates impact embryo dry weight using R
194 stats::step to compare models that included mother standard length, brood size, and collection
195 date in an AIC framework. Mother standard length and collection date were selected and
196 included as covariates in the final linear model using R stats::lm. We averaged dry weights of
197 embryos at the same stage within a brood to obtain one value per stage per brood. Thus, the

198 model used for this analysis was: average embryo dry weight ~ stage + species + collection date
199 + mother standard length. Pairwise comparisons between mean embryo dry weight for each stage
200 were made with an ANOVA and Tukey post-hoc test with R emmeans::emmeans (Table S6).
201 We visualized differences in embryo size after accounting for covariates by plotting the
202 partial residuals of average embryo dry weight for each stage with R visreg::visreg (Breheny and
203 Burchett 2017), split by species. Because it is difficult to determine whether stage 0 eggs are
204 fully-yolked (see above), we omitted these eggs from the developmental profile plots, but
205 separately plot the stage 0 eggs that appeared to have even lipid distribution and size within their
206 brood (Table S5). We also plotted the raw data (i.e. without accounting for covariates) and
207 reassuringly, the overall patterns were similar (Fig. S5).
208
209 *Assessing the relationship between genome-wide ancestry and embryo and ovary size*
210 We were curious whether genome-wide ancestry proportion in natural hybrid mothers
211 might predict embryo size and ovary weight, given that this trait is heritable in lab-raised hybrids
212 (see Results). We sampled 34 pregnant mothers from a natural hybrid population on the Río
213 Calnali (the Calnali Low population, elevation 905 m) with *X. malinche* ancestry proportions
214 ranging from 0.28 to 0.71 (see Table S7 and Supplementary Information 4). Embryos and ovary
215 tissue were dissected, staged, and dry-weighed as described above. We included all embryos
216 within a brood for this analysis, since there is variation in embryo ancestry proportion within a
217 brood. As above, we used R stats::step to choose an appropriate model for testing the
218 relationship between embryo dry weight, as well as ovary dry weight, and mother ancestry
219 proportion. The selected mixed linear model included brood size, stage, and the interaction
220 between stage and ancestry proportion as covariates, with brood ID as a random effect. We

evaluated significance with a likelihood ratio test using R stats::anova to compare the full model and a reduced model without ancestry proportion and its interaction with stage. We also evaluated whether mother mitochondrial genotype predicted embryo or ovary dry weight (see Supplementary Information 4). We calculated and plotted partial residuals of embryo dry weight after accounting for covariates (e.g. ancestry proportion) with R visreg::visreg.

Calculating Matrotrophy Index

Research has shown that the degree of post-fertilization nutrient provisioning varies along a continuum, but researchers have described two general provisioning strategies: lecithotrophy and matrotrophy. A simple quantitative metric for distinguishing these strategies and measuring the level of post-fertilization provisioning is the Matrotrophy Index, which is the dry weight of the fully-developed embryo divided by the dry weight of the fully-yolked egg. A Matrotrophy Index < 1 indicates that the embryo has decreased in size over development (lecithotrophy) while a value ≥ 1 indicates that the embryo has maintained or increased in size, indicative of some level of maternal provisioning over development (matrotrophy). Reported Matrotrophy Indices within poeciliids range from 0.45 to 103 (Furness et al. 2021). Note that a large Matrotrophy Index does not necessarily correspond to large offspring.

We calculated Matrotrophy Index for *X. malinche* and *X. birchmanni* as the dry weight of a fully-developed embryo divided by the dry weight of an early-stage embryo, averaging dry weights for embryos of the same stage within a brood. Since it is difficult to identify fully-yolked eggs, we calculated Matrotrophy Index with dry weight measurements from stage 10 embryos. We only collected one brood with stage 50 embryos for *X. birchmanni*, so instead relied on stage 45 embryos for this calculation (see Table S3 for stage morphology). These analysis choices are

conservative in the context of our study, since they will lead to an underestimate of Matrotrophy Index in matrotrophic species. Since many variables impact embryo dry weight, we performed the calculations using both the raw values (Table S4) and the partial residuals of embryo dry weights (Table S8) after accounting for developmental stage, collection date, and mother standard length as covariates (with R stats::lm as above). We describe the impact of different calculations with raw and partial residual dry weights on our estimate of Matrotrophy Index in Supplementary Information 3.

Multifactorial artificial insemination crosses

To compare results from wild-caught females to females raised in controlled lab conditions, we artificially inseminated lab-raised *X. malinche* and *X. birchmanni* mothers to create the following crosses: *X. malinche* ♀ *x X. malinche* ♂, *X. birchmanni* ♀ *x X.birchmanni* ♂, *X. malinche* ♀ *x X. birchmanni* ♂, and *X. birchmanni* ♀ *x X. malinche* ♂. Artificially inseminated females from each cross (N=10-15) were reared in 200-gallon outdoor tanks at Stanford University until females were visually determined to be late in pregnancy in April-May 2023, at which point all fish were sedated with MS-222 and euthanized by severing the spinal cord. Embryos were dissected, and each embryo was assigned a developmental stage following Table S3. Dry weight of each embryo and the ovarian tissue were measured as described above (see Table S9). Due to results suggesting that embryo size diverges between species around stage 15 (see Results), we compared stage ≥20 embryo dry weights between crosses. We used a linear model including stage and brood ID as covariates with R stats::lm, ANOVA, and Tukey post-hoc test with R emmeans::emmeans (see Table S10), and plotted partial residuals of dry weights with

266 R visreg::visreg. We also made crosses between individuals of the same species from different
267 populations (Table S11-12 and Supplementary Information 5).
268 To look for evidence of placental structures (i.e. thicker ovarian follicle and interacting
269 embryo and maternal tissue), we prepared histological slides of ovaries from both nonpregnant
270 and late-stage (stage 35-40) pregnancy females from the pure *X. malinche* and *X. birchmanni*
271 crosses, as well as both F_1 crosses. Whole ovaries containing eggs or embryos were carefully
272 dissected and fixed in 10% formalin. Ovaries were paraffin-embedded, sectioned, and stained
273 with Hematoxylin & Eosin by the Histo-Tec Laboratory (Hayward, CA). We digitally scanned
274 the stained slides through the Human Pathology Histology Services Laboratory at Stanford
275 University for morphological analysis of the ovary sections. See Supplementary Information 6
276 for methods quantifying morphological differences. Additionally, we verified the genetic origin
277 of ovary follicle tissue in *X. malinche* mothers carrying F_1 offspring (Supplementary Information
278 7).
279
280 *Differential gene expression and co-expression network analysis*
281 Previous work in livebearers has identified several key genes that are differentially
282 expressed between the ovarian tissue of pregnant lecithotrophic and matrotrophic species (Jue et
283 al. 2018; Guernsey et al. 2020). To broadly compare gene expression between *X. malinche*, *X.*
284 *birchmanni,* and *X. cortezi,* we sequenced ovarian tissue from mothers from two groups: those
285 with fully-yolked eggs and those with embryos in mid- to late-development.
286 Because we were interested in species-level rather than environmentally-dependent
287 differences, we collected both wild and lab-reared females for this analysis. For wild-caught fish,
288 we collected two *X. malinche* and two *X. birchmanni* pregnant females with stage 40-50 embryos

289 from the Chicayotla and Coacuilco localities, respectively. Additionally, we sampled at least
290 three "early" pregnancy (unfertilized fully-yolked or stage ≤ 5) and at least three "late"
291 pregnancy (stage 25-45) from our lab populations. For both sets of samples, fish were euthanized
292 by rapidly severing the spinal cord with a scalpel and dissecting the mother's body cavity from
293 anal fin to gills. Wild-caught fish were stored in RNAlater and ovaries were dissected later. For
294 lab collected fish, whole ovaries were dissected immediately following euthanasia and preserved
295 in RNAlater. See Table S13 for the full list of samples with collection dates and developmental
296 stages of embryos.

297 Ovarian follicle tissue and embryos were carefully separated. See Supplementary
298 Information 8 and Table S14 for differential gene expression analysis of embryos. RNA was
299 extracted from ovarian tissue from each mother using the Qiagen Mini RNAeasy kit. RNAseq
300 libraries were prepared with unique barcodes for each sample using a KAPA mRNA HyperPrep
301 Kit, pooled, and sequenced. The wild-caught samples and half of the lab samples were
302 sequenced on one Illumina HiSeq4000 lane and the rest of the lab samples were sequenced on
303 one Illumina NovaSeq6000 lane for an average of 28 million 150 bp paired-end reads per sample
304 (see Table S13). Raw reads are available under NCBI BioProject PRJNAXXXX. For each
305 sequencing batch, samples were paired to balance species and developmental stages so that batch
306 effects could be statistically accounted for in analysis.

307 We followed the gene expression analysis methods described in Payne et al (2022). We
308 used cutadapt (Martin 2011) and Trim Galore! (Krueger et al. 2021) to trim Illumina adapter
309 sequences and low-quality bases (Phred score < 30) from reads. Using the tool *kallisto* (Bray et
310 al. 2016), we pseudoaligned RNAseq reads to an *X. birchmanni* "pseudoreference" transcriptome
311 generated from the southern platyfish *X. maculatus* genome (Schartl et al. 2013). The *X.*

312 *maculatus* genome assembly is well-annotated and mapped to Ensembl gene IDs with associated
313 Gene Ontology terms (Wittbrodt et al. 1989; Schartl et al. 2013). Raw transcript counts were
314 converted to gene-level counts to evaluate gene expression; note that the *X. birchmanni*
315 transcriptome contains only a single transcript per gene.

316 For differential gene expression analysis, we combined all lab- and wild-collected
317 ovarian follicle samples into a single dataset, using a design formula that included species (*X.*
318 *malinche*, *X. birchmanni*, or *X. cortezi*) and pregnancy category (early or late) as a grouped
319 interaction, origin (lab or wild), and library preparation/sequencing batch. Briefly, using R
320 DESeq2::DESeq (Love et al. 2014), we normalized gene counts by library size, estimated within-
321 experimental group dispersion, fit a negative binomial generalized linear model, and tested
322 significance with a Wald test. Shrunken log-fold changes were calculated with the ashr::ashr
323 shrinkage estimator (Stephens 2017). Extreme outlier and low count genes were removed. Genes
324 were considered significantly differentially expressed between species at an FDR-adjusted p-
325 value<0.05. Out of a total of 19,176 genes in the *X. birchmanni* pseudoreference transcriptome,
326 96% are expressed in at least one species. Expression results from this analysis can be found in
327 Table S15.

328 To explore biological pathways enriched in genes that were differentially expressed
329 between species in ovarian tissue, we performed Gene Ontology enrichment analysis. We used R
330 biomaRt (Durinck et al. 2009) and GOstats (Falcon and Gentleman 2007) to match *X. maculatus*
331 Ensembl gene IDs with GO terms. We created a "universe" of all genes analyzed with DESeq2,
332 which was used as the reference set of genes for testing category enrichment. Using R
333 GSEABase::hyperGTest (Morgan, Martin et al. 2023), we ran a hypergeometric test to identify
334 overrepresented GO terms (p-value<0.05) in the set of genes that were significantly differentially

335 expressed between species and developmental stage (Table S16). We also used the R tool
336 WGCNA (Langfelder and Horvath 2008) to cluster co-expressed genes and identify clusters
337 highly correlated with species and developmental stage as a grouped variable. Detailed WGCNA
338 methods are in Supplementary Information 9 and full results are in Tables S17-18.
339
340 *Immunostaining of prolactin expression in ovarian tissue*
341 Ovaries were fixed, processed, and sectioned as described above. Briefly, sections were
342 deparaffinized and dehydrated through xylenes and a graded ethanol series. Slides were blocked
343 for 1h (at room temperature, hereafter RT) in gelatin block, then incubated with anti-prolactin
344 primary antibody (rabbit, Abcam EPR19386, 1:200) overnight (4C). Slides were washed,
345 peroxide blocked for 30m (RT) then incubated with biotinylated goat anti-rabbit secondary
346 antibody (Jackson ImmunoResearch 111-065-144, 1:5000) for 1h (RT). Slides were washed,
347 then incubated for 30m with VectaShield Elite ABC reagent (Vector Labs PK-6100; RT).
348 Prolactin signal was amplified with TSA-Cy3 (Akoya Biosciences NEL744001KT) for 6m (RT)
349 and counterstained with DAPI (Thermo Scientific 62248, 1:2000) and mounted with Prolong
350 Gold Antifade (Thermo Scientific P36930). See complete methods in Supplementary
351 Information 10.
352 Images were acquired using NIS Elements software v4.30.02 on a Nikon Ti Eclipse
353 inverted microscope equipped with an ASI MS-2000 motorized linear XY stage, Yokogawa
354 CSU-W1 single disk (50mM pinhole) spinning disk unit, Andor Zyla 4.2 (6.5mM pixel size)
355 scMOS camera, and 10x/0.45 NA or 20X/0.75 NA Nikon PlanApo Lambda air objectives (see
356 Supplementary Information 10 for complete imaging specifications). Data was saved in .nd2
357 format and manually processed in ImageJ.

358 *Exploring ecological differences in X. birchmanni* and *X. malinche habitats*

359 One striking environmental difference between *X. birchmanni* and *X. malinche* habitats is
360 water temperature, driven by elevation differences (Fig. S10). In addition, many ecological
361 factors such as primary productivity covary with temperature. While we investigated direct
362 responses to temperature in *X. birchmanni* and *X. malinche* fry (Supplementary Information 11
363 and Table S19), we were also interested in responses to the indirect effects of temperature, such
364 as food availability.

365 Given that *X. malinche* populations likely experience lower food and nutrient availability,
366 we predicted that newborn fry may be more likely to face starvation conditions than *X.*
367 *birchmanni*, and that large offspring size may have evolved as an adaptation to these conditions.
368 To directly test this, we compared starvation tolerance between newborn *X. malinche* and *X.*
369 *birchmanni* fry in the lab. We collected four broods of fry from each species and split individuals
370 in a brood evenly between one experimental and one control tank. Fry in the control tanks were
371 fed brine shrimp twice daily for three days while fry in experimental tanks were not fed for those
372 three days. Observations of physical state and behavior were recorded twice per day at 10:00 and
373 17:00 during the laboratory light period (09:00-18:00). At the end of the three-day trial, fry were
374 euthanized with MS-222, photographed so that standard length could be measured with ImageJ,
375 and prepared for total lipid extraction to measure differences in dry weight and fat content
376 between control and experimental fry (see below). After model selection, we fit a mixed linear
377 model (R nlme::lme) for standard length, including species, treatment, and their interaction as
378 fixed effects and brood number as a random effect. Pairwise comparisons between the interaction
379 of species and treatment were made with an ANOVA and Tukey post-hoc test with R

380 emmeans::emmeans and partial residuals were calculated and plotted with lme4::lmer and
381 visreg::visreg. See Table S20 for raw data and Table S21 for statistics.
382
383 *Lipid extraction and fat content analysis of wild-caught females and lab-born fry*
384 *Xiphophorus* species were thought to reproduce year-round. However, in sampling
385 natural *X. malinche* and *X. birchmanni* populations for this project we detected clear evidence for
386 seasonality in breeding in *X. malinche* populations (see Results). To more systematically
387 evaluate this, we opportunistically collected pregnancy rates for collections made in August
388 2020, May 2022, September 2022, and February 2023 from the Chicayotla *X. malinche* and
389 Coacuilco *X. birchmanni* populations (Table S22).
390 Dry weight and body fat content are indirect measures of nutrient availability in a fish's
391 environment. We dry weighed and extracted total lipids from wild-caught nonpregnant
392 Chicayotla *X. malinche* and Coacuilco *X. birchmanni* adult females caught in September 2022
393 and February 2023. We also collected dry weight and total lipid data from the fry subject to the
394 food deprivation trials described above. For adult fish, abdominal organs (i.e. digestive,
395 excretory, and reproductive tissue from the abdomen) were dissected out. Fry were left intact.
396 Fish were placed on a tray wrapped with foil and dried in a drying oven set to 65°C for five days.
397 After desiccation, dried fish were weighed to obtain dry weight prior to lipid extraction
398 ($weight_1$). To extract lipids, each dried fish was placed in a 5mL glass vial of petroleum ether.
399 After 24 hours, the petroleum ether was drained and replaced with fresh petroleum ether. This
400 process was repeated once more to ensure complete lipid extraction, for a total of three
401 petroleum ether washes. After the third wash, fish were dried at 65°C for 24 hours and then dry

402 weighed to obtain dry weight after lipid extraction ($weight_2$). Fat content percentage was
403 calculated as:

404 $$Fat\ Content\ \% = \frac{weight_1 - weight_2}{weight_1} \times 100$$

405 Differences in fat content for nonpregnant females for each season were evaluated with a
406 Student's two-sided t-test. Differences in dry weight and fat content between species and
407 treatments for food deprivation trial fry were evaluated with the same methods described for fry
408 standard length (see above), with the same model selected (Tables S20-21).
409
410 *Measuring viability by cross direction*
411 In addition to comparing embryo size in F_1 hybrids, we quantified differences in survival
412 of F_1 fry as a function of cross direction. We used additional F_1 crosses generated in the lab to do
413 so. For these crosses, we monitored females twice daily, and provided low-density and high-
414 cover conditions for females that were morphologically identified as being close to giving birth.
415 New broods were morphologically scored for being full-term or premature, and stillborn fry were
416 identified and counted. Tanks of collected fry were monitored twice daily. For crosses with high
417 mortality rates, survival to two weeks was recorded for surviving fry (Table S23).
418
419
420

421 **Results**

422 *X. malinche newborn fry are exceptionally large*

423 To evaluate variation in offspring size at birth across *Xiphophorus*, we measured the
424 standard lengths and head widths of newborn fry from five species: *X. birchmanni, X. malinche,*
425 *X. cortezi, X. pygmaeus*, and *X. variatus* (Fig. 1A, Supp. Fig. S1-2, Table S1). We found
426 significant differences in size across species (Table S2). Notably, *X. malinche* have the largest
427 fry (10.8 mm $\pm$ 1.1 mm; adjusted p-value<0.005 for all five comparisons, see Table S2). Despite
428 this difference in offspring size, *X. birchmanni* and *X. malinche* did not consistently differ in the
429 number of offspring per brood (although offspring size varied as a function of mother size and
430 collection season; Supp. Fig. S3). Moreover, adult females of the two species grown in common
431 garden conditions were similar in size (Supp. Fig. S4). Although *X. birchmanni* fry are
432 significantly smaller than *X. malinche* fry, they are larger than fry of other *Xiphophorus* species
433 (Fig. 1; Table S2).

434 We also compared the size of newborn *X. malinche x X. birchmanni* F_1 and F_2 fry. We
435 found that F_1 standard length and head width was *X. malinche*-like and F_2 and natural hybrid fry
436 were intermediate in size to their parent species (Fig. 1B, Supp. Fig. 2B, Tables S1-2). After
437 accounting for the effects of collection date and brood ID, we estimated the broad-sense
438 heritability of standard length at birth attributable to ancestry to be 0.27 (see Supplementary
439 Information 1).

440

441 *X. malinche mothers provision developing embryos*

442 Given that *X. malinche* fry are significantly larger at birth than *X. birchmanni*, we
443 hypothesized that *X. malinche* embryos are also larger throughout embryonic development. We

444 measured the dry weight of embryos dissected from wild-caught *X. malinche* and *X. birchmanni*
445 pregnant females across developmental stages. We found that *X. birchmanni* embryos show a
446 classically lecithotrophic developmental profile, where embryos lose weight overall across
447 development (Fig. 2A). In contrast, *X. malinche* embryos maintain roughly the same dry weight
448 throughout development.

449 Given that *X. birchmanni* and *X. malinche* unfertilized eggs do not differ in size at
450 fertilization (p-value=0.98, Fig. 2B), the maintenance of weight over development by *X.*
451 *malinche* embryos suggests that there is some level of post-fertilization nutrient provisioning
452 from *X. malinche* mothers. We estimated the Matrotrophy Index for each species to obtain a
453 quantitative metric of nutrient provisioning during development, using three calculations with
454 different "fully-developed" embryo stage categories: mean stage 50, mean stage 45, and mean of
455 stages 45 and 50 combined (see Supplementary Information 3). After accounting for collection
456 date and mother standard length, we calculated Matrotrophy Indices of 0.65, 0.69, and 0.69 for
457 *X. birchmanni*, respectively, and 0.99, 0.90, and 0.95 for *X. malinche*, respectively. Because we
458 collected many stage 50 *X. malinche* broods (N=7), the most accurate estimate of Matrotrophy
459 Index for *X. malinche* is 0.99. We also attempted to calculate Matrotrophy Index for the sister
460 species to the *X. malinche-X. birchmanni* clade, *X. cortezi*, but our small sample size led to
461 uncertainty in these estimates (Supplementary Information 3).

462

463 *Late-stage F_1 embryos in the X. malinche x X. birchmanni cross are intermediate in size*

464 To compare the size of mid- and late-stage embryos (stage ≥20) from the reciprocal F_1
465 crosses to pure parental crosses, we made controlled crosses between *X. malinche* and *X.*
466 *birchmanni* (see *Methods*). Consistent with our measures from wild-caught broods, lab-raised *X.*

467 *malinche* embryos are significantly larger than *X. birchmanni* embryos (p-value<0.0001). We
468 find that F_1 embryos from the *X. malinche* mother and *X. birchmanni* father cross direction trend
469 towards being smaller than *X. malinche* embryos (p-value=0.05) but are significantly larger than
470 *X. birchmanni* embryos (p-value<0.0001). This suggests that in an *X. malinche* maternal
471 environment, both maternal effects and offspring genotype contribute to offspring size at birth.
472 By contrast, F_1 embryos with *X. birchmanni* mothers are similar in size to *X. birchmanni*
473 embryos (Fig. 3C; see Table S10 for summary statistics).
474 Given that mid- and late-stage embryos are larger in *X. malinche* than *X. birchmanni*, we
475 tested whether higher genome-wide *X. malinche* ancestry in naturally occurring hybrids predicts
476 larger embryo size. We sampled pregnant mothers carrying stage ≥ 20 broods from the Calnali
477 Low natural hybrid population, since embryo size diverges between species after stage 15. After
478 accounting for stage and brood ID, we found that proportion of *X. malinche* ancestry in the
479 mother's genome and its interaction with stage are strongly positively associated with embryo
480 dry weight (L-ratio=61.6, p-value<0.0001, R=0.37; Supp. Fig. S6A). Unexpectedly, we find a
481 significant negative relationship between the mother's ancestry and ovary dry weight (F-
482 value=2.90, p-value=0.02), suggesting that greater *X. birchmanni* ancestry in a hybrid genome
483 predicts higher ovary dry weight.
484
485 *X. malinche and X. birchmanni ovaries do not have clear morphological differences*
486 To explore potential differences in ovarian structures between species, we compared
487 histological slides of sectioned ovaries. In highly matrotrophic livebearing fishes, nutrients are
488 delivered to developing embryos through the maternal follicle, which is thicker and highly
489 vascularized (Kwan et al. 2015; Guernsey et al. 2020; Ponce de León and Uribe 2021). Visually,

490 we did not observe consistent differences in maternal follicle thickness, amount of vasculature,
491 or overlap of maternal and embryonic membranes between sections of ovaries carrying
492 unfertilized eggs or late-stage embryos from *X. malinche* and *X. birchmanni* (Fig. 2D;
493 Supplementary Information 6).

494 We also sought to determine whether ovarian follicular tissue is maternal or embryonic in
495 origin (see Supplementary Information 7). Taking advantage of gene expression data from late
496 pregnancy *X. malinche* mothers carrying *X. malinche x X. birchmanni* F_1 broods, we found that
497 the *X. malinche* allele alone was expressed at nearly all ancestry informative sites, indicating that
498 the ovarian follicle is maternally-derived (Supp. Fig. S7).

499

500 *Patterns of gene expression in X. malinche, X. birchmanni, and X. cortezi ovaries*

501 To gain insight into the mechanisms underlying differences in reproductive strategy
502 between these closely related species, we compared gene expression patterns in ovarian tissue
503 from *X. malinche, X. birchmanni,* and *X. cortezi* from "early" pregnancy (fully-yolked
504 unfertilized or stage <5) and "late" pregnancy (25-45; Table S15). Principal component analysis
505 shows that PC1 explains about 31% of the variation in ovary expression and correlates with
506 species while PC2 explains about 17% of total variation and correlates with species and rearing-
507 environment (Fig. 3A). Within *X. malinche*, expression patterns in ovarian tissues separate along
508 PC1 by the developmental stage of the embryos they contained, while in *X. birchmanni* and *X.*
509 *cortezi* they do not (Fig. 3A). Of the genes expressed in the ovaries of both species (adjusted p-
510 value<0.05), *X. malinche* differs from *X. birchmanni* in expression for 13% (2,202) and 28%
511 (4,934) of genes in early and late pregnancy, respectively, and from *X. cortezi* for 45% (7,957)
512 and 32% (5,639) of genes, respectively. Notably, we see that more genes respond to embryonic

513 developmental stage in *X. malinche* ovaries (310) compared to *X. birchmanni* (63) and *X. cortezi*
514 (130) ovaries. Although we do not focus on them in the main text, we performed similar analyses
515 of genes that were differentially expressed in developing embryos across the three species
516 (Supplementary Information 8).

517 Several genes that are specifically involved in pregnancy and nutrient provisioning in
518 placental taxa, including mammals and matrotrophic poeciliids, are significantly upregulated in
519 *X. malinche* compared to its close relatives. Notably, we find that the prolactin signaling
520 pathway, which has been implicated in the evolution of matrotrophy in livebearers (Guernsey et
521 al. 2020), is strongly upregulated in *X. malinche* ovaries. Prolactin receptor and releaser genes
522 are highly expressed in *X. malinche* ovaries in both early and late pregnancy compared to *X.*
523 *birchmanni* and *X. cortezi* ovaries (adjusted p-value<0.001 for all comparisons; Fig. 3B).
524 Prolactin receptors (*prlr*) bind the hormone prolactin and are thought to be directly involved in
525 trophoblast invasion for nutrient uptake in mice (Stefanoska et al. 2013). Prolactin-releasing
526 peptide receptor (*prlhr*) regulates prolactin expression. Interestingly, *prlhr* is also upregulated in
527 late pregnancy *X. birchmanni* ovaries (adjusted p-value<0.001), while we did not detect
528 expression of this gene in *X. cortezi* ovaries (Fig. 3B).

529 Though there is transcription of prolactin receiving and releasing machinery in late-
530 pregnancy *X. malinche* and *X. birchmanni* ovaries, we do not see expression of prolactin itself
531 based on RNAseq data (as expected since prolactin is a hormone translated in the pituitary
532 gland). To evaluate whether prolactin protein appears in *X. malinche* and *X. birchmanni* ovaries,
533 we performed immunohistochemistry on sections of late pregnancy ovaries for both species (Fig.
534 3C). We find that prolactin protein is present in both *X. malinche* and *X. birchmanni* maternal

535 ovarian follicle and embryonic tissues. Additionally, as expected, prolactin is present in retinal
536 and mesenchymal tissue of embryos in both species (Fig. 3C).

537 To explore biological pathways potentially involved in nutrient transfer and provisioning
538 in *X. malinche*, we first looked for enriched Gene Ontology pathways in genes that were
539 significantly differentially expressed between *X. malinche* and *X. birchmanni* ovaries. Notably
540 artery development is enriched, and the genes in this category are upregulated in *X. malinche*
541 ovaries (p-value < 0.002; Supp. Fig. S8). We also see enrichment of several potentially relevant
542 metabolism and nutrient transport pathways, including acyl- and acetyl-CoA metabolism in late-
543 stage *X. malinche* ovaries (Table S16).

544 We also looked at groups of co-expressed genes and asked which of these groups are
545 significantly associated with late-stage pregnancy in *X. malinche* (see Supp. Fig. S10 for module-
546 trait correlation matrix). Using WGCNA, we identified 17 ovary co-expression gene clusters
547 significantly associated with *X. malinche* ancestry (p-value<0.05), 11 of which were associated
548 with late-pregnancy *X. malinche* ovaries. Gene Ontology enrichment of these clusters revealed
549 that the 'darkorange2' cluster was highly enriched for acyl- and acetyl-CoA biosynthesis and
550 metabolic pathways, as well as vascular growth and signaling pathways, like artery development
551 and vasculogenesis (Fig. 3D, Supp. Fig. S9). Many of the represented acyl- and acetyl-CoA
552 metabolism genes are strongly upregulated or downregulated in *X. malinche*, particularly in late
553 pregnancy, compared to *X. birchmanni* and *X. cortezi* ovaries (Fig. 3D, Supp. Fig. S9). In
554 mammals, fatty acids like acyl-CoA are an important source of energy for both placental and
555 fetal growth during gestation and are transferred through the placenta to the fetus during mid- to
556 later-stages of pregnancy (Chavan-Gautam et al. 2018). The 'darkorange' cluster was enriched in

557 amino acid biosynthesis and metabolism pathways, and the 'violet' cluster was enriched for
558 intracellular and transmembrane signaling pathways.
559
560 *Testing the potential role of resource availability in driving the evolution of large offspring size*
561 We tested the response of offspring of both species to two ecological factors that differ
562 between the *X. malinche* and *X. birchmanni* environments. We found that the larger offspring of
563 *X. malinche* did not have improved minimum thermal tolerance compared to *X. birchmanni* fry
564 (Supplementary Information 11). We thus turned to investigating another ecological factor that
565 correlates with temperature: food availability. Cooler, headwater habitats are generally less
566 resource-rich than warmer downstream habitat (Reznick 1982*b*). Given low winter temperatures
567 observed in *X. malinche* populations (Supp. Fig. S11; Payne et al. 2022), we asked whether cold
568 temperatures (and presumably low primary production and food availability associated with such
569 temperatures) impacted breeding cycles in *X. malinche* populations and starvation tolerance in
570 newborn fry.
571 Water temperatures at *X. malinche* and *X. birchmanni* sites are coolest from November to
572 March with peak lows in January and February and are warmest from April to October with peak
573 highs in May and June (Supp. Fig. S11). The shifts in temperature roughly correspond with the
574 rainy season and river flooding, which typically starts late June and ends in October. We
575 collected mature females from natural populations of both *X. birchmanni* and *X. malinche* across
576 seasons. We find that while many *X. birchmanni* females were reproducing regardless of
577 sampling month or season (the lowest pregnancy rate observed was 34% in February), <20% of
578 *X. malinche* mature females were pregnant from February – August, while 80% were pregnant in
579 May (Supp. Fig. S12; Table S22).

To evaluate how relative female condition may vary seasonally, we compared total lipids from wild nonpregnant Chicayotla *X. malinche* and Coachuilco *X. birchmanni* females and found temporal differences in fat content between species. In February, before the *X. malinche* breeding season, we found no significant difference in percent fat content between *X. malinche* and *X. birchmanni* females (t-value=1.64, p-value=0.12; Fig. 4A). However, in September, after the *X. malinche* breeding season, *X. birchmanni* females had significantly higher fat content than *X. malinche* (t-value=-12.45, p-value=1.30×10^{-6}), consistent with limited resource availability in *X. malinche* habitat.

To directly compare how newborn fry of the two species respond to reduced food availability, we exposed *X. malinche* and *X. birchmanni* newborn fry to abundant-food and no-food conditions and compared standard length, dry weight, and fat content between treatments and species. Surprisingly, after three days of feeding, *X. malinche* and *X. birchmanni* fry were approximately the same standard length and dry weight (ANOVA and Tukey post-hoc test p-value=0.76 and 0.91, respectively). Compared to their initial standard lengths, *X. birchmanni* fry grew 4× more rapidly (~0.32mm/day) than *X. malinche* fry (~0.08mm/day) (Supp. Fig. 13A). However, while both *X. malinche* and *X. birchmanni* unfed newborn fry weighed less than their fed counterparts, we found that on average food deprivation for three days impacted *X. malinche* fry weight less than *X. birchmanni* fry, suggesting that they may be more resilient to starvation immediately after birth (Fig. 4B). We found no significant difference in total body fat content between species for either treatment (Supp. Fig. S13B).

603 *Differences in viability by cross direction*

604 In generating F_1 crosses for this and other projects, we have found a clear difference in
605 the frequency of premature birth and stillbirth in crosses between *X. malinche* mothers and *X.*
606 *birchmanni* fathers compared to crosses between *X. birchmanni* mothers and *X. malinche* fathers.
607 Given that these crosses are genetically identical except for the Y chromosome and
608 mitochondrial haplotype (Moran et al. 2021), this raises the possibility that maternal effects may
609 impact survival of the F_1 fry.

610 We found high rates of premature birth in crosses between *X. birchmanni* mothers and *X.*
611 *malinche* fathers (Table S23). Of 25 crosses generated by artificial insemination between 2011-
612 2022, only eight F_1 fry survived (out of 64 born). In five of these crosses, the mother also
613 suffered mortality. In sixteen out of the 25 crosses, mothers gave birth to morphologically
614 premature fry (Supp. Fig. S14), which have a low probability of survival (~98% mortality,
615 Moran et al. 2021). In the ten crosses where fry were born at term, 50% contained stillborn fry,
616 versus 7% for premature broods (Fisher's exact p-value=0.04). Maternal mortality was observed
617 in 30% of these full-term crosses, versus 13% of crosses where fry were born prematurely
618 (though this difference was not significant; Fisher's exact test p-value=0.1). For cases where we
619 developmentally staged premature fry, individuals tended to be around stage 40.

620 By contrast, we have never documented a premature birth, stillbirth, or maternal
621 mortality for *X. malinche* mothers. To date we have generated >190 adult F_1 hybrids and 1,253
622 adult F_2 hybrids from intercrossing these individuals. Premature birth has also not been observed
623 in F_2 crosses between these F_1 individuals.

624

625

626 **Discussion**

627 Offspring size is a complex trait that impacts both parent and offspring fitness. Compared
628 with its sister species, *X. birchmanni*, and other *Xiphophorus* species, *X. malinche* produce
629 exceptionally large fry. Though offspring size has been shown to be sensitive to ecological
630 conditions and the maternal environment in livebearers (Reznick 1982*a*; Reznick and Yang
631 1993; Reznick et al. 1996*b*; Bashey 2006*b*, 2008), we see this size difference in lab-raised
632 individuals, and estimate that the broad-sense heritability of offspring size attributable to
633 ancestry is 0.27. Moreover, the proportion of the genome derived from *X. malinche* impacts
634 offspring size in natural hybrid populations.

635 Larger offspring size generally increases the probability of survival to adulthood across a
636 variety of species (Brockelman 1975; McGurk 1986; Tessier and Consolatti 1989; Einum and
637 Fleming 2000; Marshall and Keough 2007; Jørgensen et al. 2011; Rollinson and Hutchings 2013;
638 Bowen et al. 2015). Given that *X. malinche* live in unique environments compared to other
639 species in the genus, we predicted that larger offspring size may have evolved in response to
640 ecological pressures – specifically, limited nutrient availability. Sampling for this study revealed
641 that while *X. birchmanni* breed across seasons, *X. malinche* breed seasonally, with peak
642 productivity in the warmest months. Notably, before the *X. malinche* breeding season, we find
643 that nonpregnant *X. malinche* and *X. birchmanni* females have comparable body fat content.
644 However, after the *X. malinche* breeding season, *X. malinche* have significantly lower body fat
645 content than *X. birchmanni* females, hinting that resources are generally more limited in *X.*
646 *malinche* habitat.

647 Past work in guppies has shown that under limited food conditions, mothers produce
648 larger offspring (Reznick 1982*b*; Reznick and Yang 1993; Reznick et al. 1996*b*; Bashey 2006*b*),

649 which presumably have a fitness advantage in lower resource environments (Pettersen et al.
650 2015, 2022). We directly tested offspring performance in food-depleted conditions in a
651 controlled lab experiment. We find that starved newborn *X. malinche* retain more weight than
652 newborn *X. birchmanni*. This suggests that *X. malinche* fry may be more resilient to a low
653 resource environment, consistent with resource limitation (or high competition) as a driver of the
654 evolution of large offspring in *X. malinche* (see also Bashey 2006*b*, 2008). Notably, in scenarios
655 where resources were not limited, newborn *X. birchmanni* fry grew more rapidly than *X.*
656 *malinche* and matched their size within days. This pattern could hint that there is an advantage to
657 being large early, consistent with previous work in other taxa (Chambers et al. 1989; Gliwicz and
658 Guisande 1992). The relationship between energetics and offspring size is an exciting area for
659 future research.

660 There are two mechanisms through which livebearing fish can produce larger fry. First,
661 mothers may produce larger eggs. Second, mothers may continue to provision nutrients to
662 offspring over embryonic development (note that matrotrophy does not predictably lead to large
663 offspring at birth across taxa; Furness et al. 2021). We evaluated evidence for both mechanisms
664 in *X. malinche*. We measured dry weights for embryos across ten embryonic stages ranging from
665 unfertilized fully-yolked eggs to fully developed juveniles for *X. malinche, X. birchmanni,* and
666 their close relative, *X. cortezi*. Notably, unfertilized fully-yolked eggs and early stage embryos
667 are similar in size between *X. malinche* and *X. birchmanni* (Fig. 2). However, *X. malinche* and *X.*
668 *birchmanni* embryos diverge in size around stage 20 of embryonic development. While *X.*
669 *birchmanni* embryos undergo a decline in dry weight after stage 15, *X. malinche* embryos
670 maintain roughly the same weight over development. We summarize this difference in
671 developmental profile with the first estimates of Matrotrophy Index for *X. malinche* (0.99) and *X.*

672 *birchmanni* (~0.65). This estimate for *X. malinche* is consistent with *X. malinche* embryos
673 receiving nutrients from their mother over development and explains larger offspring size at birth
674 in *X. malinche*. Moreover, this represents a recent shift in the degree of reproductive investment
675 since the *X. birchmanni* and *X. malinche* lineages diverged only ~250,000 generations ago
676 (Schumer et al. 2018). It is notable that although *X. birchmanni* do not appear to provision
677 nutrients during development, *X. birchmanni* also has fry that are much larger at birth than other
678 *Xiphophorus* species. We were unable to collect sufficient samples to accurately quantify
679 Matrotrophy Index from these species but we predict that they may produce smaller eggs than *X.*
680 *birchmanni*, and indeed the few samples we have are consistent with this prediction (Fig. S17).
681 In matrotrophic species, maternal and embryonic tissues interact to form an interface for
682 nutrient transfer, and some species form placental structures of varying complexity. To look for
683 evidence of simple placental structures in *X. malinche*, we quantified characteristics of the
684 maternal-embryo interface in histological sections of late-stage pregnancy in *X. malinche* and *X.*
685 *birchmanni* ovaries. In extremely matrotrophic poeciliids, embryonic and maternal blood vessels
686 range from apposed to fused (Ponce de León and Uribe 2021) and the ovarian follicular
687 epithelium is dense and maximizes surface area contact with embryos for nutrient transfer (Kwan
688 et al. 2015). We did not see consistent differences in ovarian follicle thickness or vascularization
689 between stained sections of late-stage *X. malinche* and *X. birchmanni* ovaries. However, we
690 found it difficult to compare these traits between 2-dimensional slices of ovary. Because post-
691 fertilization nutrient provisioning in *X. malinche* arose recently, we might predict differences in
692 ovary morphology between these species to be subtle. Future work should focus on obtaining 3-
693 dimensional images suitable for quantitative comparisons of the whole ovary.

694 To further investigate differences in maternal nutrient provisioning at the molecular level,
695 we quantified gene expression of early- and late-stage pregnant *X. malinche* and *X. birchmanni*
696 ovaries. The observed expression patterns raise multiple possible mechanisms through which
697 post-fertilization nutrient provisioning may be occurring in *X. malinche*. Receptor and releaser
698 genes for prolactin, a pituitary hormone important during pregnancy in mammals and in
699 matrotrophic poecilids (Menzies et al. 2011; Guernsey et al. 2020), are highly expressed in the *X.*
700 *malinche* ovarian follicle compared to *X. birchmanni* (and *X. cortezi*) follicles both before and
701 during pregnancy. Combined with evidence that *X. malinche* mothers provision their embryos
702 over development to a greater extent than *X. birchmanni* mothers, this result suggests that
703 prolactin may be a key candidate for the evolution of matrotrophy in livebearers (as proposed by
704 Guernsey et al. 2020). Immunostains of prolactin in sections of late-stage pregnant *X. malinche*
705 and *X. birchmanni* ovaries confirms that prolactin is present in the ovarian follicle of both
706 species. Given these results, we predict that the striking differences in abundance of prolactin
707 releasing and receiving machinery that we observe at the gene expression level (Fig. 3) may
708 regulate the downstream transfer and function of prolactin in the two species, which includes
709 nutrient transport.

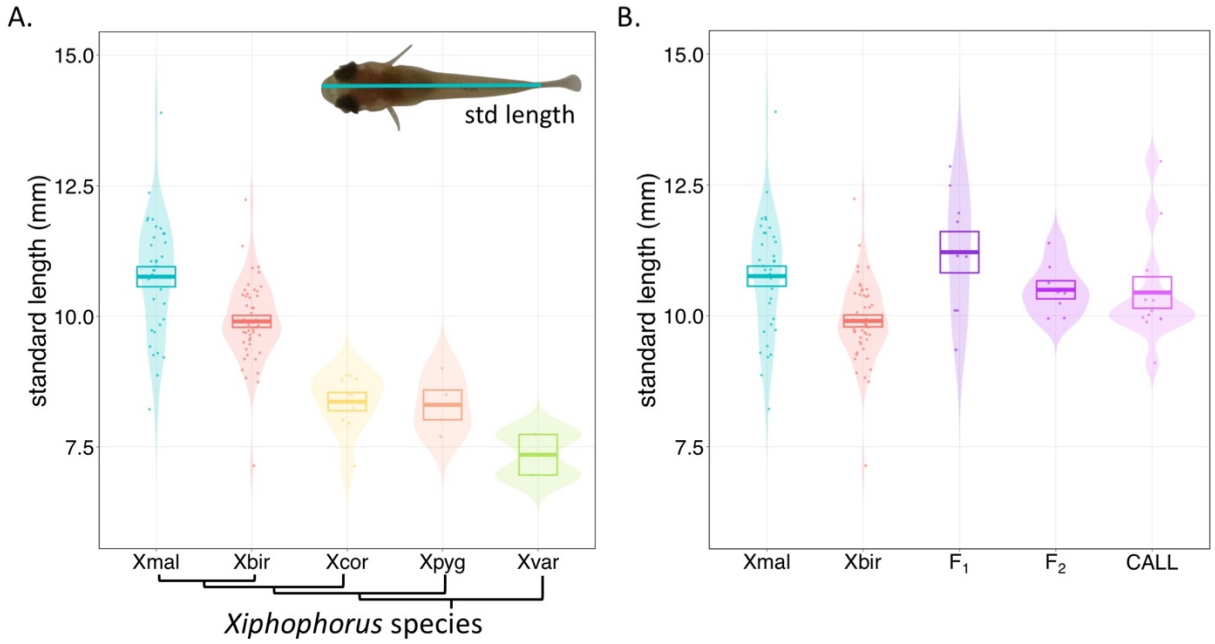
710 While offspring almost universally benefit from being larger at birth, producing large
711 offspring can be costly for parents (Einum and Fleming 2000; Walker et al. 2008; Ljungström et
712 al. 2016) and constrained by environmental conditions (Reznick and Endler 1982; Hutchings
713 1991; Janzen et al. 2000; Allen et al. 2008; Marshall and Keough 2008; Pettersen et al. 2022).
714 These pressures can drive parent-offspring conflict in the evolution of offspring size (Trivers
715 1974; Crespi and Semeniuk 2004; Moore 2012). Similarly, if males and females differ in their
716 level of resource investment in offspring, conflict between the maternal and paternal genomes

717 can drive similar dynamics (Moore and Haig 1991). In mammals where females tend to invest
718 more than males in their offspring, studies have documented paternally contributed factors that
719 increase offspring growth and maternally contributed factors that suppress them (Moore and
720 Haig 1991; Barlow and Bartolomei 2014). Given these findings in other taxa and the large
721 difference in *X. malinche* and *X. birchmanni* embryos during development, we were curious to
722 understand whether this trait impacts reproductive isolation between these species. In the lab, *X.*
723 *malinche* mothers and *X. birchmanni* fathers successfully produce viable F_1 offspring and
724 subsequent early-generation crosses, whereas the reverse cross is typically aborted late in
725 pregnancy (by stage 40). This asymmetry in reciprocal F_1 cross viability has been shown to stem
726 from differences in nutrient provisioning over development in other poeciliid species (Schrader
727 et al. 2013). We initially hypothesized that differences in provisioning strategy might result in
728 embryos that are too large for *X. birchmanni* mothers to sustain. However, we found no
729 differences in stage-matched offspring weight between pure *X. birchmanni* crosses and F_1 hybrid
730 crosses with *X. birchmanni* mothers. Alternatively, the energetic demands of F_1 embryos may
731 outpace the nutrients stored in yolk from *X. birchmanni* mothers, which could lead to
732 malnourishment. Consistent with this hypothesis, we find that many offspring from this cross are
733 stillborn and the majority are born prematurely.

734 Our data also shows that offspring size is a complex trait, and the size of any individual
735 offspring is impacted by many variables, including developmental stage, brood size, mother size,
736 and mother environment. Accounting for all of these effects make this trait difficult to
737 characterize, complicating comparisons of offspring size and embryonic development profiles
738 between livebearing species. Recently, Skalkos et al. (2023) reviewed the current limitations in
739 the way matrotrophy has been studied in livebearers. Our findings underscore several of their

740 points, including that direct measures of early and late stage embryo weight, rather than indirect
741 measures using regression-based approaches, should be used to estimate Matrotrophy Index
742 where possible (since stages are morphological categories that do not linearly track
743 developmental time). Moreover, if provisioning varies over development (as hinted at by our
744 data), these methods could suffer from reduced accuracy. Our results further suggest that a single
745 collection may not be sufficient to capture the complex factors impacting the estimate of
746 Matrotrophy Index for an entire species. Future work should consider these factors. We note that
747 in our work using lab populations served as a powerful resource for confirming patterns inferred
748 from wild populations (Fig. 2), as they allowed us to control for many of the environmental
749 variables that impact wild collections.
750 Using a combination of morphometric and molecular data, we provide the first
751 description of incipient matrotrophy and post-fertilization nutrient provisioning mechanism in
752 the *Xiphophorus* clade and document immense variation in offspring size across species.
753 Importantly, because *X. malinche* and *X. birchmanni* are recently diverged (~250,000
754 generations ago), this is an example of rapid evolution of reproductive strategy. Parent of origin
755 incompatibility phenotypes associated with spontaneous abortion late in development hint that
756 this difference in reproductive strategy could contribute to the diverse reproductive barriers
757 present in these young sister species.
758
759
760
761
762
763

764 **Figures**



765

766 **Figure 1**. Comparisons of offspring size at birth across *Xiphophorus* species and their hybrids.

767 **A)** Violin plots of standard length (mm) of newborn fry from five *Xiphophorus* species – *X.*

768 *malinche*, *X. birchmanni*, *X. cortezi*, *X. pygmaeus*, and *X. variatus*. Phylogenetic relationships

769 between species are shown schematically on the x-axis (following Preising et al. 2022). **B)**

770 Violin plots of standard length (mm) of newborn fry of different groups, including *X. malinche*,

771 *X. birchmanni*, F_1 hybrids, F_2 hybrids, and natural hybrids ("CALL" – Calnali Low hybrid

772 population). For both **A** & **B**, each data point represents the average standard length of newborn

773 fry from one brood. For each group, boxes show the mean ± 1 standard error.

774

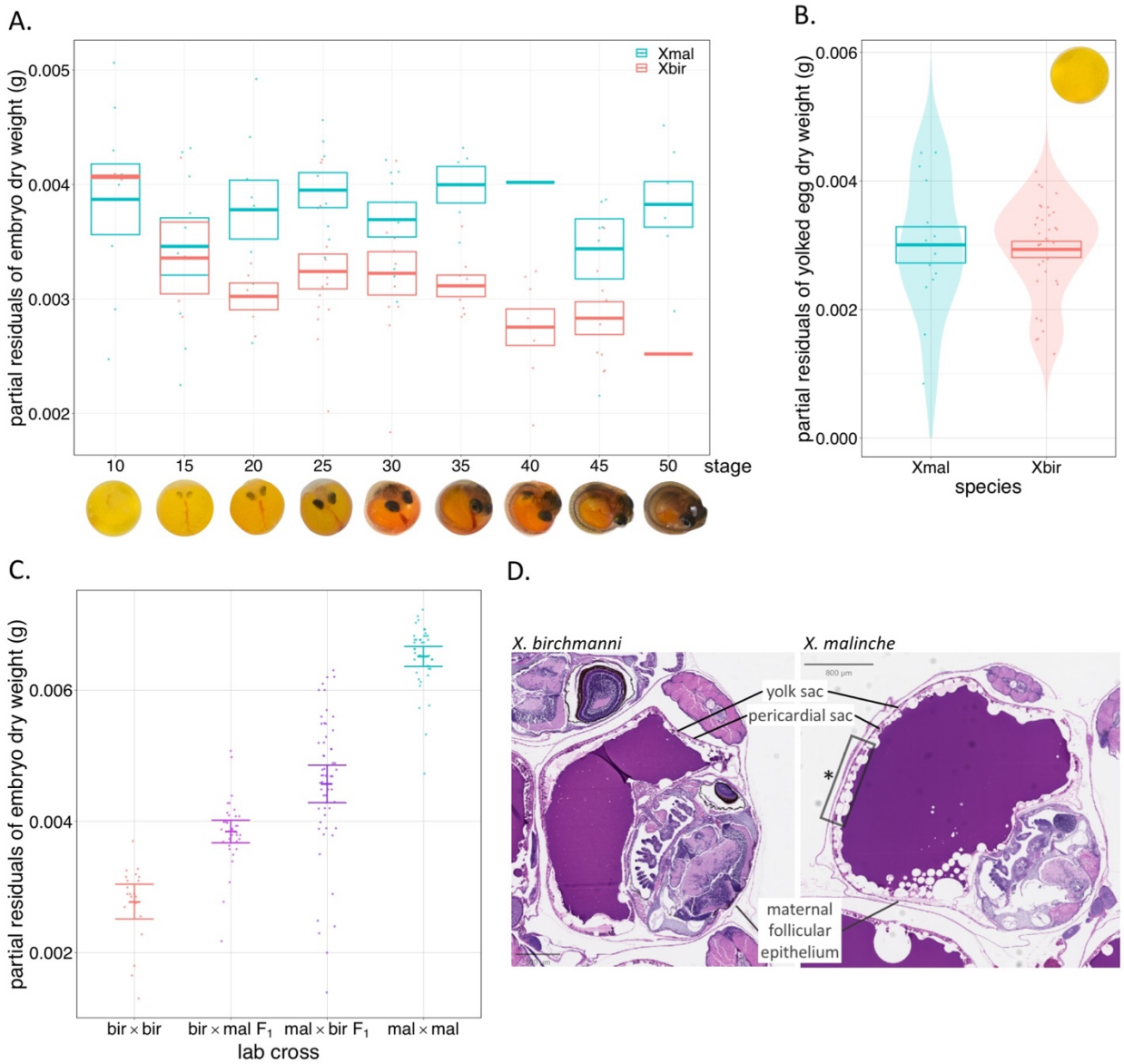


Figure 2. Comparisons of embryo weight throughout development and ovarian structures between *X. birchmanni* and *X. malinche*. **A**) Embryo size developmental profiles for *X. birchmanni* (Xbir – pink) and *X. malinche* (Xmal – blue). Developmental stage is plotted on the x-axis (with images of *X. malinche* embryos at corresponding stages) and the partial residuals of average embryo dry weight per brood (after correcting for maternal size and collection date) is plotted on the y-axis. Points show the partial residuals colored by species. For each species, boxes show mean ±1 standard error. These profiles were generated from wild-caught fish. *X.*

783 *birchmanni* and *X. malinche* have similar weights early in development but diverge in weight
784 after stage 15. **B**) Violin plot comparing stage 0 (fully-yolked but unfertilized) eggs between *X.*
785 *birchmanni* and *X. malinche*. Points show the partial residuals of average egg dry weight per
786 brood and boxes show mean and ±1 standard error. *X. birchmanni* and *X. malinche* eggs are not
787 significantly different in size at stage 0. These profiles were generated from wild-caught fish. **C**)
788 Embryo dry weights from multifactorial crosses between species. Patterns observed in **A** are
789 replicated in stage ≥20 lab-crossed parental species raised under common conditions, with *X.*
790 *birchmanni* embryos being significantly smaller than *X. malinche* later in development. We find
791 that F_1 hybrids from *X. birchmanni* mothers (bir×mal F_1) do not differ significantly in size from
792 pure *X. birchmanni*, while F_1 hybrids from *X. malinche* mothers (mal×bir F_1) are significantly
793 larger than the alternate cross (p-value=0.02) but trend towards being smaller than pure *X.*
794 *malinche* fry (p-value=0.05), hinting that the trait is impacted by both genetic and maternal
795 effects. **D)** Histological slides stained with Hematoxylin & Eosin of *X. birchmanni* (left) and *X.*
796 *malinche* (right) ovaries derived from females pregnant with late-stage embryos (stage 35 and
797 40, respectively) are structurally similar.
798

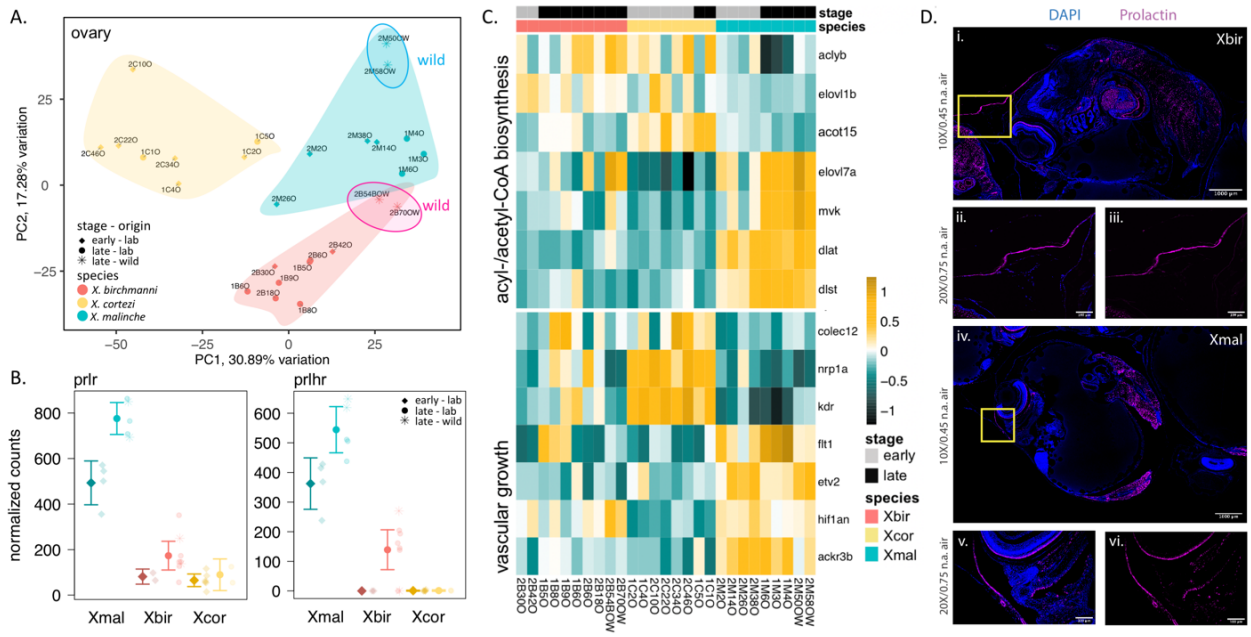


Figure 3. Gene expression analysis hints at a role for prolactin, fatty acid biosynthesis, and vascular growth pathways in nutrient provisioning post-fertilization in *X. malinche*. **A**) Principal component analysis of transformed gene abundance counts from ovary RNAseq data from *X. birchmanni* (pink), *X. malinche* (blue), and *X. cortezi* (yellow). For each species, samples included ovarian tissue from both early- and late-stage pregnancies. For *X. birchmanni* and *X. malinche,* two wild-caught samples were also analyzed (denoted by stars and labels). Samples cluster by species, and for *X. malinche*, by developmental stage. Colored envelops show the space in PC1 and PC2 occupied by all samples of a given species. **B**) Prolactin receptor (*prlr*) and releaser (*prlhr*) genes are upregulated in *X. malinche* ovaries, regardless of pregnancy status or sample origin. For each species, mean normalized read count ±2 standard error is shown for early-pregnancy samples on the left (denoted with diamonds) and late-pregnancy samples on the right (denoted with circles). Semi-transparent points show data from each individual (stars for wild-collected). **C)** Expression heatmap of genes from acyl- and acetyl-CoA biosynthesis and vasculature development GO pathways that were enriched in the "darkorange2" WGCNA

814 cluster, which was significantly associated with late pregnancy *X. malinche* ovaries. Sample IDs
815 appear on the x-axis, gene annotations appear on the y-axis. The blue to yellow color bar
816 indicates the difference in expression, with yellow colors indicating greater expression than
817 average and blue colors indicating lower expression than average. **D**) Immunofluorescence
818 demonstrates the presence of the prolactin protein (magenta) in sections from both pregnant *X.*
819 *birchmanni* females (i.-iii.) and *X. malinche* (iv.-vi.). As expected, prolactin positive cells are
820 identified throughout the developing embryo in mesenchymal and neuronal tissues (such as the
821 eye, i., iv.). Both species also express prolactin in the maternal membranes surrounding the ovary
822 and embryos. Yellow boxes identify the area highlighted in the higher magnification images. iii.
823 and vi. show prolactin signal only, without DAPI counterstain.
824

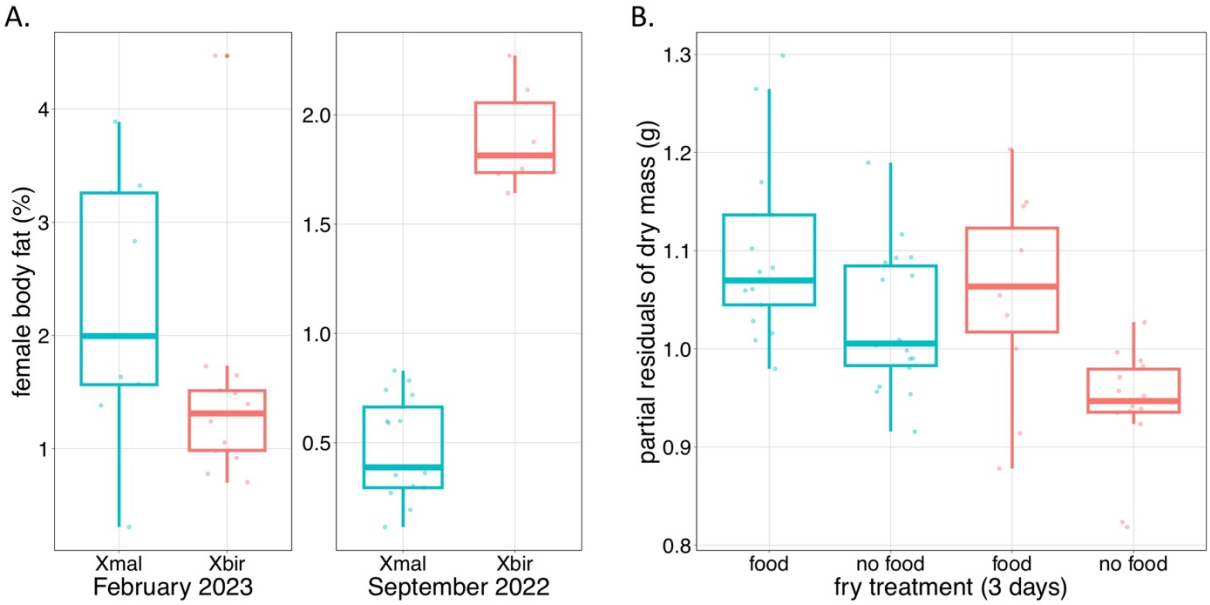


Figure 4. Comparisons of responses to food availability in *X. malinche* and *X. birchmanni*. **A**) Boxplots showing fat content of non-pregnant *X. malinche* and *X. birchmanni* females collected from wild populations in different seasons. *X. malinche* females have much lower fat content before their breeding season than *X. birchmanni*. Note that fat content is not directly comparable across the two collections given that samples were processed in different batches. **B**) Results of starvation experiments in newborn *X. birchmanni* and *X. malinche* fry. *X. birchmanni* (pink) and *X. malinche* (blue) did not significantly differ in weight three days after birth when both were fed, indicating that *X. birchmanni* fry gain weight rapidly after birth (see Fig. 1A). However, in starvation conditions, *X. birchmanni* fry weighed significantly less than *X. malinche* fry, suggesting that *X. malinche* fry have improved tolerance to starvation.

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Data Availability

All data is publicly available. Raw sequences are available under NCBI BioProject PRJNAXXXX. All code and data files can be found in the GitHub repository: https://github.com/cypayne/swordtail-offspring-size.

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