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

Cheyenne Y. Payne[1,2,3,4], Derek Ly[5], Rebecca A. Rodriguez-Soto[1], Daniel L. Powell[1,2], Nim D. Robles[1,2], Theresa Gunn[1], John J Bazcenas[1], Abby J. Bergman[6], Alexa Pollock[1], Ben M. Moran[1,2], Julie C. Baker[6], David Reznick[5], Molly Schumer[1,2,7]

[1]Department of Biology, Stanford University

[2]Centro de Investigaciones Científicas de las Huastecas "Aguazarca", A.C.

[3]Department of Ecology and Evolutionary Biology, University of California, Santa Cruz

[4]Southwest Fisheries Science Center, National Marine Fisheries Service, National Oceanic and Atmospheric Administration

[5]Department of Biology, UC Riverside

[6]Department of Genetics, Stanford University

[7]Freeman Hrabowski Fellow, Howard Hughes Medical Institutes

*Correspondence to: cypayne@ucsc.edu and schumer@stanford.edu

Key words: swordtail fish, matrotrophy, offspring size, hybridization

Abstract

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 evidence 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.

Another mechanism for increasing offspring survival that has recurrently evolved is viviparity, or internal development of embryos. Viviparity reduces size-dependent mortality of

61 eggs and embryos (Jørgensen et al. 2011) and can offer improved environmental conditions for
62 developing offspring. For some species, it provides an opportunity to directly provision nutrients
63 to developing offspring (Griffith and Wagner 2017). While viviparous species typically provide
64 some level of nutrition to their offspring in the form of yolk in the egg, a strategy known as
65 lecithotrophy, some provision nutrients both before and after fertilization, a strategy known as
66 matrotrophy (Wourms et al. 1988). This post-fertilization provisioning is mediated through
67 physical interfaces between parent and offspring tissue, such as the mammalian placenta
68 (Meredith et al. 2011). While the complex mammalian placenta evolved once over 100 million
69 years ago (Meredith et al. 2011), maternal provisioning structures of varying complexity have
70 evolved over 130 times across vertebrates (Blackburn 2015; Whittington et al. 2022). Lineages
71 in which nutrient provisioning evolved recently may be especially useful in understanding the
72 pressures that drive post-fertilization nutrient provisioning.

73 Poeciliid fishes offer an opportunity to study the evolutionary drivers of viviparity, nutrient
74 provisioning strategies, and variation in offspring size. The common ancestor of poeciliids
75 evolved internal fertilization and live birth of juvenile fish, or fry (Pollux et al. 2009), and
76 species vary widely in the degree of post-fertilization nutrient provisioning (Reznick et al. 2002;
77 Pires et al. 2010). Counterintuitively, the evolution of offspring size and matrotrophy are often
78 decoupled in poeciliids. While it might be expected that provisioning more nutrients during
79 development would lead to larger offspring at birth, empirical data have yielded no consistent
80 association across taxa (Olivera-Tlahuel et al. 2015; Meiri et al. 2020; Furness et al. 2021).
81 However, potential links between offspring size and matrotrophy have been difficult to
82 disentangle because offspring size is often strongly impacted by maternal traits like age, size, and
83 condition (Berkeley et al. 2004; Marshall and Keough 2007; Hagmayer et al. 2018; but see

84 Marshall et al. 2010). Ecological factors are also important drivers of offspring size, independent
85 of post-fertilization nutrient provisioning (Reznick et al. 1996*a*; Jennions et al. 2006; Riesch et
86 al. 2010; Pollux and Reznick 2011; Schrader and Travis 2012; Leips et al. 2013). For example,
87 offspring of lecithotrophic *Poecilia* from environments with low predation and high competition
88 are 50% larger at birth than those from high predation, low competition environments (Reznick
89 1982*a*; Reznick and Endler 1982; Reznick and Bryga 1987; Reznick et al. 1996*c*; Bashey 2006*a*,
90 2008; Jørgensen et al. 2011), presumably as a result of mothers investing in larger eggs. Similar
91 environmental conditions appear to promote larger offspring size in some matrotrophic species
92 (Schrader and Travis 2012; Leips et al. 2013; but see Reznick et al. 1996*b*) but not in others
93 (Pollux and Reznick 2011).

94 Here, we address questions about the evolution of offspring size and matrotrophy in a
95 group of livebearing fish that are an important evolutionary and behavioral model system.
96 *Xiphophorus* species inhabit dramatically different ecological environments, from valley rivers
97 nearly at sea level to mountain streams in the Sierra Madre Oriental in central México. Past work
98 on a handful of species has suggested that although all *Xiphophorus* species are livebearing, they
99 do not invest in their offspring post-fertilization (i.e. they are lecithotrophic; Constantz et al.
100 1989). However, this hypothesis has not been evaluated on a genus-wide scale, and data is
101 particularly lacking in species living in extreme environments for the group (Morris and Ryan
102 1992; Pollux et al. 2014). There is no data available on variation in offspring size within or
103 between species.

104 We find that *X. malinche* have evolved exceptionally large offspring and explore the
105 mechanisms and pressures potentially driving the evolution of this trait. By characterizing the
106 change in embryo size over development in the lab and wild for the closely related northern

107 swordtail species *X. malinche* and *X. birchmanni*, we find evidence that *X. malinche* achieves
108 larger offspring size in part through post-fertilization maternal nutrient provisioning. Using a
109 combination of approaches, including evaluating expression at genes that have been repeatedly
110 co-opted for post-fertilization maternal nutrient provisioning (Guernsey et al. 2020), we
111 investigate some of the potential mechanisms underlying this reproductive strategy in *X.*
112 *malinche*. Notably, *X. malinche* and *X. birchmanni* naturally hybridize (Culumber et al. 2011),
113 which we leverage to study the link between genome-wide ancestry and offspring size. Finally,
114 we explore the possibility that a conflict between maternal nutrient provisioning and offspring
115 nutrient demands over development underlies asymmetric hybrid inviability between these two
116 species. This observation is particularly exciting given ongoing hybridization between these two
117 species in nature.
118
119

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 (~2 cm deep) 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 (*X. birchmanni* and *X. malinche* experience high levels of stress when singly housed), we were not able to collect covariates such as mother standard length. However, because average mother size is similar between species (on average, 4.04 cm for *X. malinche* and 3.94 for *X. birchmanni* reared in common conditions; 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 *X. birchmanni*. *X. malinche,* and hybrid fry, we also

142 obtained an estimate of broad-sense heritability (H^2) of offspring standard length at birth (see
143 Supplementary Information 2 for methods).
144
145 *Measuring embryo weight across developmental stages*
146 To compare embryo size throughout development in each species, we measured the dry
147 weights of embryos across developmental stages from wild-caught females, collected with baited
148 minnow traps. We collected a total of 38 pregnant and 11 nonpregnant females (with fully-
149 yolked eggs) from the *X. malinche* source population Chicayotla on the Río Xontla (1,003 m
150 elevation; 20°55′27.24″N 98°34′34.50″W) across two seasons (45 in May 2022 and 4 in August
151 2020), 59 pregnant and 51 nonpregnant females from the *X. birchmanni* source population
152 Coacuilco on the Río Coacuilco (320 m elevation; 21°5'50.85 N, 98°35'19.46 W) across four
153 seasons (31 in October 2023, 22 in February 2023, 30 in September 2022, and 27 in August
154 2020), and 19 pregnant females from the *X. cortezi* source population Puente de Huichihuayán
155 on the Río Huichihuayán (89 m elevation; 21°26'9.95 N 98°56'0.00 W) in one season (February
156 2023). In practice, the sample of 19 females from *X. cortezi* was insufficient to capture
157 developmental profiles because we had ≤1 brood for many stages, so we limit our discussion of
158 these results to the supplement (Supplementary Information 3). Each mother was euthanized
159 with MS-222, cut from the anal pore to the gills so as to expose the brood for fixation, and stored
160 in 95% ethanol. Consistent with previous reports for *Xiphophorus* (Kindsvater et al. 2012;
161 Furness et al. 2019), we found that all embryos in a brood were at roughly the same
162 developmental stage.
163 Embryos were staged following the numerical scoring guidelines described by Reznick
164 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. It is typically difficult to discern whether stage 0 eggs are fully yolked or are still yolking. Therefore, we noted whether unfertilized eggs had an even distribution of lipid droplets on the surface of the egg and were evenly sized within their brood, which are the best visual indicators of completed yolking.

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

Comparison of embryo weight over development between species

Maternal size has previously been shown to strongly correlate with offspring size in poeciliids, including in *X. birchmanni* (Jørgensen et al. 2011; Kindsvater et al. 2012; Hagmayer et al. 2018), as have brood size and environmental variables like collection season (Kindsvater et al. 2012). In our wild collections, pregnant mothers ranged in length from 3.4-5.7cm for *X. malinche* and 2.8-5.2cm for *X. birchmanni*, and brood size ranged from 3-36 and 4-44 embryos, respectively. Therefore, to evaluate which variables impact embryo dry weight other than our variables of interest (species, stage, and the interaction between species and stage), we used R stats::step to compare models that included mother standard length, brood size, and season in an AIC framework. Collection dates were binned into two seasonal categories describing water

temperature (Supp. Fig. S11): the "warm" season includes April-October months and the "cold" season includes November-March months. Mother standard length and season were selected and included as covariates with fixed effects. We confirmed that there was not a significant interaction between species and mother standard length before proceeding with analysis. We also note that, reassuringly, the overall patterns in our results are similar with and without standard length and season covariates included in the model. For the full analysis, we also included brood ID as a random effect, which is unique to each female and is species-specific. Therefore, we fit the following mixed linear model with R lme4::lmer: embryo dry weight (g) ~ species + stage + species:stage + season + mother standard length + (1 | brood ID). Pairwise comparisons between mean embryo dry weight for each stage were made with an ANOVA and Tukey post-hoc test with R emmeans::emmeans (Table S6).

We visualized differences in embryo size after accounting for covariates by plotting the partial residuals of embryo dry weight for each stage with R visreg::visreg (Breheny and Burchett 2017), split by species. We also plotted the raw data (i.e. without accounting for covariates) and reassuringly, the overall patterns were similar (Fig. S5).

As noted above, because it is difficult to determine whether stage 0 eggs are fully-yolked, we omitted these eggs from the developmental profile plots, but separately plot the stage 0 eggs that appeared to have even lipid distribution and size within their brood (Table S5). We used the subset of unfertilized eggs that met these criteria to compare pre-fertilized egg weight between species but note that the distribution of these dry weights likely skews lower than the true pre-fertilized egg weights for both species (see Table S5). We fit a mixed linear model of egg dry weight by species with mother standard length and season as selected covariates and brood ID as

210 a random effect, performed pairwise comparisons with an ANOVA and Tukey post-hoc, and
211 plotted partial residuals of egg dry weights as above.
212
213 *Assessing the relationship between genome-wide ancestry and embryo and ovary size*
214 We were curious whether genome-wide ancestry proportion in natural hybrid mothers
215 might predict embryo size and ovary weight, given that this trait is heritable in lab-raised hybrids
216 (see Results). We sampled 34 pregnant mothers from a natural hybrid population on the Río
217 Calnali on a single collection date (the Calnali Low population, elevation 905 m). *X. malinche*
218 ancestry proportions ranged from 0.28 to 0.71 in these individuals (see Table S7 and
219 Supplementary Information 4). Embryos and ovary tissue were dissected, staged, and dry-
220 weighed as described in the previous section. As above, we used R stats::step to choose an
221 appropriate model for testing the relationship between embryo dry weight, as well as ovary dry
222 weight, and mother ancestry proportion. The selected mixed linear model included brood size,
223 stage, and the interaction between stage and ancestry proportion, with brood ID as a random
224 effect. We evaluated significance with a likelihood ratio test using R stats::anova to compare the
225 full model and a reduced model without ancestry proportion and its interaction with stage. We
226 also evaluated whether mother mitochondrial genotype predicted embryo or ovary dry weight
227 (see Supplementary Information 4). We calculated and plotted partial residuals of embryo dry
228 weight after accounting for covariates (e.g. ancestry proportion) with R visreg::visreg.
229
230 *Calculating Matrotrophy Index*
231 The degree of post-fertilization nutrient provisioning varies along a continuum, but
232 researchers have described two general provisioning strategies: lecithotrophy and matrotrophy

233 (Pollux et al. 2009). A simple quantitative metric for distinguishing these strategies and
234 measuring the level of post-fertilization provisioning is the Matrotrophy Index, which is the dry
235 weight of the fully-developed embryo divided by the dry weight of the fully-yolked egg. A
236 Matrotrophy Index < 1 indicates that the embryo has decreased in size over development
237 (lecithotrophy) while a value ≥ 1 indicates that the embryo has maintained or increased in size,
238 indicative of some level of maternal provisioning over development (matrotrophy). Reported
239 Matrotrophy Indices within poeciliids range from 0.45 to 103 (Furness et al. 2021). Note that a
240 large Matrotrophy Index does not necessarily correspond to large offspring size at birth.
241 We calculated Matrotrophy Index for *X. malinche* and *X. birchmanni* as the dry weight of
242 a fully-developed embryo divided by the dry weight of an early-stage embryo, averaging dry
243 weights for embryos of the same stage within a brood. Since it is difficult to identify fully-yolked
244 eggs, we calculated Matrotrophy Index with dry weight measurements from stage 10 embryos.
245 This analysis choice is conservative in the context of our study, since it will give an
246 underestimate of Matrotrophy Index in matrotrophic species. Since multiple variables impact
247 embryo dry weight, we calculated Matrotrophy Index using both the raw averages (Table S4)
248 and the partial residuals of average embryo dry weights (Table S8) after accounting for season
249 and mother standard length as covariates as above (with R stats::lm). We describe the impact of
250 different calculations with raw and partial residual dry weights on our estimate of Matrotrophy
251 Index in Supplementary Information 3.
252
253 *Multifactorial artificial insemination crosses*
254 To compare results from wild-caught females to females raised in controlled lab
255 conditions, we artificially inseminated lab-raised *X. malinche* and *X. birchmanni* mothers

following the *Xiphophorus* Genetic Stock Center Protocol to create the following crosses: *X. malinche* ♀ × *X. malinche* ♂, *X. birchmanni* ♀ × *X.birchmanni* ♂, *X. malinche* ♀ × *X. birchmanni* ♂, and *X. birchmanni* ♀ × *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). Because we were interested in evaluating cross-level differences in late-stage embryo size, we subsampled stage 30-50 embryos (from 2 broods for all crosses except *X. birchmanni* ♀ × *X. malinche* ♂, for which there was only 1 brood; see Table S9). We found that for this dataset, due to the small sample size, mother standard length and brood ID were perfectly correlated, and including brood ID as a random effect caused singularity in the model. Therefore, we fit a simple linear model including species, stage, brood size, and mother standard length as selected 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 R visreg::visreg. Reassuringly, the partial residual results are the same whether brood ID is included as a random effect in the model or not. We also made crosses between individuals of the same species from different populations for comparison (Table S11-12 and Supplementary Information 5).

To look for evidence of anatomical differences between species at the maternal-offspring interface that could be associated with nutrient provisioning (i.e. thicker ovarian follicle and interacting embryo and maternal tissue), we prepared histological slides of ovaries from both nonpregnant and late-stage (stage 35-40) pregnancy females from the pure *X. malinche* and *X.*

279 *birchmanni* crosses, as well as both F_1 crosses. Whole ovaries containing eggs or embryos were
280 carefully dissected and fixed in 10% formalin. Ovaries were paraffin-embedded, sectioned, and
281 stained with Hematoxylin & Eosin by the Histo-Tec Laboratory (Hayward, CA). We digitally
282 scanned the stained slides through the Human Pathology Histology Services Laboratory at
283 Stanford University for morphological analysis of the ovary sections. See Supplementary
284 Information 6 for methods quantifying morphological differences. Additionally, we verified the
285 genetic origin of ovary follicle tissue in *X. malinche* mothers carrying F_1 offspring
286 (Supplementary Information 7).
287
288 *Differential gene expression and co-expression network analysis*
289 Previous work in livebearers has identified several key genes that are differentially
290 expressed between the ovarian tissue of pregnant lecithotrophic and matrotrophic species (Jue et
291 al. 2018; Guernsey et al. 2020). To broadly compare gene expression between *X. malinche*, *X.*
292 *birchmanni,* and *X. cortezi,* we sequenced ovarian tissue from mothers from two groups: those
293 with fully-yolked eggs and those with embryos in mid- to late-development.
294 Because we were interested in species-level rather than environmentally-dependent
295 differences, we collected both wild and lab-reared females for this analysis. For wild-caught fish,
296 we collected two *X. malinche* and two *X. birchmanni* pregnant females with stage 40-50 embryos
297 from the Chicayotla and Coacuilco localities, respectively. Additionally, we sampled at least
298 three "early" pregnancy females (unfertilized fully-yolked or stage ≤ 5) and at least three "late"
299 pregnancy females (stage 25-45) from our lab populations. For both sets of samples, fish were
300 euthanized by rapidly severing the spinal cord with a scalpel and dissecting the mother's body
301 cavity from anal fin to gills. Wild-caught fish were stored in RNAlater and ovaries were

302 dissected later. For lab collected fish, whole ovaries were dissected immediately following
303 euthanasia and preserved in RNAlater. See Table S13 for the full list of samples with collection
304 dates and developmental stages of embryos.

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

315 We followed the gene expression analysis methods described in Payne et al (2022). We
316 used cutadapt (Martin 2011) and Trim Galore! (Krueger et al. 2021) to trim Illumina adapter
317 sequences and low-quality bases (Phred score < 30) from reads. Using the tool *kallisto* (Bray et
318 al. 2016), we pseudoaligned RNAseq reads to an *X. birchmanni* "pseudoreference" transcriptome
319 generated from the southern platyfish *X. maculatus* genome (Schartl et al. 2013). The *X.*
320 *maculatus* genome assembly is well-annotated and mapped to Ensembl gene IDs with associated
321 Gene Ontology terms (Wittbrodt et al. 1989; Schartl et al. 2013). Raw transcript counts were
322 converted to gene-level counts to evaluate gene expression; note that the *X. birchmanni*
323 transcriptome contains only a single transcript per gene.

324 For differential gene expression analysis, we combined all lab- and wild-collected
325 ovarian follicle samples into a single dataset, using a design formula that included species (*X.*
326 *malinche*, *X. birchmanni*, or *X. cortezi*) and pregnancy category (early or late) as a grouped
327 interaction, origin (lab or wild), and library preparation/sequencing batch. Briefly, using R
328 DESeq2::DESeq (Love et al. 2014), we normalized gene counts by library size, estimated within-
329 experimental group dispersion, fit a negative binomial generalized linear model, and tested
330 significance with a Wald test. Shrunken log-fold changes were calculated with the ashr::ashr
331 shrinkage estimator (Stephens 2017). Extreme outlier and low count genes were removed. Genes
332 were considered significantly differentially expressed between species at an FDR-adjusted p-
333 value<0.05. Out of a total of 19,176 genes in the *X. birchmanni* pseudoreference transcriptome,
334 96% are expressed in at least one species. Expression results from this analysis can be found in
335 Table S15.
336 To explore biological pathways enriched in genes that were differentially expressed
337 between species in ovarian tissue, we performed Gene Ontology enrichment analysis. We used R
338 biomaRt (Durinck et al. 2009) and GOstats (Falcon and Gentleman 2007) to match *X. maculatus*
339 Ensembl gene IDs with GO terms. We created a "universe" of all genes analyzed with DESeq2,
340 which was used as the reference set of genes for testing category enrichment. Using R
341 GSEABase::hyperGTest (Morgan, Martin et al. n.d.), we ran a hypergeometric test to identify
342 overrepresented GO terms (p-value<0.05) in the set of genes that were significantly differentially
343 expressed between species and developmental stage (Table S16). We also used the R tool
344 WGCNA (Langfelder and Horvath 2008) to cluster co-expressed genes and identify clusters
345 highly correlated with species and developmental stage as a grouped variable. Detailed WGCNA
346 methods are in Supplementary Information 9 and full results are in Tables S17-18.

347

348 *Immunostaining of prolactin expression in ovarian tissue*

349 Ovaries were fixed, processed, and sectioned as described above. Briefly, sections were
350 deparaffinized and dehydrated through xylenes and a graded ethanol series. Slides were blocked
351 for 1h (at room temperature, hereafter RT) in gelatin block, then incubated with anti-prolactin
352 primary antibody (rabbit, Abcam EPR19386, 1:200) overnight (4C). Slides were washed,
353 peroxide blocked for 30m (RT) then incubated with biotinylated goat anti-rabbit secondary
354 antibody (Jackson ImmunoResearch 111-065-144, 1:5000) for 1h (RT). Slides were washed,
355 then incubated for 30m with VectaShield Elite ABC reagent (Vector Labs PK-6100; RT).
356 Prolactin signal was amplified with TSA-Cy3 (Akoya Biosciences NEL744001KT) for 6m (RT)
357 and counterstained with DAPI (Thermo Scientific 62248, 1:2000) and mounted with Prolong
358 Gold Antifade (Thermo Scientific P36930). See complete methods in Supplementary
359 Information 10.

360 Images were acquired using NIS Elements software v4.30.02 on a Nikon Ti Eclipse
361 inverted microscope equipped with an ASI MS-2000 motorized linear XY stage, Yokogawa
362 CSU-W1 single disk (50mM pinhole) spinning disk unit, Andor Zyla 4.2 (6.5mM pixel size)
363 scMOS camera, and 10x/0.45 NA or 20X/0.75 NA Nikon PlanApo Lambda air objectives (see
364 Supplementary Information 10 for complete imaging specifications). Data was saved in .nd2
365 format and manually processed in ImageJ.

366

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

368 One striking environmental difference between *X. birchmanni* and *X. malinche* habitats is
369 water temperature, driven by elevation differences (Fig. S10). In addition, many ecological

370 factors such as primary productivity covary with temperature. While we investigated direct
371 responses to temperature in *X. birchmanni* and *X. malinche* fry (Supplementary Information 11
372 and Table S19), we were also interested in responses to the indirect effects of temperature, such
373 as food availability.

374 Given that *X. malinche* populations likely experience lower food and nutrient availability,
375 we predicted that newborn fry may be more likely to face starvation conditions than *X.*
376 *birchmanni*, and that large offspring size may have evolved as an adaptation to these conditions.
377 To directly test this, we compared starvation tolerance between newborn *X. malinche* and *X.*
378 *birchmanni* fry in the lab. We collected four broods of fry from each species and split individuals
379 in a brood evenly between one experimental and one control tank. Fry in the control tanks were
380 fed brine shrimp twice daily for three days while fry in experimental tanks were not fed for those
381 three days. Observations of physical state and behavior were recorded twice per day at 10:00 and
382 17:00 during the laboratory light period (09:00-18:00). At the end of the three-day trial, fry were
383 euthanized with MS-222, photographed so that standard length could be measured with ImageJ,
384 and prepared for total lipid extraction to measure differences in dry weight and fat content
385 between control and experimental fry (see below). After model selection, we fit a mixed linear
386 model (R nlme::lme) for standard length, including species, treatment, and their interaction as
387 fixed effects and brood ID as a random effect. Pairwise comparisons between the interaction of
388 species and treatment were made with an ANOVA and Tukey post-hoc test with R
389 emmeans::emmeans and partial residuals were calculated and plotted with lme4::lmer and
390 visreg::visreg. See Table S20 for raw data and Table S21 for statistics.
391

392 *Lipid extraction and fat content analysis of wild-caught females and lab-born fry*

393 *Xiphophorus* species were thought to reproduce year-round. However, in sampling
394 natural *X. malinche* and *X. birchmanni* populations for this project we detected clear evidence for
395 seasonality in breeding in *X. malinche* populations (see Results). To more systematically
396 evaluate this, we opportunistically collected pregnancy rates for collections made in August
397 2020, May 2022, September 2022, and February 2023 from the Chicayotla *X. malinche* and
398 Coacuilco *X. birchmanni* populations (Table S22).
399 Dry weight and body fat content are indirect measures of nutrient availability in a fish's
400 environment. We dry weighed and extracted total lipids from wild-caught nonpregnant
401 Chicayotla *X. malinche* and Coacuilco *X. birchmanni* adult females caught in September 2022
402 and February 2023. We also collected dry weight and total lipid data from the fry subject to the
403 food deprivation trials described above. For adult fish, abdominal organs (i.e. digestive,
404 excretory, and reproductive tissue from the abdomen) were dissected out to avoid extracting
405 lipids from undigested food. Fry were left intact. Fish were placed on a tray wrapped with foil
406 and dried in a drying oven set to 65°C for five days. After desiccation, dried fish were weighed to
407 obtain dry weight prior to lipid extraction ($weight_1$). To extract lipids, each dried fish was placed
408 in a 5mL glass vial of petroleum ether. After 24 hours, the petroleum ether was drained and
409 replaced with fresh petroleum ether. This process was repeated once more to ensure complete
410 lipid extraction, for a total of three petroleum ether washes. After the third wash, fish were dried
411 at 65°C for 24 hours and then dry weighed to obtain dry weight after lipid extraction ($weight_2$).
412 Fat content percentage was calculated as:

413 $$Fat\ Content\ \% = \frac{weight_1 - weight_2}{weight_1} \times 100$$

414 Differences in fat content for nonpregnant females for each season were evaluated with a
415 Student's two-sided t-test. Differences in dry weight and fat content between species and

416 treatments for food deprivation trial fry were evaluated with the same methods described for fry
417 standard length (see above), with the same model selected (Tables S20-21).
418
419
420 *Measuring viability by cross direction*
421 In addition to comparing embryo size in F_1 hybrids, we quantified differences in survival
422 of F_1 fry as a function of cross direction. We used additional F_1 crosses generated in the lab to do
423 so. For these crosses, we monitored females twice daily, and to reduce the risk of fry predation,
424 we provided low-density and high-cover conditions for females that were morphologically
425 identified as being close to giving birth. New broods were morphologically scored for being full-
426 term or premature, and stillborn fry were identified and counted. Tanks of collected fry were
427 monitored twice daily. For crosses with high mortality rates, survival to two weeks was recorded
428 for surviving fry (Table S23).
429
430
431

432 **Results**

433 *X. malinche newborn fry are exceptionally large*

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

445 We also compared the size of newborn *X. malinche x X. birchmanni* F_1 and F_2 fry. We
446 found that F_1 standard length and head width was *X. malinche*-like and F_2 and natural hybrid fry
447 were intermediate in size to their parent species (Fig. 1B, Supp. Fig. 2B, Tables S1-2). After
448 accounting for the effects of season and brood ID, we estimated the broad-sense heritability of
449 standard length at birth attributable to ancestry to be 0.25 (see Supplementary Information 1).

450

451 *X. malinche mothers provision developing embryos*

452 Given that *X. malinche* fry are significantly larger at birth than *X. birchmanni*, we
453 hypothesized that *X. malinche* embryos are also larger throughout embryonic development. We
454 measured the dry weight of embryos dissected from wild-caught *X. malinche* and *X. birchmanni*

455 pregnant females across developmental stages. First, we estimated the Matrotrophy Index for
456 each species to obtain a quantitative metric of nutrient provisioning during development using
457 stage 10 and stage 50 embryos (Fig. 2A; see Supplementary Information 3). On average by
458 brood, stage 10 embryos weighed 0.0038g for *X. birchmanni* and 0.0040g for *X. malinche*, while
459 stage 50 embryos weighed 0.0025g and 0.0038g, respectively. We note, however, that in
460 practice, embryo weight is impacted by season and mother standard length (see Methods). After
461 accounting for season and mother standard length, we calculated Matrotrophy Indices of 0.66 for
462 *X. birchmanni* and 0.98 for *X. malinche*, respectively. We also attempted to calculate
463 Matrotrophy Index for the sister species to the *X. malinche-X. birchmanni* clade, *X. cortezi*, but
464 our small sample size led to uncertainty in these estimates (Supplementary Information 3).
465 Consistent with their Matrotrophy Index estimate, we found that *X. birchmanni* embryos
466 show a lecithotrophic developmental profile, where embryos lose weight overall across
467 development (Fig. 2B). Notably, *X. birchmanni* embryonic size appears to change nonlinearly
468 through development. By contrast, *X. malinche* embryos maintain roughly the same dry weight
469 throughout development. These species do not differ in embryo dry weight early in development
470 (p-value=0.38 at stage 10; Fig. 2A) nor at the time of fertilization (p-value=0.18, Fig. 2C; but
471 note methodological issues with this stage discussed above). However, they significantly diverge
472 in embryo dry weight at stage 20 (p-value=0.008), and ultimately differ substantially by late
473 development (p-value=0.002 at stage 50; Fig. 2A-B; see Table S5 for statistical comparisons by
474 stage). Therefore, the maintenance of weight over development by *X. malinche* embryos suggests
475 that there is some level of post-fertilization nutrient provisioning from *X. malinche* mothers.
476
477

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

479 To compare the size of late-stage embryos (stage ≥30) from the reciprocal F_1 crosses to
480 pure parental crosses, we made controlled crosses between *X. malinche* and *X. birchmanni* (see
481 *Methods*). Consistent with our measures from wild-caught broods, lab-bred *X. malinche* embryos
482 are significantly larger than *X. birchmanni* embryos (p-value<0.0005). We find that F_1 embryos
483 from the *X. malinche* mother and *X. birchmanni* father cross direction trend towards being
484 smaller than *X. malinche* embryos (p-value=0.08) but span the size range of both within-species
485 crosses. This hints that in a *X. malinche* maternal environment, both maternal effects and
486 offspring genotype could contribute to offspring size at birth. For F_1 embryos with *X. birchmanni*
487 mothers, we were unable to sample sufficient numbers of embryos to confidently compare size
488 across groups (Fig. 3C; see Table S10 for summary statistics).

489 Given that late-stage embryos are larger in *X. malinche* than *X. birchmanni*, we tested
490 whether higher genome-wide *X. malinche* ancestry in naturally occurring hybrids predicts larger
491 embryo size. We sampled pregnant mothers carrying stage ≥ 20 broods from the Calnali Low
492 natural hybrid population, since embryo size diverges between species after stage 15. After
493 accounting for stage and brood ID, we found that proportion of *X. malinche* ancestry in the
494 mother's genome and its interaction with stage are strongly positively associated with embryo
495 dry weight (L-ratio=61.6, p-value<0.0001, R=0.37; Supp. Fig. S6A). Unexpectedly, we find a
496 significant negative relationship between the mother's ancestry and ovary dry weight (F-
497 value=2.90, p-value=0.02), suggesting that greater *X. birchmanni* ancestry in a hybrid genome
498 predicts higher ovary dry weight.

499

500

501 *X. malinche and X. birchmanni ovaries do not have clear morphological differences*

502 To explore potential differences in ovarian structures between species, we compared
503 histological slides of sectioned ovaries. In highly matrotrophic livebearing fishes, nutrients are
504 delivered to developing embryos through the maternal follicle, which is thicker and highly
505 vascularized (Kwan et al. 2015; Guernsey et al. 2020; Ponce de León and Uribe 2021). Visually,
506 we did not observe consistent differences in maternal follicle thickness, amount of vasculature,
507 or overlap of maternal and embryonic membranes between sections of ovaries carrying
508 unfertilized eggs or late-stage embryos from *X. malinche* and *X. birchmanni* (Fig. 2D;
509 Supplementary Information 6). Given that Matrotrophy Indices for *X. malinche* and *X.*
510 *birchmanni* both fall below 1, clear morphological differences in reproductive morphology may
511 not be expected (unlike those observed in other studies; e.g. Guernsey et al. 2020).

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

517

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

519 To gain insight into the mechanisms underlying differences in reproductive strategy
520 between these closely related species, we compared gene expression patterns in ovarian tissue
521 from *X. malinche, X. birchmanni,* and *X. cortezi* from "early" pregnancy (fully-yolked
522 unfertilized or stage <5) and "late" pregnancy (25-45; Table S15). Principal component analysis
523 shows that PC1 explains about 31% of the variation in ovary expression and correlates with

524 species while PC2 explains about 17% of total variation and correlates with species and rearing-
525 environment (Fig. 3A). Within *X. malinche*, expression patterns in ovarian tissues separate along
526 PC1 by the developmental stage of the embryos they contained, while in *X. birchmanni* and *X.*
527 *cortezi* they do not (Fig. 3A). Of the genes expressed in the ovaries of both species (adjusted p-
528 value<0.05), *X. malinche* differs from *X. birchmanni* in expression for 13% (2,202) and 28%
529 (4,934) of genes in early and late pregnancy, respectively, and from *X. cortezi* for 45% (7,957)
530 and 32% (5,639) of genes, respectively. Notably, we see that more genes respond to embryonic
531 developmental stage in *X. malinche* ovaries (310) compared to *X. birchmanni* (63) and *X. cortezi*
532 (130) ovaries. Although we do not focus on them in the main text, we performed similar analyses
533 of genes that were differentially expressed in developing embryos across the three species
534 (Supplementary Information 8).

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

547 Though there is transcription of prolactin receiving and releasing machinery in late-
548 pregnancy *X. malinche* and *X. birchmanni* ovaries, we do not see expression of prolactin itself
549 based on RNAseq data (as expected since prolactin is a hormone translated in the pituitary
550 gland). To evaluate whether prolactin protein appears in *X. malinche* and *X. birchmanni* ovaries,
551 we performed immunohistochemistry on sections of late pregnancy ovaries for both species (Fig.
552 3C). We find that prolactin protein is present in both *X. malinche* and *X. birchmanni* maternal
553 ovarian follicle and embryonic tissues. Additionally, as expected, prolactin is present in retinal
554 and mesenchymal tissue of embryos in both species (Fig. 3C).

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

562 We also looked at groups of co-expressed genes and asked which of these groups are
563 significantly associated with late-stage pregnancy in *X. malinche* (see Supp. Fig. S10 for module-
564 trait correlation matrix). Using WGCNA, we identified 17 ovary co-expression gene clusters
565 significantly associated with *X. malinche* ancestry (p-value<0.05), 11 of which were associated
566 with late-pregnancy *X. malinche* ovaries. Gene Ontology enrichment of these clusters revealed
567 that the 'darkorange2' cluster was highly enriched for acyl- and acetyl-CoA biosynthesis and
568 metabolic pathways, as well as vascular growth and signaling pathways, like artery development
569 and vasculogenesis (Fig. 3D, Supp. Fig. S9). Many of the represented acyl- and acetyl-CoA

570 metabolism genes are strongly upregulated or downregulated in *X. malinche*, particularly in late
571 pregnancy, compared to *X. birchmanni* and *X. cortezi* ovaries (Fig. 3D, Supp. Fig. S9). In
572 mammals, fatty acids like acyl-CoA are an important source of energy for both placental and
573 fetal growth during gestation and are transferred through the placenta to the fetus during mid- to
574 later-stages of pregnancy (Chavan-Gautam et al. 2018). The 'darkorange' cluster was enriched in
575 amino acid biosynthesis and metabolism pathways, and the 'violet' cluster was enriched for
576 intracellular and transmembrane signaling pathways.
577
578 *Testing the potential role of resource availability in driving the evolution of large offspring size*
579 We tested the response of offspring of both species to two ecological factors that differ
580 between the *X. malinche* and *X. birchmanni* environments. We found that the larger offspring of
581 *X. malinche* did not have improved minimum thermal tolerance compared to *X. birchmanni* fry
582 (Supplementary Information 11). We thus turned to investigating another ecological factor that
583 correlates with temperature: food availability. Cooler, headwater habitats are generally less
584 resource-rich than warmer downstream habitat (Reznick 1982*b*). Given low winter temperatures
585 observed in *X. malinche* populations (Supp. Fig. S11; Payne et al. 2022), we asked whether cold
586 temperatures (and presumably low primary production and food availability associated with such
587 temperatures) were linked to seasonal breeding in *X. malinche* populations and starvation
588 tolerance in newborn fry.
589 Water temperatures at *X. malinche* and *X. birchmanni* sites are coolest from November to
590 March with peak lows in January and February and are warmest from April to October with peak
591 highs in May and June (Supp. Fig. S11). The shifts in temperature roughly correspond with the
592 rainy season and river flooding, which typically starts late June and ends in October. We

593 collected mature females from natural populations of both *X. birchmanni* and *X. malinche* across
594 seasons. We found that while many *X. birchmanni* females were reproducing regardless of
595 sampling month or season (the lowest pregnancy rate observed was 34% in February), <20% of
596 *X. malinche* mature females were pregnant from February – August, while 80% were pregnant in
597 May (Supp. Fig. S12; Table S22).

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

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

616 immediately after birth (Fig. 4B). We found no significant difference in total body fat content
617 between species for either treatment (Supp. Fig. S13B).
618
619 *Differences in viability by cross direction*
620 In generating F_1 crosses for this and other projects, we have found a clear difference in
621 the frequency of premature birth and stillbirth in crosses between *X. malinche* mothers and *X.*
622 *birchmanni* fathers compared to crosses between *X. birchmanni* mothers and *X. malinche* fathers.
623 Given that these crosses are genetically identical except for the Y chromosome and
624 mitochondrial haplotype (Moran et al. 2021), this raises the possibility that maternal effects may
625 impact survival of the F_1 fry.
626 We found high rates of premature birth in crosses between *X. birchmanni* mothers and *X.*
627 *malinche* fathers (Table S23). Of 25 crosses generated by artificial insemination between 2011-
628 2022, only eight F_1 fry survived (out of 64 born). In five of these crosses, the mother also
629 suffered mortality. In sixteen out of the 25 crosses, mothers gave birth to morphologically
630 premature fry (Supp. Fig. S14), which have a low probability of survival (~98% mortality,
631 (Moran et al. 2021). In the ten crosses where fry were born at term, 50% contained stillborn fry,
632 versus 7% for premature broods (Fisher's exact p-value=0.04). Maternal mortality was observed
633 in 30% of these full-term crosses, versus 13% of crosses where fry were born prematurely
634 (though this difference was not significant; Fisher's exact test p-value=0.1). For cases where we
635 developmentally staged premature fry, individuals tended to be around stage 40.
636 By contrast, we have never documented a premature birth, stillbirth, or maternal
637 mortality for *X. malinche* mothers. To date we have generated >190 adult F_1 hybrids and 1,253

638 adult F_2 hybrids from intercrossing these individuals. Premature birth has also not been observed

639 in F_2 crosses between these F_1 individuals.

640

641

642 **Discussion**

643 Offspring size is a complex trait that impacts both parent and offspring fitness. Compared
644 with its sister species, *X. birchmanni*, and other *Xiphophorus* species, *X. malinche* produce
645 exceptionally large fry. Though offspring size has been shown to be sensitive to ecological
646 conditions and the maternal environment in livebearers (Reznick 1982*a*; Reznick and Yang
647 1993; Reznick et al. 1996*b*; Bashey 2006*b*, 2008), we see this size difference in the offspring of
648 lab-raised individuals, and estimate that the broad-sense heritability of offspring size attributable
649 to ancestry is 0.25. Moreover, the proportion of the genome derived from *X. malinche* impacts
650 offspring size in natural hybrid populations.

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

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

665 which presumably have a fitness advantage in lower resource environments (Pettersen et al.
666 2015, 2022). We directly tested offspring performance in food-depleted conditions in a
667 controlled lab experiment. We find that starved newborn *X. malinche* retain more weight than
668 newborn *X. birchmanni*. This suggests that *X. malinche* fry may be more resilient to a low
669 resource environment, consistent with resource limitation (or high competition) as a driver of the
670 evolution of large offspring in *X. malinche* (Bashey 2006*b*, 2008). Notably, in scenarios where
671 resources were not limited, newborn *X. birchmanni* fry grew more rapidly than *X. malinche* and
672 matched their size within days. This pattern could hint that there is an advantage to being large
673 early, consistent with previous work in other taxa (Chambers et al. 1989; Gliwicz and Guisande
674 1992; Bashey 2008).

675 There are two mechanisms through which livebearing fish can produce larger fry. First,
676 mothers may produce larger eggs. Second, mothers may continue to provision nutrients to
677 offspring over embryonic development (note that matrotrophy does not predictably lead to large
678 offspring at birth across taxa; (Furness et al. 2021). We evaluated evidence for both mechanisms
679 in *X. malinche*. We measured dry weights for embryos across ten embryonic stages ranging from
680 unfertilized fully-yolked eggs to fully developed juveniles for *X. malinche, X. birchmanni,* and
681 their close relative, *X. cortezi*. Notably, unfertilized fully-yolked eggs and early stage embryos
682 are similar in size between *X. malinche* and *X. birchmanni* (Fig. 2). However, *X. malinche* and *X.*
683 *birchmanni* embryos diverge in size around stage 20 of embryonic development. While *X.*
684 *birchmanni* embryos undergo an overall decline in dry weight after stage 15, *X. malinche*
685 embryos maintain roughly the same weight over development. We summarize this difference in
686 developmental profile with the first estimates of Matrotrophy Index for *X. malinche* (0.98) and *X.*
687 *birchmanni* (0.66). This estimate for *X. malinche* is consistent with *X. malinche* embryos

688 receiving some nutrients from their mother over development, contributing to larger offspring
689 size at birth in *X. malinche*. It is notable that although *X. birchmanni* do not appear to provision
690 nutrients during development, *X. birchmanni* also has fry that are much larger at birth than other
691 *Xiphophorus* species. We were unable to collect sufficient samples to accurately quantify
692 Matrotrophy Index from these species but we predict that they may produce smaller eggs than *X.*
693 *birchmanni*, and indeed the few samples we have are consistent with this prediction (Fig. S17).
694 Research on highly matrotrophic species (e.g. with Matrotrophy Indices>40) has shown
695 that maternal and embryonic tissues interact to form an interface for nutrient transfer, with some
696 species forming placental structures of varying complexity (Guernsey et al. 2020). To look for
697 evidence of a more complex maternal-offspring interface in *X. malinche*, we quantified
698 characteristics of the maternal-embryo interface in histological sections of late-stage pregnancy
699 in *X. malinche* and *X. birchmanni* ovaries. In highly matrotrophic poeciliids, embryonic and
700 maternal blood vessels range from apposed to fused (Ponce de León and Uribe 2021) and the
701 ovarian follicular epithelium is dense and maximizes surface area contact with embryos for
702 nutrient transfer (Kwan et al. 2015). We did not see consistent differences in ovarian follicle
703 thickness or vascularization between stained sections of late-stage *X. malinche* and *X.*
704 *birchmanni* ovaries. However, we found it difficult to compare these traits between 2-
705 dimensional slices of ovary. Because post-fertilization nutrient provisioning in *X. malinche* arose
706 recently and is less substantial than that observed in other species, we might predict differences
707 in ovary morphology between these species to be subtle. Future work should focus on obtaining
708 3-dimensional images suitable for quantitative comparisons of the whole ovary.
709 To further investigate differences in maternal nutrient provisioning at the molecular level,
710 we quantified gene expression patterns in early- and late-stage ovarian tissue from pregnant *X.*

711 *malinche* and *X. birchmanni*. The observed expression patterns raise multiple possible
712 mechanisms through which post-fertilization nutrient provisioning may be occurring in *X.*
713 *malinche*. Receptor and releaser genes for prolactin, a pituitary hormone important during
714 pregnancy in mammals and in matrotrophic poecilids (Menzies et al. 2011; Guernsey et al.
715 2020), are highly expressed in the *X. malinche* ovarian follicle compared to *X. birchmanni* (and
716 *X. cortezi*) follicles both before and during pregnancy. Combined with evidence that *X. malinche*
717 mothers appear to provision their embryos over development to a greater extent than *X.*
718 *birchmanni* mothers, this result suggests that prolactin may be a key candidate for the evolution
719 of matrotrophy in livebearers (as proposed by Guernsey et al. 2020). Immunostains of prolactin
720 in sections of late-stage pregnant *X. malinche* and *X. birchmanni* ovaries confirms that prolactin
721 is present in the ovarian follicle of both species. Given these results, we predict that the striking
722 differences in abundance of prolactin releasing and receiving machinery that we observe at the
723 gene expression level (Fig. 3) may regulate the downstream transfer and function of prolactin in
724 the two species, which includes nutrient transport.

725 While offspring almost universally benefit from being larger at birth, producing large
726 offspring can be costly for parents (Einum and Fleming 2000; Walker et al. 2008; Ljungström et
727 al. 2016) and constrained by environmental conditions (Reznick and Endler 1982; Hutchings
728 1991; Janzen et al. 2000; Allen et al. 2008; Marshall and Keough 2008; Pettersen et al. 2022).
729 These pressures can result in parent-offspring conflict in the evolution of offspring size (Trivers
730 1974; Crespi and Semeniuk 2004; Moore 2012). Similarly, if males and females differ in their
731 level of resource investment in offspring, conflict between the maternal and paternal genomes
732 can drive similar dynamics (Moore and Haig 1991), driving evolutionary arms races between
733 paternally contributed factors that increase offspring growth and maternally contributed factors

734 that suppress them (Moore and Haig 1991; Barlow and Bartolomei 2014). In hybrids between
735 species, misregulation of these interactions can result in larger or smaller offspring than expected
736 (i.e. depending on the cross direction) and impact viability of hybrid offspring (Shi et al. 2004;
737 Brekke & Good 2014), including in livebearing fish (Schrader et al. 2013). Given these findings
738 and the large difference in *X. malinche* and *X. birchmanni* embryos during development, we
739 were curious to understand whether this trait impacts reproductive isolation between these
740 species, which naturally hybridize in the wild (Culumber et al. 2011). In the lab, *X. malinche*
741 mothers and *X. birchmanni* fathers successfully produce viable F_1 offspring and subsequent
742 early-generation crosses, whereas the reverse cross is typically aborted late in pregnancy (by
743 stage 40). Asymmetric reciprocal F_1 cross viability has been shown to stem from differences in
744 nutrient provisioning over development in other poeciliid species (Turcotte et al. 2008; Schrader
745 et al. 2013). However, this case is unique in that F_1s are inviable when the mother is
746 lecithotrophic and therefore completely controls nutrient provisions (i.e. the matrotrophic
747 paternal genome cannot affect nutrient allotment). We initially hypothesized that differences in
748 provisioning strategy might result in embryos that grow too large too quickly for *X. birchmanni*
749 mothers to sustain. However, we found it difficult to collect stage matched offspring in F_1 hybrid
750 crosses with *X. birchmanni* mothers, resulting in a low sample size or this comparison (Fig. 2D).
751 While initial results do not suggest size differences compared to stage matched *X. birchmanni*
752 embryos, rigorously testing this hypothesis is an important direction for future work.
753 Alternatively, the energetic demands of F_1 embryos may outpace the nutrients stored in yolk
754 from *X. birchmanni* mothers early on, which could lead to malnourishment. Consistent with this
755 hypothesis, we find that many offspring from this cross are stillborn and the majority are born
756 prematurely.

757 Our data also shows that offspring size is a complex trait, and the size of any individual
758 offspring is impacted by many variables, including developmental stage, mother size, and mother
759 environment. Accounting for all of these effects make this trait difficult to characterize,
760 complicating comparisons of offspring size and embryonic development profiles between
761 livebearing species. Recently, Skalkos et al. (2023) reviewed current limitations in how
762 matrotrophy is studied for teleost fish. Our findings underscore some of their points, including
763 that direct measures of early- and late-stage embryo weight, rather than predictive measures
764 using regression-based approaches, should be used to estimate Matrotrophy Index where possible
765 (since stages are morphological categories that do not linearly track developmental time).
766 Moreover, if provisioning varies over development (as hinted at by some of our data), more
767 nuanced analysis methods may be more appropriate. Future work should consider these factors.
768 We note that in our work using lab populations served as a powerful resource for confirming
769 patterns inferred from wild populations (Fig. 2), as they allowed us to control for many of the
770 environmental variables that impact wild collections.
771 Using a combination of morphometric and molecular data, we document immense
772 variation in offspring size across *Xiphophorus* species and provide the first evidence of incipient
773 matrotrophy and post-fertilization nutrient provisioning mechanism in the *Xiphophorus*.
774 Importantly, because *X. malinche* and *X. birchmanni* are recently diverged (~250,000
775 generations ago), this is an example of recent shift in reproductive strategy. Parent of origin
776 incompatibility phenotypes associated with spontaneous abortion late in development hint that
777 this difference in reproductive strategy could contribute to the diverse reproductive barriers
778 present in these young sister species.
779

780 **Data Availability**

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

784

785 **Acknowledgements**

786 We thank Moi Exposito-Alonso and members of the Schumer lab for helpful feedback on
787 previous versions of this manuscript. We are grateful to the Mexican federal government for
788 permission to collect samples. We thank Stanford University and the Stanford Research
789 Computing Center for providing computational support for this project. This study was
790 supported by a Society for Integrative & Comparative Biology GIAR grant to CYP, an American
791 Society of Naturalists Student Research Award to CYP, and a Human Frontiers in Science
792 Programme grant (RGY0081), HHMI Freeman-Hrabowski award, Pew Biomedical Scholars,
793 and Searle Scholars Award to MS.

794

Figures

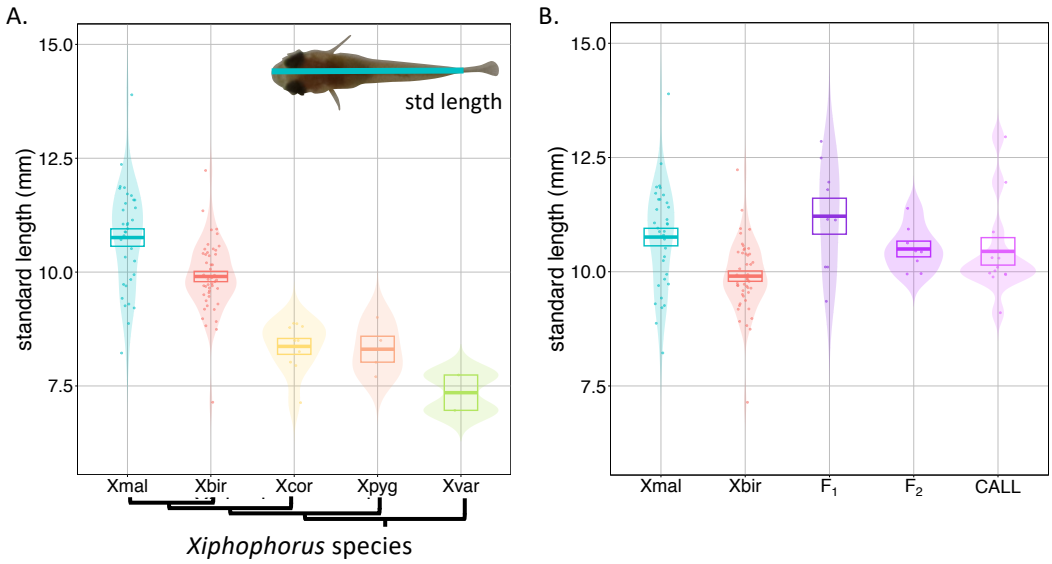

Figure 1. Comparisons of offspring size at birth across *Xiphophorus* species and their hybrids. **A)** Violin plots of standard length (mm) of newborn fry from five *Xiphophorus* species – *X. malinche*, *X. birchmanni*, *X. cortezi*, *X. pygmaeus*, and *X. variatus*. Phylogenetic relationships between species are shown schematically on the x-axis (following (Preising et al. 2022). **B)** Violin plots of standard length (mm) of newborn fry of different groups, including *X. malinche*, *X. birchmanni*, F_1 hybrids, F_2 hybrids, and natural hybrids ("CALL" – Calnali Low hybrid population). For both **A** & **B**, each data point represents the average standard length of newborn fry from one brood. For each group, boxes show the mean ± 1 standard error.

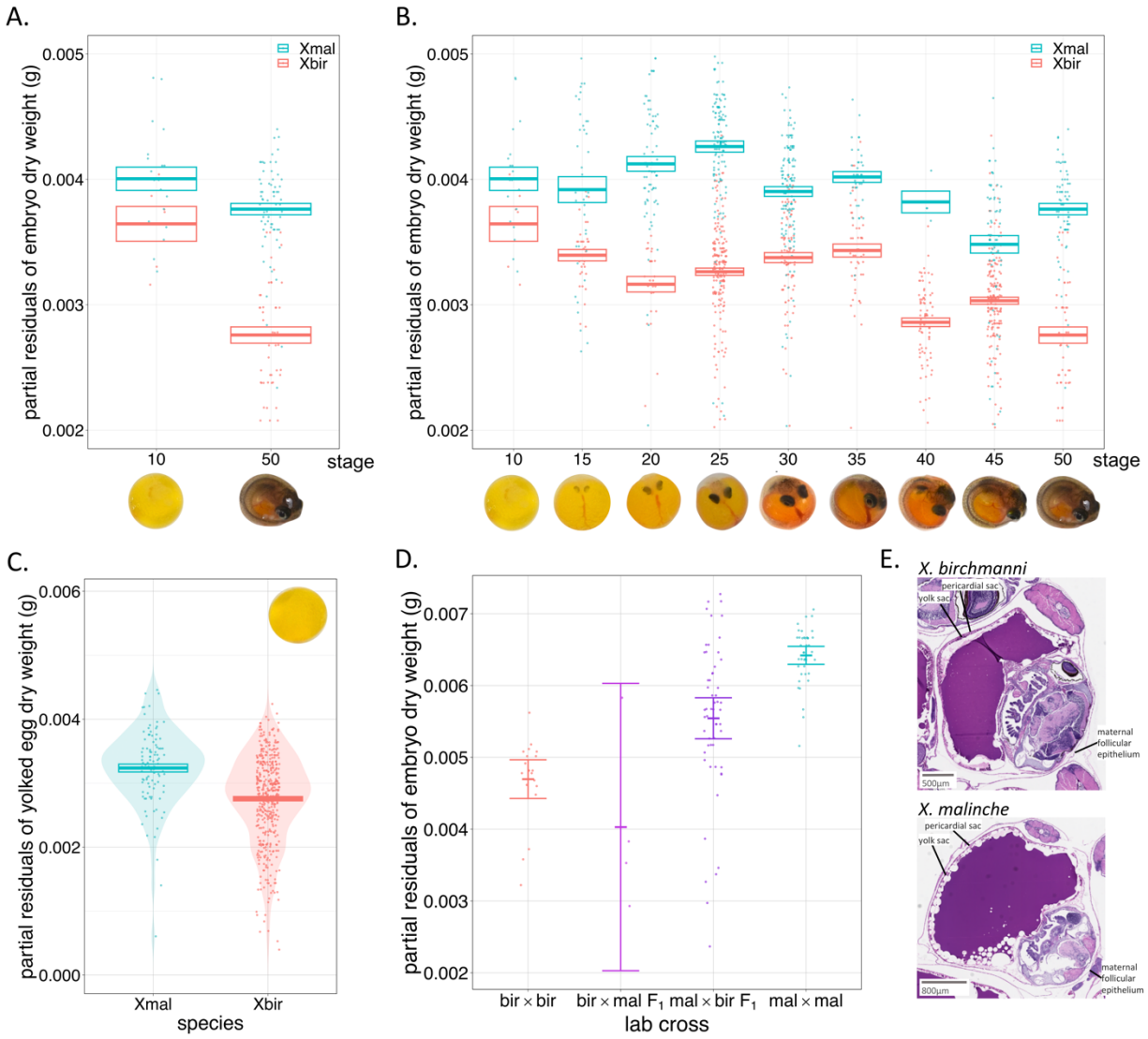

Figure 2. Comparisons of embryo weight throughout development and ovarian structures between *X. birchmanni* and *X. malinche*. **A)** Dry weights of wild-caught *X. birchmanni* and *X. malinche* embryos do not significantly differ shortly after fertilization but are substantially different in weight just before birth (as well as at birth, see Fig. 1). Plot shows partial residuals of stage 10 and stage 50 embryo dry weights correcting for season and mother standard length as covariates and brood ID as a random effect. Points show the partial residuals and boxes show mean ±1 standard error, colored by species (*X. birchmanni*/Xbir – pink and *X. malinche*/Xmal – blue). The y-axis was limited to 0.002-0.005g to show the mean differences between species by

814 stage more clearly; however, several points were cut out of the plot frame. The full plot including
815 all partial residual points is shown in Fig. S5A. **B**) Developmental size profiles for wild-caught
816 *X. birchmanni* (Xbir – pink) and *X. malinche* (Xmal – blue) show that the difference in embryo
817 size between these species may fluctuate throughout development (or be impacted by variation in
818 sampling), but appears to diverge after stage 15. Developmental stage is plotted on the x-axis
819 (with images of *X. malinche* embryos at corresponding stages) and the partial residuals of
820 average embryo dry weight per brood (after correcting for maternal size and collection season) is
821 plotted on the y-axis. Points show the partial residuals and boxes show mean ±1 standard error,
822 colored by species. **C**) Though difficult to accurately stage (see Methods), unfertilized, fully-
823 yolked eggs do not significantly differ in size between species (p-value=0.18). Violin plot
824 compares stage 0 (fully-yolked but unfertilized) eggs between wild-caught *X. birchmanni* and *X.*
825 *malinche*. Points show the partial residuals and boxes show mean ±1 standard error, colored by
826 species. **D**) Patterns observed in **B** are replicated in stage ≥30 lab-crossed parental species raised
827 under common conditions, with *X. birchmanni* embryos being significantly smaller than *X.*
828 *malinche* later in development (p-value=0.0005). Given the small sample size of late-stage F_1
829 hybrids from *X. birchmanni* mothers (bir×mal F_1), we have limited power to evaluate whether
830 they differ in size from the other crosses. F_1 hybrids from *X. malinche* mothers (mal×bir F_1)
831 trend towards being smaller than pure *X. malinche* fry (p-value=0.08), hinting that the trait could
832 be impacted by both genetic and maternal effects. **E)** Histological slides stained with
833 Hematoxylin & Eosin of *X. birchmanni* (left) and *X. malinche* (right) ovaries derived from
834 females pregnant with late-stage embryos (stage 35 and 40, respectively) are structurally similar.

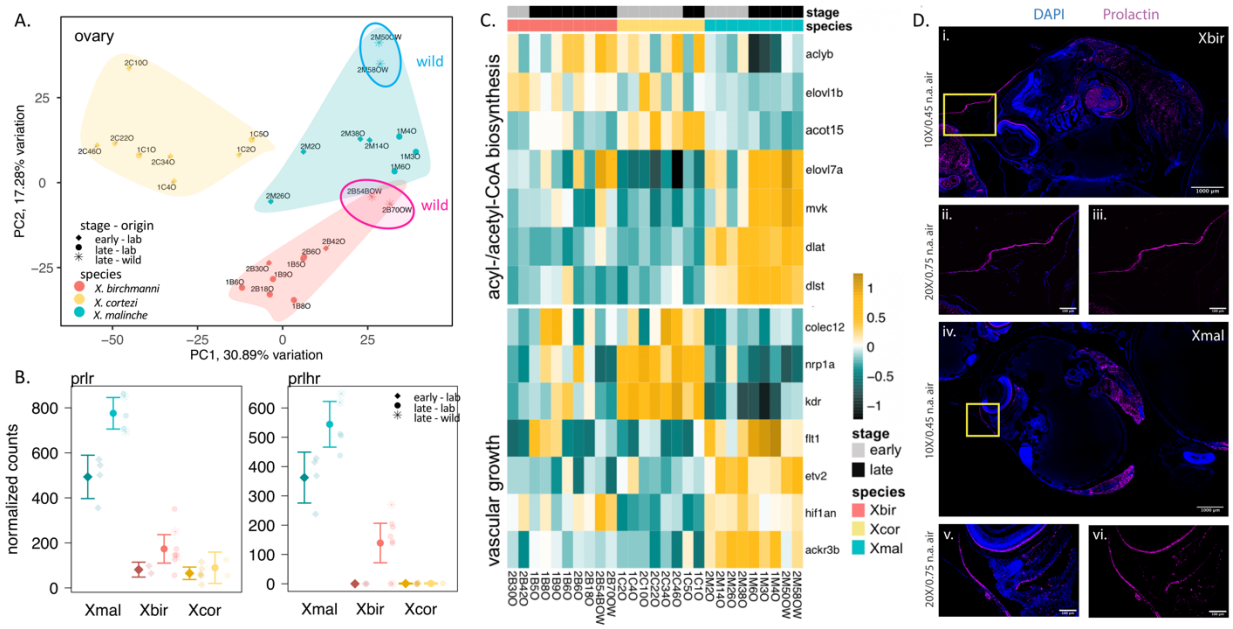

Figure 3. Gene expression analysis hints at a role for prolactin, fatty acid biosynthesis, and vascular growth pathways in nutrient provisioning post-fertilization in *X. malinche*. **A**) Principal component analysis of transformed gene abundance counts from ovary RNAseq data from *X. birchmanni* (pink), *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

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

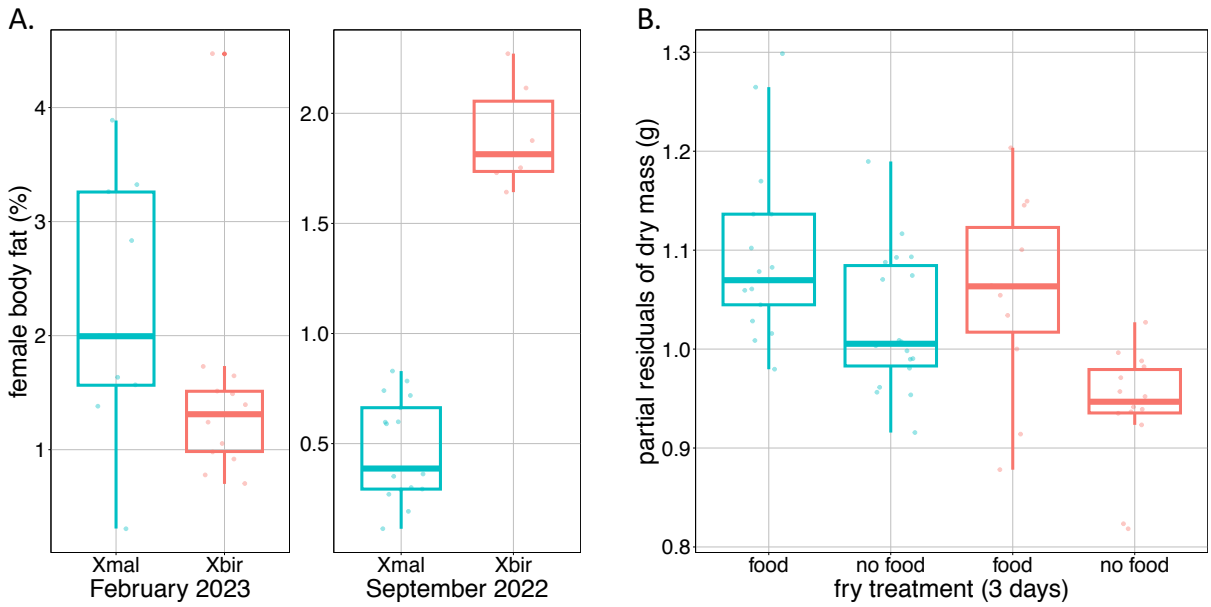

Figure 4. Comparisons of responses to food availability in *X. malinche* and *X. birchmanni*. **A**) Boxplots showing fat content of non-pregnant *X. malinche* and *X. birchmanni* females collected from wild populations in different seasons. *X. malinche* females have much lower fat content before their 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.

References

Allen, R. M., Y. M. Buckley, and D. J. Marshall. 2008. Offspring Size Plasticity in Response to Intraspecific Competition: An Adaptive Maternal Effect across Life-History Stages. The American Naturalist 171:225–237.

Barlow, D. P., and M. S. Bartolomei. 2014. Genomic Imprinting in Mammals. Cold Spring Harbor Perspectives in Biology 6:a018382.

Bashey, F. 2006*a*. Cross-Generational Environmental Effects and the Evolution of Offspring Size in the Trinidadian Guppy Poecilia Reticulata. Evolution 60:348–361.

———. 2006*b*. Cross-Generational Environmental Effects and the Evolution of Offspring Size in the Trinidadian Guppy Poecilia Reticulata. Evolution 60:348–361.

———. 2008. Competition as a Selective Mechanism for Larger Offspring Size in Guppies. Oikos 117:104–113.

Berkeley, S. A., C. Chapman, and S. M. Sogard. 2004. Maternal Age as a Determinant of Larval Growth and Survival in a Marine Fish, Sebastes Melanops. Ecology 85:1258–1264.

Blackburn, D. G. 2015. Evolution of vertebrate viviparity and specializations for fetal nutrition: A quantitative and qualitative analysis. Journal of Morphology 276:961–990.

Bowen, W. D., C. E. den Heyer, J. I. McMillan, and S. J. Iverson. 2015. Offspring size at weaning affects survival to recruitment and reproductive performance of primiparous gray seals. Ecology and Evolution 5:1412–1424.

Bray, N. L., H. Pimentel, P. Melsted, and L. Pachter. 2016. Near-optimal probabilistic RNA-seq quantification. Nature Biotechnology 34:525–527.

Breheny, P., and W. Burchett. 2017. Visualization of Regression Models Using visreg. The R Journal 9:56.

Brockelman, W. Y. 1975. Competition, the Fitness of Offspring, and Optimal Clutch Size. The American Naturalist 109:677–699.

Chambers, R. C., W. C. Leggett, and J. A. Brown. 1989. Egg Size, Female Effects, and the Correlations Between Early Life History Traits of Capelin, Mallotus villosus: An Appraisal at the Individual Levell. FISHERY BULLETIN 87.

Chavan-Gautam, P., A. Rani, and D. J. Freeman. 2018. Chapter Six - Distribution of Fatty Acids and Lipids During Pregnancy. Pages 209–239 *in* G. S. Makowski, ed. Advances in Clinical Chemistry (Vol. 84). Elsevier.

Constantz, G., G. Meffe, and F. Snelson. 1989. Ecology and evolution of livebearing fishes (Poeciliidae). Reproductive Biology of Poeciliid Fishes. Prentice Hall, Englewood Cliffs, NJ.

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

Culumber, Z. W., H. S. Fisher, M. Tobler, M. Mateos, P. H. Barber, M. D. Sorenson, and G. G. Rosenthal. 2011. Replicated hybrid zones of Xiphophorus swordtails along an elevational gradient. Molecular Ecology 20:342–356.

Durinck, S., P. T. Spellman, E. Birney, and W. Huber. 2009. Mapping identifiers for the integration of genomic datasets with the R/Bioconductor package biomaRt. Nature Protocols 4:1184–1191.

Einum, S., and I. A. Fleming. 2000. Selection Against Late Emergence and Small Offspring in Atlantic Salmon (salmo Salar). Evolution 54:628–639.

Falcon, S., and R. Gentleman. 2007. Using GOstats to test gene lists for GO term association. Bioinformatics (Oxford, England) 23:257–258.

Furness, A. I., J. C. Avise, B. J. A. Pollux, Y. Reynoso, and D. N. Reznick. 2021. The evolution of the placenta in poeciliid fishes. Current Biology 31:2004-2011.e5.
Furness, A. I., and I. Capellini. 2019. The evolution of parental care diversity in amphibians. Nature Communications 10:4709.
Furness, A. I., B. J. A. Pollux, R. W. Meredith, M. S. Springer, and D. N. Reznick. 2019. How conflict shapes evolution in poeciliid fishes. Nature Communications 10:3335.
Gliwicz, Z. M., and C. Guisande. 1992. Family Planning in Daphnia: Resistance to Starvation in Offspring Born to Mothers Grown at Different Food Levels. Oecologia 91:463–467.
Griffith, O. W., and G. P. Wagner. 2017. The placenta as a model for understanding the origin and evolution of vertebrate organs. Nature Ecology & Evolution 1:1–10.
Gross, M. R. 2005. The Evolution of Parental Care. The Quarterly Review of Biology 80:37–45.
Guernsey, M. W., H. van Kruistum, D. N. Reznick, B. J. A. Pollux, and J. C. Baker. 2020. Molecular Signatures of Placentation and Secretion Uncovered in Poeciliopsis Maternal Follicles. Molecular Biology and Evolution 37:2679–2690.
Hagmayer, A., A. I. Furness, D. N. Reznick, and B. J. A. Pollux. 2018. Maternal size and body condition predict the amount of post-fertilization maternal provisioning in matrotrophic fish. Ecology and Evolution 8:12386–12396.
Hutchings, J. A. 1991. FITNESS CONSEQUENCES OF VARIATION IN EGG SIZE AND FOOD ABUNDANCE IN BROOK TROUT SALVELINUS FONTINALIS. Evolution; International Journal of Organic Evolution 45:1162–1168.
Janzen, F. J., J. K. Tucker, and G. L. Paukstis. 2000. Experimental analysis of an early life-history stage: avian predation selects for larger body size of hatchling turtles. Journal of Evolutionary Biology 13:947–954.
Jennions, M. D., B. B. M. Wong, A. Cowling, and C. Donnelly. 2006. Life-history phenotypes in a live-bearing fish Brachyrhaphis episcopi living under different predator regimes: seasonal effects? Environmental Biology of Fishes 76:211–219.
Jørgensen, C., S. K. Auer, and D. N. Reznick. 2011. A Model for Optimal Offspring Size in Fish, Including Live-Bearing and Parental Effects. The American Naturalist 177:E119–E135.
Jue, N. K., R. J. Foley, D. N. Reznick, R. J. O'Neill, and M. J. O'Neill. 2018. Tissue-Specific Transcriptome for Poeciliopsis prolifica Reveals Evidence for Genetic Adaptation Related to the Evolution of a Placental Fish. G3: Genes|Genomes|Genetics 8:2181–2192.
Kaplan, R. H. 1992. Greater Maternal Investment Can Decrease Offspring Survival in the Frog Bombina Orientalis. Ecology 73:280–288.
Kindsvater, H. K., G. G. Rosenthal, and S. H. Alonzo. 2012. Maternal size and age shape offspring size in a live-bearing fish, Xiphophorus birchmanni. PloS One 7:e48473.
Klug, H., and M. B. Bonsall. 2014. What are the benefits of parental care? The importance of parental effects on developmental rate. Ecology and Evolution 4:2330–2351.
Krueger, F., F. James, P. Ewels, E. Afyounian, and B. Schuster-Boeckler. 2021. FelixKrueger/TrimGalore: v0.6.7 - DOI via Zenodo. Zenodo.
Kwan, L., M. Fris, F. H. Rodd, L. Rowe, L. Tuhela, and T. M. Panhuis. 2015. An examination of the variation in maternal placentae across the genus Poeciliopsis (Poeciliidae). Journal of Morphology 276:707–720.
Langfelder, P., and S. Horvath. 2008. WGCNA: an R package for weighted correlation network analysis. BMC Bioinformatics 9:559.

Leips, J., F. Helen Rodd, and J. Travis. 2013. The adaptive significance of population differentiation in offspring size of the least killifish, Heterandria formosa. Ecology and Evolution 3:948–960.
Ljungström, G., M. Stjernstedt, E. Wapstra, and M. Olsson. 2016. Selection and constraints on offspring size-number trade-offs in sand lizards (Lacerta agilis). Journal of Evolutionary Biology 29:979–990.
Love, M. I., W. Huber, and S. Anders. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biology 15:550.
Marshall, D. J., S. S. Heppell, S. B. Munch, and R. R. Warner. 2010. The relationship between maternal phenotype and offspring quality: Do older mothers really produce the best offspring? Ecology 91:2862–2873.
Marshall, D. J., and M. J. Keough. 2007. The Evolutionary Ecology of Offspring Size in Marine Invertebrates. Pages 1–60 *in*Advances in Marine Biology (Vol. 53). Academic Press.
Marshall, D. J., and M. J. Keough. 2008. The Relationship between Offspring Size and Performance in the Sea. The American Naturalist 171:214–224.
Martin, M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.journal 17:10–12.
McGurk, M. 1986. Natural mortality of marine pelagic fish eggs and larvae: role of spatial patchiness. Marine Ecology Progress Series 34:227–242.
Meiri, S., A. Feldman, R. Schwarz, and R. Shine. 2020. Viviparity does not affect the numbers and sizes of reptile offspring. Journal of Animal Ecology 89:360–369.
Menzies, B. R., A. J. Pask, and M. B. Renfree. 2011. Placental expression of pituitary hormones is an ancestral feature of therian mammals. EvoDevo 2:16.
Meredith, R. W., J. E. Janečka, J. Gatesy, O. A. Ryder, C. A. Fisher, E. C. Teeling, A. Goodbla, et al. 2011. Impacts of the Cretaceous Terrestrial Revolution and KPg Extinction on Mammal Diversification. Science 334:521–524.
Moore, T. 2012. Review: Parent-offspring conflict and the control of placental function. Placenta 33 Suppl:S33-36.
Moore, T., and D. Haig. 1991. Genomic imprinting in mammalian development: a parental tug-of-war. Trends in Genetics 7:45–49.
Moran, B. M., C. Y. Payne, D. L. Powell, E. N. K. Iverson, S. M. Banerjee, Q. K. Langdon, T. R. Gunn, et al. 2021. A Lethal Genetic Incompatibility between Naturally Hybridizing Species in Mitochondrial Complex I. bioRxiv 2021.07.13.452279.
Morgan, Martin, S. Falcon, and R. Gentleman. n.d. GSEABase: Gene set enrichment data structures and methods version 1.52.1 from Bioconductor.
Morris, M. R., and M. J. Ryan. 1992. Breeding Cycles in Natural Populations of Xiphophorus nigrensis, X. multilineatus, and X. pygmaeus. Copeia 1992:1074–1077.
Olivera-Tlahuel, C., A. G. Ossip-Klein, H. S. Espinosa-Pérez, and J. J. Zúñiga-Vega. 2015. Have superfetation and matrotrophy facilitated the evolution of larger offspring in poeciliid fishes? Biological journal of the Linnean Society. Linnean Society of London 116:787–804.
Payne, C., R. Bovio, D. L. Powell, T. R. Gunn, S. M. Banerjee, V. Grant, G. G. Rosenthal, et al. n.d. Genomic insights into variation in thermotolerance between hybridizing swordtail fishes. Molecular Ecology n/a.
Pettersen, A. K., L. Schuster, and N. B. Metcalfe. 2022. The Evolution of Offspring Size: A Metabolic Scaling Perspective. Integrative and Comparative Biology 62:1492–1502.

Pettersen, A. K., C. R. White, and D. J. Marshall. 2015. Why does offspring size affect performance? Integrating metabolic scaling with life-history theory. Proceedings of the Royal Society B: Biological Sciences 282:20151946.

Pires, M. N., J. Arendt, and D. N. Reznick. 2010. The evolution of placentas and superfetation in the fish genus Poecilia (Cyprinodontiformes: Poeciliidae: subgenera Micropoecilia and Acanthophacelus). Biological Journal of the Linnean Society 99:784–796.

Pollux, B. J. A., R. W. Meredith, M. S. Springer, T. Garland, and D. N. Reznick. 2014. The evolution of the placenta drives a shift in sexual selection in livebearing fish. Nature 513:233–236.

Pollux, B. J. A., M. N. Pires, A. I. Banet, and D. N. Reznick. 2009. Evolution of Placentas in the Fish Family Poeciliidae: An Empirical Study of Macroevolution. Annual Review of Ecology, Evolution, and Systematics 40:271–289.

Pollux, B. J. A., and D. N. Reznick. 2011. Matrotrophy limits a female's ability to adaptively adjust offspring size and fecundity in fluctuating environments. Functional Ecology 25:747–756.

Ponce de León, J. L., and M. C. Uribe. 2021. Morphology of yolk and pericardial sacs in lecithotrophic and matrotrophic nutrition in poeciliid fishes. Journal of Morphology 282:887–899.

Preising, G. A., T. Gunn, J. J. Baczenas, A. Pollock, D. L. Powell, T. O. Dodge, J. A. M. Kairuz, et al. 2022. Recurrent evolution of small body size and loss of the sword ornament in Northern Swordtail fish. bioRxiv.

Reznick, D. 1981. "Grandfather Effects": The Genetics of Interpopulation Differences in Offspring Size in the Mosquito Fish. Evolution 35:941–953.

———. 1982*a*. Genetic Determination of Offspring Size in the Guppy (Poecilia reticulata). The American Naturalist 120:181–188.

———. 1982*b*. The Impact of Predation on Life History Evolution in Trinidadian Guppies: Genetic Basis of Observed Life History Patterns. Evolution 36:1236–1250.

Reznick, D., H. Callahan, and R. Llauredo. 1996*a*. Maternal Effects on Offspring Quality in Poeciliid Fishes. American Zoologist 36:147–156.

———. 1996*b*. Maternal Effects on Offspring Quality in Poeciliid Fishes. American Zoologist 36:147–156.

Reznick, D., and J. A. Endler. 1982. The Impact of Predation on Life History Evolution in Trinidadian Guppies (Poecilia reticulata). Evolution 36:160–177.

Reznick, D. N., and H. Bryga. 1987. Life-History Evolution in Guppies (poecilia Reticulata): 1. Phenotypic and Genetic Changes in an Introduction Experiment. Evolution 41:1370–1385.

Reznick, D. N., M. J. Butler, F. H. Rodd, and P. Ross. 1996*c*. LIFE-HISTORY EVOLUTION IN GUPPIES (POECILIA RETICULATA) 6. DIFFERENTIAL MORTALITY AS A MECHANISM FOR NATURAL SELECTION. Evolution; International Journal of Organic Evolution 50:1651–1660.

Reznick, D. N., M. Mateos, and M. S. Springer. 2002. Independent Origins and Rapid Evolution of the Placenta in the Fish Genus Poeciliopsis. Science 298:1018–1020.

Reznick, D., and A. P. Yang. 1993. The Influence of Fluctuating Resources on Life History: Patterns of Allocation and Plasticity in Female Guppies. Ecology 74:2011–2019.

Riesch, R., M. Plath, F. J. García de León, and I. Schlupp. 2010. Convergent life-history shifts: toxic environments result in big babies in two clades of poeciliids. Die Naturwissenschaften 97:133–141.

Rollinson, N., and J. A. Hutchings. 2013. The relationship between offspring size and fitness: integrating theory and empiricism. Ecology 94:315–324.
Roney, N. E., R. A. Oomen, H. Knutsen, E. M. Olsen, and J. A. Hutchings. 2018. Temporal variability in offspring quality and individual reproductive output in a broadcast-spawning marine fish. ICES Journal of Marine Science 75:1353–1361.
Schartl, M., R. B. Walter, Y. Shen, T. Garcia, J. Catchen, A. Amores, I. Braasch, et al. 2013. The genome of the platyfish, Xiphophorus maculatus , provides insights into evolutionary adaptation and several complex traits. Nature Genetics 45:567–572.
Schrader, M., R. C. Fuller, and J. Travis. 2013. Differences in offspring size predict the direction of isolation asymmetry between populations of a placental fish. Biology Letters 9:20130327.
Schrader, M., and J. Travis. 2012. Assessing the roles of population density and predation risk in the evolution of offspring size in populations of a placental fish. Ecology and Evolution 2:1480–1490.
Skalkos, Z. M. G., J. U. Van Dyke, and C. M. Whittington. 2023. Distinguishing Between Embryonic Provisioning Strategies in Teleost Fishes Using a Threshold Value for Parentotrophy. Biomolecules 13:166.
Smith, C. C., and S. D. Fretwell. 1974. The Optimal Balance between Size and Number of Offspring. The American Naturalist 108:499–506.
Stearns, S. C. 1989. Trade-Offs in Life-History Evolution. Functional Ecology 3:259–268.
Stefanoska, I., M. Jovanović Krivokuća, S. Vasilijić, D. Ćujić, and L. Vićovac. 2013. Prolactin stimulates cell migration and invasion by human trophoblast in vitro. Placenta 34:775–783.
Stephens, M. 2017. False discovery rates: a new deal. Biostatistics 18:275–294.
Tessier, A. J., and N. L. Consolatti. 1989. Variation in Offspring Size in Daphnia and Consequences for Individual Fitness. Oikos 56:269–276.
Trivers, R. L. 1974. Parent-Offspring Conflict. American Zoologist 14:249–264.
Turcotte, M. M., M. N. Pires, R. C. Vrijenhoek, and D. N. Reznick. 2008. Pre- and post-fertilization maternal provisioning in livebearing fish species and their hybrids (Poeciliidae: Poeciliopsis). Functional Ecology 22:1118–1124.
Walker, R. S., M. Gurven, O. Burger, and M. J. Hamilton. 2008. The trade-off between number and size of offspring in humans and other primates. Proceedings of the Royal Society B: Biological Sciences 275:827–834.
Weiss, M. N., S. Ellis, D. W. Franks, M. L. K. Nielsen, M. A. Cant, R. A. Johnstone, D. K. Ellifrit, et al. 2023. Costly lifetime maternal investment in killer whales. Current Biology 33:744-748.e3.
Whittington, C. M., A. L. Buddle, O. W. Griffith, and A. M. Carter. 2022. Embryonic specializations for vertebrate placentation. Philosophical Transactions of the Royal Society B: Biological Sciences 377:20210261.
Wittbrodt, J., D. Adam, B. Malitschek, W. Mäueler, F. Raulf, A. Telling, S. M. Robertson, et al. 1989. Novel putative receptor tyrosine kinase encoded by the melanoma-inducing Tu locus in Xiphophorus. Nature 341:415–421.
Wourms, J. P., B. D. Grove, and J. Lombardi. 1988. 1 The Maternal-Embryonic Relationship in Viviparous Fishes. Pages 1–134 *in* W. S. Hoar and D. J. Randall, eds. Fish Physiology, The Physiology of Developing Fish (Vol. 11). Academic Press.