Genetics and resource availability shape divergence in life history and behavior between locally-adapted populations of Atlantic mollies (*Poecilia mexicana*, Poeciliidae)

John L. Coffin[1,2], Bethany L. Williams[3,4] & Michael Tobler[3,4,5]

[1]Division of Biology, Kansas State University, 104 Ackert Hall, Manhattan, KS 66506
[2]Current address: Washburn Rural High School, Topeka, KS 66619, USA
[3]Department of Biology, University of Missouri—St. Louis, St. Louis, MO 63121, USA
[4]Whitney R. Harris World Ecology Center, University of Missouri—St. Louis, St. Louis, MO, USA
[5]WildCare Institute, St. Louis Zoo, St. Louis, MO 63110, USA

Correspondence: tobler@umsl.edu

Abstract

Phenotypic variation is common along environmental gradients, but it is often unknown to what extent it results from genetic differentiation between populations or phenotypic plasticity. We studied populations of a livebearing fish that have colonized streams rich in toxic hydrogen sulfide (H_2S). In nature, there is strong phenotypic differentiation between adjacent sulfidic and nonsulfidic populations. In this study, we varied food availability to pregnant mothers from different populations to induce maternal effects, a form of plasticity, and repeatedly measured life-history and behavioral traits throughout the offspring's ontogeny. Genetic differentiation affected most of the traits we measured, as sulfidic offspring tended to be born larger, mature later, have lower burst swimming performance, be more exploratory, and feed less accurately. In contrast, maternal effects impacted few traits and at a smaller magnitude, even though offspring from poorly provisioned mothers tended to be born larger and be more exploratory. Population differences and maternal effects (when both were present) acted synergistically, and there was no evidence for population differences in plasticity. Overall, our study suggests that phenotypic divergence between these populations in nature is primarily caused by genetic differentiation, and that plasticity mediated by maternal effects accentuates—but does not cause—differences between populations.

Keywords: adaptation; extreme environments; hydrogen sulfide; maternal effects; resource availability; Poeciliidae

Introduction

Phenotypic variation is at the heart of evolutionary analyses because it links the cause (natural selection) to the consequence (genotypic change) of adaptive evolution (Lande & Arnold, 1983). We have known how inheritance causes resemblance between parents and their offspring for well over a century, reflecting a genetic component to phenotypic variation (Stenseth et al. 2022). However, trait variation can also be influenced by phenotypic plasticity, where a single genotype can give rise to alternate phenotypes in response to internal or environmental cues (West-Eberhard, 1989; Pigliucci, 2001). In addition, the environment experienced by parents can affect phenotypes of their offspring (*i.e.*, parental effects; Uller 2008; Badyaev and Uller 2009), representing a case of plasticity that spans generational boundaries. Phenotypic variation in nature can therefore arise from genetic differences among individuals, plasticity induced by individual exposure to different environmental conditions, plasticity induced by parental effects, and their interactions (Scheiner 1993; Dingemanse and Araya-Ajoy 2015). For many natural systems, we know little about the origins of phenotypic variation, even though it critically shapes our inference of adaptation in natural populations.

Plasticity induced by parental effects is particularly strong from mothers due to their higher reproductive investment and, in viviparous species, the physically intimate relationship with their developing young (Lindholm, Hunt, & Brooks, 2006; Wolf & Wade, 2009). Such maternal effects are widespread in nature (Mousseau and Fox 1998) and can impact trait expression and evolution (Rossiter, 1996; Wilson *et al.*, 2005; Beckerman *et al.*, 2006). Maternal effects can be adaptive if the expression of offspring traits is biased to match the environment experienced by the mother (Marshall and Uller 2007), or if mothers in good condition are able to endow phenotypes that provide a competitive advantage to their offspring in any environment (Grafen, 1988; Monaghan, 2008; Van Allen *et al.*, 2021). However, maternal effects can also be maladaptive and produce mismatches between offspring phenotype and environment, as documented in some organisms

59 responding to anthropogenic climate change (Schuler and Orrock 2012; Leonard and Lancaster
60 2020). Maladaptive maternal effects can also be related to stress, where physiological stress
61 responses in mothers have unintended negative side effects on offspring (MacLeod, While, & Uller,
62 2021). Regardless of whether maternal effects are adaptive, they are important biological phenomena
63 that warrant careful attention and explicit accounting in evolutionary analyses due to the non-genetic
64 effects on phenotypic expression.

65 Phenotypic variation in nature is common along environmental gradients, but it is often
66 unclear whether it is caused by genetic differentiation among populations or plastic effects that arise
67 from population-specific environmental exposure histories experienced by mothers or directly by
68 their offspring. For example, freshwater springs rich in toxic hydrogen sulfide (H_2S) in the Grijalva
69 River basin of southern Mexico are extreme environments that are connected to adjacent nontoxic
70 streams, and stark phenotypic gradients can be observed in fish occupying these habitats in as little
71 as a few meters. Sulfide springs are complex ecosystems with several correlated sources of selection
72 (Tobler *et al.*, 2016b). H_2S is toxic because it disrupts aerobic ATP production (Reiffenstein, Hulbert,
73 & Roth, 1992; Cooper & Brown, 2008), but habitats rich in H_2S also differ from nonsulfidic habitats
74 in other physical and chemical water parameters (dissolved oxygen concentrations, pH, and salinity),
75 and the presence of competitors and predators (Riesch *et al.*, 2010a; Tobler *et al.*, 2011; Greenway *et*
76 *al.*, 2014). Resource availability also differs greatly between habitat types; fish in nonsulfidic
77 environments eat primarily algae and detritus, while fish in sulfidic environments have shifted to
78 eating primarily sulfide bacteria and invertebrates (Tobler *et al.*, 2015). Populations of Atlantic
79 mollies (*Poecilia mexicana*), a species of livebearing fish of the family Poeciliidae, have independently
80 colonized and adapted to sulfidic streams across multiple river drainages, and previous studies have
81 documented that colonization of sulfide springs has been associated with convergent changes in
82 morphology, locomotion, and respiration (Tobler & Hastings, 2011; Camarillo, Arias Rodriguez, &

83 Tobler, 2020), behavior (Plath *et al.*, 2007; Lukas *et al.*, 2021; Doran *et al.*, 2022), physiology (Tobler *et*
84 *al.*, 2011; Barts *et al.*, 2018; Greenway *et al.*, 2020), and life history traits (Riesch *et al.*, 2011a; Riesch,
85 Plath, & Schlupp, 2011b; Riesch *et al.*, 2014). Phenotypic divergence between sulfidic and nonsulfidic
86 mollies likely has a significant genetic component, because it coincides with strong genetic
87 differentiation between populations, even though there are no physical barriers separating
88 populations in the different habitat types (Palacios *et al.*, 2013; Plath *et al.*, 2013; Riesch *et al.*, 2016b).
89 However, trait variation between populations likely also has an environmental component; while
90 population differentiation persists in captive populations reared in common garden conditions in the
91 lab (Tobler *et al.*, 2016a; Greenway *et al.*, 2020), there is also evidence for plasticity caused by short-
92 term exposure to different environmental conditions (Bierbach *et al.*, 2011; Passow *et al.*, 2017a). In
93 addition, the impact of maternal effects on offspring trait expression remains to be investigated in
94 these livebearing fish. Hence, we tested how genetic differentiation, maternal effects, and their
95 interactions shape phenotypic expression in *P. mexicana* populations from sulfidic and nonsulfidic
96 habitats. To induce maternal effects, we manipulated resource availability to pregnant mothers,
97 because natural populations vary substantially in nutritional state. Fish in sulfidic habitats are
98 consistently under food stress, exhibiting significantly reduced body condition (both when inferred
99 through length-weight regression and body fat content analysis; Plath *et al.*, 2005; Tobler *et al.*, 2006;
100 Tobler, 2008). Food stress arises as a consequence of constraints associated with resource
101 acquisition; because H_2S coincides with and exacerbates hypoxia, fish from sulfidic habitats have to
102 trade off performing aquatic surface respiration, a compensatory behavior to access better-
103 oxygenated surface waters, with benthic foraging (Tobler *et al.*, 2009). Accordingly, populations in
104 sulfide springs have adaptations to low resource availability, including reductions in routine
105 metabolic rates and energetically expensive tissues like the brain (Schulz-Mirbach *et al.*, 2016;
106 Passow, Arias-Rodriguez, & Tobler, 2017b). In other species, including some poeciliids, resource

107 availability experienced by mothers has been shown to impact offspring trait expression (Reznick,
108 Callahan, & Llauredo, 1996a; Altmann & Alberts, 2005; Boots & Roberts, 2012), and different
109 population histories in terms of exposure to food stress in *P. mexicana* may have caused changes in
110 resource-induced maternal effects.

111 To quantify population differentiation and maternal effects in populations of *P. mexicana*, we
112 followed families of offspring from birth to the onset of maturation and repeatedly quantified a host
113 of complex phenotypic traits. Focal traits included brood size, size at birth, and age at maturity as
114 well as ontogenetic trajectories in growth rates, burst swimming, exploratory behavior, and feeding
115 accuracy. We chose these traits because they likely affect fitness, and we have prior knowledge for
116 many of them from natural populations, providing us with a framework to make *a priori* predictions.
117 Specifically, our experiments sought to address four specific questions: (1) Is there evidence for
118 phenotypic trait differences between populations from sulfidic and nonsulfidic habitats that persist
119 in fish reared in a common-garden environment for multiple generations? Divergence in phenotypic
120 traits between populations regardless of maternal food treatments would indicate that trait
121 differentiation is due to genetic variation between populations. (2) Is there evidence for maternal
122 effects in response to resource availability? Significant differences in offspring traits between
123 maternal food treatments, irrespective of population of origin, would suggest resource-induced
124 maternal effects. (3) How do functional traits vary throughout ontogeny, and how do population
125 differences and maternal effects interact with ontogeny? Age is a major determinant in the
126 expression of many traits (Hegyi *et al.*, 2006; Zhang *et al.*, 2015), but how population differences and
127 maternal effects impact trait expression through ontogeny is less clear. In other poeciliids, maternal
128 effects tend to be present at birth and decline with age (Lindholm *et al.*, 2006). In contrast,
129 population differences between sulfidic and nonsulfidic *P. mexicana* are stark in adults, suggesting
130 differences may emerge early in life and even increase throughout ontogeny (Riesch *et al.*, 2011a).

131 Accordingly, we predicted that age would impact most of the traits measured, that population
132 differentiation would increase with age, and that maternal effects would diminish with age. (4) How
133 do population differences interact with maternal effects? A difference in how each population
134 responds to variation in maternal resource availability would indicate genotype-by-environment
135 interactions. Fish from sulfidic habitats generally face constraints in resource levels, while those
136 from nonsulfidic habitats have access to more abundant resources (Tobler *et al.*, 2006, 2009; Tobler,
137 2008). Hence, we predicted that trait variation induced by the low-food treatment would occur in
138 the same direction as trait variation produced by differences between the nonsulfidic and sulfidic
139 populations (i.e., maternal effects would be aligned with population differences). In this case,
140 maternal effects would accentuate divergence between populations that resembles patterns of
141 variation found in the wild. Alternatively, low-resource traits may be canalized in the sulfidic
142 population because sulfidic individuals are constantly food stressed in nature. In this case, maternal
143 effects may be weaker in the sulfidic population.
144

145 **Methods**

146 *Experimental overview*

147 For our experiments, we used two lab-reared populations of *Poecilia mexicana* originating from wild-
148 caught relatives in the Tacotalpa River drainage. One population originated from a sulfide spring
149 complex called El Azufre I (according to Plath *et al.*, 2013; hereafter referred to as the sulfidic
150 population or ecotype), and the other population was from a nonsulfidic stream connected to the
151 mainstem of the Tacotalpa River, called Arroyo Bonita (according to Plath *et al.*, 2013; hereafter
152 referred to as the nonsulfidic population or ecotype). Both populations were reared in 680-liter stock
153 tanks filled with filtered tap water. Tanks were fed *ad libitum* twice daily with commercial dry fish
154 food (Purina), and ~50% of the water was exchanged weekly. Mothers used in this experiment were

155 raised under common-garden conditions for at least three generations (*i.e.*, they were at least great-
156 grandchildren from individuals originally collected in the wild).

157 From each tank, 30 females were caught with a dipnet and isolated in a 20-liter tank with an
158 aerating filter and a bundle of plastic mesh as shelter for newborn fry. Females were fed twice daily,
159 once with aquatic gel diet for omnivorous fish (Mazuri) and once with freshly hatched *Artemia*
160 nauplii (Brine Shrimp Direct). Females were randomly assigned to either a "high-food" diet (0.32 ml
161 per feeding) or a "low-food" diet (0.08 ml per feeding), with equal numbers of females from each
162 population being assigned to each group (Figure 1). Each tank was checked daily for newborn fry.
163 By the end of the experiment, 18 sulfidic females gave birth (12 from the high food treatment and 6
164 from the low food treatment) and 22 nonsulfidic females produced a brood (12 from the high food
165 treatment and 10 from the low food treatment). The amount of time each female was in the food
166 treatment before giving birth varied (mean 28.2 days, range 0-99 days). As some females gave birth
167 during the acclimation period or immediately after beginning the food treatments, we included
168 treatment length as a potential covariate in all analytical models. Additionally, for a subset of models
169 we tested whether using females that were in the treatment for at least seven days would affect the
170 resulting top models (Table S1). Because the models were largely unaffected, we chose to include all
171 broods to increase statistical power to detect population differences throughout ontogeny.

172 Whenever a brood was born, we recorded the brood size, date of birth for each family, the
173 number of days that the female was in the food treatment, and the mother's standard length
174 (distance from the anterior tip of the snout to the posterior end of the caudal peduncle, mm).
175 Mothers were then removed from the tanks, and we randomly selected 15 newborn fry (if available)
176 from each family to remain in the experimental tanks to minimize density-dependent effects.

177 From that point on, we followed the developing families through ontogeny. All fry—
178 irrespective of the mother's food treatment—received the same amount of food; they were fed *ad*

179 *libitum* twice daily with a mixture of decapsulated brine shrimp eggs (Brine Shrimp Direct) and dry
180 food. We assessed offspring phenotypes at approximately weekly intervals by measuring life history
181 traits (size at birth, weekly growth rate, and age at maturity) and behavioral traits (burst swimming,
182 exploratory behavior, and feeding accuracy; see Figure 1). We specifically tested whether there were
183 differences in these phenotypes between maternal food treatments (*i.e.*, maternal effects), between
184 populations (*i.e.*, sulfidic *vs.* nonsulfidic, indicating effects of evolved population differences), or
185 both. All analyses were conducted in R 4.0.5 (R Core Team, 2023). Code and data to reproduce all
186 analyses can be found on GitHub (https://github.com/michitobler/common-garden).
187
188 *Size at birth and growth rate*
189 To measure size at birth, we photographed each family from above on the day of their birth with a
190 Nikon D90 digital camera fitted with an AF-S Micro NIKKOR 105mm f/2.8 lens. A ruler was
191 included in the background of each image. Images were imported to ImageJ v. 1.53 (Schneider,
192 Rasband, & Eliceiri, 2012) and calibrated by setting the scale. We measured the standard length
193 (mm) of each offspring in the family. These measurements were averaged across all individuals to
194 obtain a single mean size at birth for each family. This measurement was completed weekly, and the
195 measurement for each family was subtracted from that family's measurement from the week prior to
196 obtain an average growth rate [mm/week]. To account for allometric differences in growth rate, we
197 converted the average weekly growth rates to a proportional growth rate by dividing the average
198 weekly growth rate by the mean body size the week prior.
199
200 *Age at maturity*
201 We estimated the minimum age at maturity for each family using morphological characteristics of
202 male sexual maturity. While the sexes of juvenile livebearers are difficult to distinguish, at the onset

203 of sexual maturity, the male anal fin is modified into an intromittent organ (gonopodium), while it
204 remains unmodified in females (Rosen & Gordon, 1953; Chambers, 1987). We therefore measured
205 minimum age at maturity (days) as the time it took to for the first male in a group to develop its
206 complete gonopodium as judged by the presence of a fleshy palp on anal fin ray 3.
207
208 *Burst swimming*
209 Most fish avoid predation with a highly conserved, reflexive escape response that causes the head to
210 move away from the stimulus, bending the body into a 'C' shape (Eaton, Bombardieri, & Meyer,
211 1977). Then, a strong stroke of the caudal fin starts the movement away from the stimulus
212 (Domenici & Blake, 1997). This process (bending into a 'C' and propelling away from the stimulus)
213 is known as a C-start response and is frequently used as a metric of escape performance in fish
214 (Walker, 1997; Ghalambor, Reznick, & Walker, 2004; Langerhans *et al.*, 2004; Camarillo *et al.*, 2020).
215 To quantify this burst swimming behavior, we adopted the methods and metrics used by prior
216 studies (Langerhans *et al.*, 2004; Ingley *et al.*, 2016; Camarillo *et al.*, 2020). We placed a haphazardly
217 chosen individual from each family in a glass petri dish (9 cm in diameter, containing 2 cm of water)
218 with opaque sides suspended above an angled mirror, providing a ventral view of each fish. After 5
219 min of acclimation, we struck the surface of the water within a body length of the fish with a probe
220 and recorded the movement of the fish from below with a Sony NEX-FS700R camcorder at 60
221 frames per second (fps) and 1080p resolution. We converted the resulting .mts files into .mp4 files
222 (to enhance compatibility with downstream applications) with FFmpeg (Tomar, 2006).
223 We used DLTdv8 (Hedrick, 2008) to digitize the 2-D location of the isthmus (*i.e.*, the area
224 on the ventral surface of the head where the opercula converge) of the fish in each frame. Digitized
225 points were then used to calculate the maximum velocity (v_{max}, mm/s), maximum acceleration (a_{max},
226 mm/s^2), and net distance traveled (d_{net}, mm of displacement within 1/12th of a second after the C-

227 start). To calculate v_{max}, we calculated the straight-line distance between each pair of successive
228 digitized points, divided this distance by the inverse of the frame rate (60 fps), and found the
229 maximum value between any two points. a_{max} was calculated by subtracting the velocity value at each
230 point from the velocity value at the point immediately preceding it and finding the maximum value.
231 d_{net} was calculated by recording the 2-D position immediately after the fish ends the C-start with a
232 single stroke of the caudal fin, and then recording the 2-D position $1/12^{th}$ of a second later (5
233 frames later) and calculating the straight-line distance between the two points. To reduce the
234 dimensionality of this dataset, we conducted a principal component analysis (PCA) using the
235 prcomp() function with a correlation matrix. There was one principal component with an eigenvalue
236 greater than 1 (explaining 85.6 % of the total variance), which was retained as a compound metric of
237 burst swimming performance. Positive scores along this principal component axis were associated
238 with higher velocity, acceleration, and distance traveled (Table S2A). All mathematical operations
239 were conducted using packages contained in the base distribution of R.
240
241 *Exploratory behavior*
242 We used an open field test to quantify the exploratory tendencies of fry. We filled a Styrofoam cup
243 (9 cm in diameter) with 3 cm of water and covered the arena with a sheet of glass. We haphazardly
244 selected one individual from each family and placed it in the arena undisturbed for a 5-min
245 acclimation period, after which we recorded 5 min of video from above with a GoPro Hero 4
246 (1080p resolution, linear field of view, 30 fps).
247 We used idTracker (v. 2.1, bundled with 64-bit Matlab Compiler Runtime 8.3; Pérez-
248 Escudero *et al.*, 2014) to track the 2-D location of the fish automatically for the entire 5-min
249 recording. We set the number of individuals to 1 and manually determined the intensity threshold

250 (0.5–0.8) and minimum size (40–250 pixels) for each video. We also imported a still frame from each
251 video into ImageJ to measure the centroid coordinates and arena radius.
252 Using the 2-D coordinates in each frame, we calculated several metrics of motion that we
253 used as proxies for exploratory behavior. We calculated distance traveled between each pair of
254 successive points, the velocity, the acceleration, and the total cumulative distance traveled (d_{total},
255 mm), as described above. We also calculated average velocity (v_{avg}, mm/s), maximum velocity (v_{max},
256 mm/s), and maximum acceleration (a_{max}, mm/s^2) by finding the means and maxima of all velocity
257 and acceleration values. Finally, we calculated the proportional average distance from the center of
258 the arena (d_{center}, dimensionless) by calculating the distance from the fish's location to the centroid of
259 the arena and dividing this value by the arena radius. Videos were excluded if the fish was
260 completely still in all frames. To reduce the dimensionality of and observe the correlation structure
261 within this dataset, we ran a PCA on v_{avg}, v_{max}, a_{max}, d_{total}, and d_{center} with a correlation matrix. We
262 retained scores along the first PC axis (explaining 56.9 % of the total variance) as a composite
263 exploratory behavior score. As shown in Table S2B, higher PC1 scores were associated with more
264 exploratory behavior (positive loadings for all variables).
265
266 *Feeding accuracy*
267 To measure feeding accuracy, we withheld food from individuals of each tank for 24 h prior to the
268 experiment and placed one haphazardly selected fry in a viewing tank for a 5-min acclimation
269 period. The viewing tank was constructed by connecting two 10 cm × 10 cm sheets of glass to either
270 side of 1 cm diameter glass rods with silicone. The rear wall of the viewing tank was covered with a
271 black sheet of plastic to enhance contrast between the background and the fish in the tank. The
272 feeding solution consisted of 1 g of freshly hatched, live *Artemia* nauplii diluted into 100 ml of
273 filtered tap water. After 5-min of acclimation, we added 0.08 ml of the feeding solution to the

viewing tank and recorded 5 min of video with the camcorder. We analyzed the video frame-by-frame using BORIS v. 7.13 (Friard & Gamba, 2016) and recorded the number of successful strikes (a feeding strike that ends in consumption of the food item) and unsuccessful strikes (a feeding strike that either misses the food item or results in regurgitation of the food item). We divided the number of successful strikes by the total number of strikes to get an overall proportional estimate of feeding accuracy, which was arcsine-square-root transformed prior to analysis.

Statistical analyses of individual traits

There were many potential sources of variation in our experiment. Other than the effects of interest for our study (population, maternal food treatment, and their interaction), the observed variation in traits could also have arisen from differences in fry age, maternal body size (standard length), the duration of her food treatment, and brood size. Consequently, we used a model selection approach to find the models that were best supported by our data for each experiment separately. For each phenotype, we created a global model that contained all possible effects. For phenotypes that were only measured once for each family (size at birth, brood size, and age at maturity), we used a general linear model using the lm() function from the STATS package v. 3.6.2, and for phenotypes that were measured repeatedly through development (growth rate, burst swimming, exploratory behavior, and feeding accuracy), we used a linear mixed model implemented with the lmer() function from the LME4 package v. 1.1-26 (Bates *et al.*, 2015) that included 'family' as a random effect. We then used the dredge() function from the MUMIN package v. 1.47.1 (Bartón 2009) to create a model selection table based on the effects contained in the global model, with different models ranked and weighted based on AIC_c (Johnson & Omland, 2004). Full model selection tables are available for each phenotype in Table S3. To avoid overfitting, we limited the models to a maximum of four terms. We chose the top-supported model for each trait and analyzed it with either type-III analysis of variance

298 (ANOVA) or Wald's chi-square tests (see Table 1). Quantification and visualization of effects was
299 accomplished by calculating and plotting estimated marginal means for the effects of 'Population'
300 and/or 'Food Treatment', depending on the best-supported model, using the Effect() function from
301 the EFFECTS package v. 4.2-2 (Fox & Weisberg, 2018a,b). To aid in drawing inferences from our
302 models, we generated 95% confidence intervals (CI) for model coefficients in our top models using
303 the confint() function form the base R distribution. Effect sizes were calculated as partial eta-
304 squared (η_p^2), which represents the proportion of variance explained by a particular variable after
305 accounting for the variance explained by all other variables. We calculated η_p^2 with the etasq()
306 function (Fox et al. 2021) for general linear models or the eta_squared() function (Ben-Shachar,
307 Lüdecke, & Makowski, 2020) for linear mixed models.
308
309 *Multivariate analysis*
310 Since selection ultimately acts on complex, multivariate phenotypes (Lande & Arnold, 1983), we
311 sought to understand how the traits measured for our analyses vary and covary to jointly shape
312 multivariate phenotypes. To do so, we averaged each phenotype across all ages for each tank. We
313 used this approach rather than including age as a covariate and analyzing raw phenotypic scores
314 because of timing and logistical constraints that made it impossible to conduct each experiment on
315 offspring that were exactly the same age. We analyzed the averaged phenotypes with a PCA
316 (correlation matrix) and used scores along the first two principal components—which had
317 eigenvalues greater than 1—as dependent variables. We analyzed principal component scores along
318 each axis separately because the axes, by definition, were orthogonal. For each axis, we created a
319 global model containing all possible effects and selected the best-supported model based on AIC_c, as
320 explained above.
321

322 **Results**

323 We measured seven functional traits in offspring from a sulfidic and a nonsulfidic population of *P.*
324 *mexicana* throughout ontogeny. Model selection tables for the analysis of each trait at birth and
325 separately across all ages can be found in Table S3, and the best-supported models are summarized
326 in Table 1. For brevity, we will present results in the context of our hypotheses outlined in the
327 Introduction, focusing on how all traits vary through ontogeny in terms of population
328 differentiation, maternal effects, and their interaction, rather than presenting the results for each trait
329 separately.

330

331 *Is there evidence for population differentiation?*

332 We found evidence for significant population differences in five of the seven traits measured: size at
333 birth, age at maturity, burst swimming, exploratory behavior, and feeding accuracy (but not brood
334 size or growth rate). Age at maturity, burst swimming, and feeding accuracy were significantly
335 different between populations, but not between food treatments, whereas there were significant
336 effects of 'Population' and 'Food Treatment' (but no interactions) on size at birth and exploratory
337 behavior (see Table 1 and below). Sulfidic individuals were born 9.2 % larger [$F_{1,35}$ = 20.032; P <
338 0.001; estimated marginal mean for sulfidic fish (EMM_S) = 10.03 mm, estimated marginal mean for
339 nonsulfidic fish (EMM_{NS}) = 9.18 mm; η_p^2 = 0.36; Figure 2A], matured an average of 10.5 days later
340 ($F_{1,26}$ = 4.420; P = 0.045; EMM_S = 54.2 days, EMM_{NS} = 43.7 days; η_p^2 = 0.15), had significantly
341 lower burst swimming performance (χ^2_1= 4.308; P = 0.038; CI: –0.94, –0.03; η_p^2 = 0.03), were more
342 exploratory (χ^2_1 = 7.513; P = 0.006; CI: 0.29, 1.70; η_p^2 = 0.28), and were 7.2% less successful during
343 feeding (χ^2_1 = 4.645; P = 0.031; EMM_S = 0.72, EMM_{NS} = 0.80; η_p^2 = 0.04; Figure 3).

344

345 *Is there evidence for maternal effects in response to resource availability?*

346 To test whether maternal effects induced by resource availability during pregnancy impact functional
347 traits in offspring, we compared each phenotype between offspring born to mothers who
348 experienced a high-food environment and mothers who experienced a low-food environment.
349 Mothers in low-food treatments produced offspring that were 3.4% larger at birth [$F_{1,35}$ = 4.354; P =
350 0.044; estimated marginal mean for fish in the low-food treatment (EMM_{low}) = 9.8 mm, estimated
351 marginal mean for fish in the high-food treatment (EMM_{high}) = 9.4 mm; η_p^2 = 0.11; Figure 2A] and
352 were more exploratory (χ^2_1 = 4.245; P = 0.039; CI: –0.04, –1.42; η_p^2 = 0.18; Figure 3E). For all
353 remaining traits (brood size, age at maturity, growth rate, burst swimming, and feeding accuracy),
354 'Food Treatment' was not included in the best-supported model (see Table 1), suggesting that
355 maternal effects did not affect the expression of those traits.

356

357 *How do population differences and maternal effects interact with ontogeny?*

358 To determine how traits changed through offspring development, we compared each phenotype
359 across age groups. 'Age' was a significant predictor of growth rate and burst swimming, indicating
360 that these traits changed throughout ontogeny, while the other phenotypes were not affected by age.
361 Across populations and food treatments, fry grew at a slower relative rate (χ^2_1 = 147.91; P < 0.001,
362 CI: –0.010, –0.007; η_p^2 = 0.50) and performed better in burst swimming trials (χ^2_1 = 30.178; P <
363 0.001; CI: 0.024, 0.049; η_p^2 = 0.16) as they got older (Table 1). Burst swimming scores were also
364 lower in sulfidic individuals (see above), but the interaction term 'Population × Age' was not
365 significant, demonstrating that both populations exhibited similar changes in burst swimming
366 throughout ontogeny.

367 To ensure that signals of population differentiation and maternal effects occurring at birth
368 were not obscured by measurements later in life, we also subset our dataset for each phenotype to
369 obtain only the earliest data point for each family. As mentioned above, fry from the sulfidic
370 population and the low-food maternal treatment had significantly larger size at birth (Figure 2; Table
371 1). However, there were no population differences or maternal effects on brood size, which was
372 higher in larger mothers (Mother SL; $F_{1,37}$ = 23.342; P < 0.001; CI: 0.73, 1.79; η_p^2 = 0.38) and
373 mothers who spent longer in the food treatment (Treatment Length; $F_{1,37}$ = 8.926; P = 0.005; CI:
374 0.07, 0.40; η_p^2 = 0.19). Growth rate at birth was significantly lower in sulfidic fry ($F_{1,37}$ = 5.025; P =
375 0.032; CI: –0.12, –0.006; η_p^2 = 0.12; Table 1; Figure 3A), but there was no evidence for maternal
376 effects on growth rate at birth ('Food Treatment' was not included in best-supported model). Note
377 that the population difference in growth rate at birth disappeared as fry developed (Figure 3B). All
378 other traits that were measured throughout ontogeny (burst swimming, exploratory behavior, and
379 feeding accuracy) did not exhibit significant population differences or maternal effects at birth.
380 Interestingly, the maternal effect that we detected on exploratory behavior across all ages (see above)
381 was not evident at birth ($F_{1,31}$ = 2.727; P = 0.108; CI: –0.29, 2.81). These results collectively
382 demonstrate that—contrary to our hypothesis regarding ontogenetic variation of functional traits—
383 population differentiation did not increase with age, and maternal effects were not always observable
384 at birth, nor did they decline with age.
385
386 *How do population differences interact with maternal effects?*
387 We hypothesized that variation from maternal effects would be aligned with population differences,
388 but that there would be a significant interaction between population differences and maternal effects
389 (*i.e.*, different magnitudes of maternal effects in each population due to different evolutionary
390 histories associated with resource stress). Contrary to our predictions, no 'Population × Food

391 Treatment' interactions were included in the best-supported model for any of the traits we measured
392 (Table 1), suggesting a general lack of support for significant interactions between population
393 differences and maternal effects. Therefore, maternal effects—if present—were similar in direction
394 and magnitude between populations.

395 To further address our hypothesis, we asked whether population differences and maternal
396 effects were, in fact, aligned, and whether they explained a similar proportion of phenotypic variance
397 when they acted in unison. We compared the signs (positive *vs.* negative coefficient estimates) and
398 effect sizes (η_p^2) of population differences and maternal effects for the two traits with evidence of
399 both effects simultaneously impacting trait expression—size at birth and exploratory behavior. For
400 both traits, the difference between the nonsulfidic and sulfidic population occurred in the same
401 direction as the trait shifts between the high- and low-food treatments, indicating that the effects
402 were aligned (Figures 2A and 3E). Effect size estimates for 'Population' and 'Food Treatment' for
403 both traits indicated that 'Population' had a larger effect on trait expression than 'Food Treatment'
404 (η_p^2 = 0.36 *vs.* 0.11 for size at birth and 0.28 *vs.* 0.18 for exploratory behavior).

405

406 *Multivariate analysis*

407 In addition to the univariate analyses of trait variation, we were also interested in understanding how
408 the traits covaried with one another, and whether and how multivariate phenotypes were impacted
409 by population differences and maternal effects. We averaged each phenotype across ages for each
410 family, conducted a PCA, and then analyzed principal component scores along the first two
411 principal component axes. The first principal component accounted for 28.5% of variance in
412 multivariate phenotypes, and scores along the first principal component were positively correlated
413 with brood size, growth rate, and feeding accuracy, and they were negatively correlated with size at
414 birth, age at maturity, burst swimming, and exploratory behavior (see Table S2C). The second

415 principal component explained 21.1% of variance and was positively correlated with burst
416 swimming and size at birth and negatively correlated with brood size, growth rate, feeding accuracy,
417 exploratory behavior, and age at maturity (Table S2). After conducting model selection, the best-
418 supported model for PC1 contained the main effects of 'Population', 'Food Treatment', and
419 'Treatment Length', and the best-supported model for PC2 contained the main effects of
420 'Population', 'Mother SL', and 'Treatment Length'.

421 Sulfidic offspring had significantly lower scores along PC1 ($F_{1,24}$ = 16.266; P < 0.001; CI: –
422 2.40, –0.78; η_p^2 = 0.40), as did offspring from low-food mothers ($F_{1,24}$ = 4.746; P = 0.039; CI: –1.59,
423 –0.04), indicating population differences and maternal effects on PC1 scores. As for the univariate
424 analyses, maternal effects were aligned with population differences in PC1 scores (Figure 4),
425 'Population' had a larger effect than 'Food Treatment' (η_p^2 for 'Population' = 0.40; η_p^2 for 'Food
426 Treatment' = 0.17), and there was no evidence for a significant 'Population × Food Treatment'
427 interaction (the term was not included in best-supported model). We also found that sulfidic
428 individuals exhibited marginally lower PC2 scores ($F_{1,24}$ = 3.321; P = 0.081; CI: –1.45, 0.09; η_p^2 =
429 0.12) but detected no evidence for maternal effects (the 'Food Treatment' term was not included in
430 the best-supported model) on PC2.

431

432 **Discussion**

433 Phenotypic variation can be shaped by multiple genetic and nongenetic factors, but the interplay of
434 genes and environmental effects is rarely disentangled in natural systems, even though it
435 fundamentally impacts our inference of adaptation. We examined how genetic variation (*i.e.*,
436 population differences), phenotypic plasticity mediated by maternal effects, and their interactions
437 shape trait expression in two populations of *Poecilia mexicana* that are exposed to strong divergent
438 selection in nature. We found significant trait differences between populations even though fish

439 were housed under common-garden conditions in the laboratory for at least three generations. In
440 contrast, exposure of mothers to different food treatments impacted relatively few traits in their
441 offspring and, if they occurred, had weaker effects than the population differences. In addition,
442 maternal effects tended to be aligned with population differences and act in the same way across
443 populations. Principal component analysis supported these overall conclusions in multivariate trait
444 space. We also found no evidence for interactions between populations and food treatments,
445 suggesting that while populations have diverged in phenotypic traits, they have retained similar
446 maternal influences on those same traits. Overall, we found that the stark phenotypic differences
447 between populations of *P. mexicana* that are evident in nature are largely a consequence of genetic
448 divergence, likely representing local adaptation to the distinct ecological conditions of their habitats.
449 Maternal effects in response to resource availability, while present in some traits, appear to
450 accentuate population differences, but not cause them.
451
452 *Trait variation across populations and maternal food treatments*
453 There is a rich history integrating field-based studies that quantify trait variation of poeciliid fishes in
454 nature with laboratory-based studies that isolate causative environmental factors (Endler, 1980,
455 1995; Reznick & Bryga, 1987; Reznick, Bryga, & Endler, 1990; Langerhans, Gifford, & Joseph, 2007;
456 Tobler *et al.*, 2008; Ghalambor *et al.*, 2015; Ingley & Johnson, 2016). While most studies focus on
457 single or a few related traits and take a snapshot at a single ontogenetic stage, our study
458 demonstrated that multiple complex trait differences quantified in common-garden conditions—
459 across food treatments and across ontogeny—closely mirror trait differences between populations in
460 nature.
461 First, our study corroborates a genetic basis for population divergence in reproductive life
462 history traits. We documented significant population divergence in size at birth, with sulfidic mollies

463 giving birth to larger offspring (see Figures 2A and 4). This finding is consistent with life history
464 studies of sulfide spring populations in *P. mexicana* and other poeciliid species (Riesch *et al.*, 2010b;
465 Riesch, Plath, & Schlupp, 2010c; Riesch *et al.*, 2014), suggesting that divergence in offspring size
466 between sulfidic and nonsulfidic populations is a repeatable evolutionary consequence of sulfide
467 spring colonization that is driven by natural selection for larger offspring size. Among-population
468 variation in size at birth has also been documented in other poeciliids that are exposed to other
469 sources of divergent selection, including variation in predation (Weeks & Gaggiotti, 1993; Reznick,
470 Rodd, & Cardenas, 1996b; Schrader & Travis, 2012; Belk, Ingley, & Johnson, 2020). Our
471 multivariate results (Figure 4) also indicated that, like other poeciliids, the nonsulfidic population of
472 *P. mexicana* closely resembles an opportunistic life history strategy (Winemiller & Rose, 1992), which
473 places a premium on earlier maturity and higher fecundity, at the expense of lower juvenile
474 survivorship. The population differences we found in the sulfidic population may represent a shift in
475 the direction of the equilibrium strategy, which is energetically costly to parents but should
476 ultimately benefit offspring competitive ability. In natural populations, maternal effects induced by
477 variation in resource availability likely accentuate genetic differences in offspring size across sulfidic
478 and nonsulfidic habitats. This pattern was also evident across a plethora of life history traits in
479 guppies; in all ten life history traits with evidence for significant genetic divergence and maternal
480 effects, these effects always occurred in the same direction (Felmy *et al.*, 2022).

481 Second, we found patterns of population differentiation in age at maturity that match with
482 previous work in livebearing fishes. Prior studies have demonstrated that guppies in streams with
483 high predation pressure on adults mature earlier than conspecifics from low-predation populations
484 (Reznick & Endler, 1982). Several studies have also documented genetic differences in age at
485 maturity between populations of *P. mexicana*. For example, cave mollies mature significantly later
486 than surface populations, and there was also significant plasticity in age at maturity due to resource

487 availability (Riesch *et al.*, 2016a). Another study of surface mollies that compared size at maturity in
488 sulfidic and nonsulfidic populations found that sulfidic individuals reached maturity at a significantly
489 smaller size than nonsulfidic individuals (Riesch *et al.*, 2011b), likely because energy limitation
490 imposed by constraints associated with compensatory behaviors in sulfidic habitats (Tobler *et al.*,
491 2009) selects for reduced growth rates and smaller size at maturity (Passow *et al.*, 2017b). Since
492 poeciliid males exhibit determinate growth that ends at maturity (Stearns & Sage, 1980), size at
493 maturity should closely mirror age at maturity, assuming growth rates are the same as we found in
494 our study. However, we found that sulfidic mollies matured at a significantly later age than
495 nonsulfidic individuals, despite identical growth rates across ontogeny (Figure 3B). This discrepancy
496 between our findings and those of previous studies suggests that plasticity in age at maturity may be
497 strong and that variation in experimental design and rearing conditions matters.

498 Third, we found significant behavioral differences between populations from sulfidic and
499 nonsulfidic habitats, including exploratory behavior, feeding accuracy, and burst swimming. Previous
500 work has shown that nonsulfidic mollies—and ones that were better fed—were bolder in their
501 natural habitat, but behavioral differences also disappeared in the laboratory (Riesch *et al.*, 2009).
502 While we did not measure boldness *per se*, exploratory behavior as measured in our experiment (*i.e.*,
503 activity levels in a novel arena) is often characterized as part of a behavioral syndrome that correlates
504 with boldness (Conrad *et al.*, 2011). Unlike previous work, our study found that individuals from the
505 sulfidic population and the low-food treatment were more exploratory (Table 1 and Figure 3E).
506 These results support that maternal resource environment affects exploratory behavior, but also
507 imply heritable differences between populations. Similar heritable population differences were also
508 found for feeding accuracy and burst swimming but without any evidence for maternal effects. At
509 least for burst swimming, the pattern of population differentiation in our experiment again mirrors
510 findings from adult fish in natural habitats (Camarillo *et al.*, 2020).

511

512 *Trait variation and adaptive function*

513 Sulfide springs and adjacent nonsulfidic habitats do not only differ in the presence and absence of
514 H_2S, but they also vary in numerous abiotic and biotic factors that are often unaddressed in studies
515 of adaptation. Sulfidic habitats have lower dissolved oxygen concentrations, higher temperature,
516 higher specific conductivity, and lower pH (Tobler *et al.*, 2011), which in turn affect the biotic
517 communities (Greenway *et al.*, 2014) and selection associated with resource exploitation,
518 competition, predation, and parasites (Riesch *et al.*, 2010a; Tobler *et al.*, 2014, 2015). Adaptation in
519 sulfide springs is therefore not solely in response to selection from H_2S but is instead in response to
520 a multifarious selective regime that has caused multivariate phenotypic differentiation between
521 populations (Tobler *et al.*, 2018). The complexity of selective regimes and evolutionary responses
522 make disentangling cause-and-effect relationships difficult, especially because theoretical predictions
523 for the effects of different, covarying sources of selection are not mutually exclusive. For example,
524 the evolution of large offspring size at birth could be explained by (1) selection from H_2S, which
525 should favor larger offspring with a lower surface-to-volume ratio to reduce the influx of toxic H_2S
526 (Riesch et al. 2014); (2) selection from resource constraints, which favors larger offspring with
527 higher energy stores (Reznick *et al.*, 1996a); or (3) relaxation of selection from predation, which also
528 favors larger offspring (Reznick & Endler, 1982; Johnson & Belk, 2001; Jennions *et al.*, 2006).
529 Similarly, resource constraints and low predation also favor more exploratory individuals that are
530 better able to locate and exploit resources under those conditions (Teska, Smith, & Novak, 1990;
531 Kaun *et al.*, 2007; Huang, Sieving, & Mary, 2012). Assessing the adaptive value of trait differences
532 between sulfidic and nonsulfidic population is consequently nontrivial and remains a work in
533 progress.

534 Variation in some traits investigated in our study may also be the consequence of genetic,
535 developmental, or functional tradeoffs with other traits. Such tradeoffs are common in organisms
536 inhabiting contrasting environments because divergent selection acting to optimize one trait can
537 inadvertently influence other traits due to constraints (Ghalambor *et al.*, 2004; Kawecki & Ebert,
538 2004; Garland, Downs, & Ives, 2022). For example, reductions in burst swimming performance—as
539 documented in our study for fish from the sulfidic population—may arise as a consequence of
540 selection for increased steady swimming efficiency, as different body shapes are associated with
541 optimization of steady *vs.* unsteady swimming (Langerhans, 2007, 2009; Tokić & Yue, 2012). Indeed,
542 a tradeoff between burst speed and sustained swimming performance has been documented in adult
543 fish from the same populations we studied here. The tradeoff is likely balanced by the need for
544 energy-efficient swimming in sulfidic habitats with resource constraints (but low predation) and
545 selection for efficient predator avoidance in nonsulfidic habitats with high abundances of natural
546 enemies (but abundant resources) (Camarillo *et al.*, 2020). Our study found a similar reduction in
547 burst swimming performance in the sulfidic population, even among individuals that had not yet
548 reached maturity, suggesting population differences in burst swimming arise early in ontogeny. Our
549 results also matched *a priori* predictions regarding tradeoffs between respiration and feeding.
550 Habitats rich in H_2S also experience rampant hypoxia, which has selected for the evolution of
551 craniofacial traits (larger heads and jaws and longer gill filaments) that increase ventilation efficiency
552 (Camarillo *et al.*, 2020). In addition to changes in morphology, sulfidic individuals also exhibit
553 decreased foraging efficiency compared to nonsulfidic individuals (Tobler *et al.*, 2009), which was
554 supported by our findings related to feeding accuracy (Figure 3F). The decreases in feeding
555 efficiency noted in this and other studies may therefore be a consequence of the craniofacial
556 modifications that accompany colonization of sulfidic habitats.
557

558 *Plasticity accentuates genetic trait differentiation in natural populations*

559 Phenotypes in nature are the sum of genetic and environmental effects, but, surprisingly, we found
560 no evidence for canalization or the evolution of plasticity by genotype × environment interactions.
561 Our work demonstrated that maternal effects were aligned with population differences, accentuating
562 trait divergence between populations, and that the trait shifts induced by maternal effects were of a
563 similar magnitude in both populations. For both traits in which we observed significant population
564 differentiation and maternal effects (size at birth and exploratory behavior), the lowest phenotypic
565 scores were found in nonsulfidic fry from high-food mothers, and the highest scores were found in
566 sulfidic fry from low-food mothers (Figures 2A and 3E). Because sulfidic mollies exhibit reduced
567 foraging efficiency and body condition as a consequence of hypoxia (Tobler, 2008; Tobler *et al.*,
568 2009), sulfidic habitats are naturally analogous to our low-food treatment, and nonsulfidic habitats
569 are similar to the high-food treatment. If maternal effects enhance population differences in natural
570 populations similarly as in our experiments, then it could explain why stronger trait divergence is
571 typically observed in nature than in common garden reared fish (Tobler *et al.*, 2008; Passow *et al.*,
572 2015). Likewise, it is important to note that our experiments only captured a small aspect of
573 plasticity—the portion controlled by the mother. Future work needs to address genetic effects,
574 maternal effects, and plasticity in response to variation in environmental factors directly experienced
575 by the offspring to better understand the forces driving trait variation in nature. We also caution that
576 the shifts found in our study are only from comparisons of a single population pair. Our
577 understanding of the relative roles of genetic and plastic effects on phenotypic variation would
578 benefit greatly from studies that expand to other sulfidic and nonsulfidic population pairs in *P.*
579 *mexicana* and other species.

580

581 **Conflict of Interest Statement**

The authors declare no conflict of interest.

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Data Availability Statement

All datasets and R scripts used to analyze the data, with associated documentation, have been archived on GitHub (https://github.com/michitobler/common-garden).

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Table 1. Model terms in the best-supported model for each dependent variable. Empty cells represent terms that were not present in the best-supported model for that dependent variable. Type-III ANOVA or Wald's Chi-square tests were used to determine whether model terms were significantly different from zero. *F*-statistics or χ^2 statistics are presented for each included model term, with the degrees of freedom in a subscript. *P*-values are presented underneath each test statistic.

Dependent Variable	**Brood Size**	**Treatment Length**	**Mother SL**	**Age**	**Population**	**Food Treatment**
Size at Birth	$F_{1,35} = 20.876$ $P < 0.001$		$F_{1,35} = 16.593$ $P < 0.001$		$F_{1,35} = 20.032$ $P < 0.001$	$F_{1,35} = 4.354$ $P = 0.044$
Brood Size		$F_{1,37} = 8.926$ $P = 0.005$	$F_{1,37} = 23.342$ $P < 0.001$			
Age at Maturity					$F_{1,26} = 4.420$ $P = 0.045$	
Growth Rate at Birth					$F_{1,37} = 5.025$ $P = 0.031$	
Overall Growth Rate				$\chi^2_1 = 147.91$ $P < 0.001$		
Burst Swimming at Birth			$F_{1,39} = 5.025$ $P = 0.121$			
Overall Burst Swimming				$\chi^2_1 = 30.178$ $P < 0.001$	$\chi^2_1 = 4.308$ $P = 0.038$	
Exploratory Behavior at Birth						$F_{1,31} = 2.727$ $P = 0.108$
Overall Exploratory Behavior					$\chi^2_1 = 7.513$ $P = 0.006$	$\chi^2_1 = 4.245$ $P = 0.039$
Feeding Accuracy at Birth		$F_{1,38} = 0.775$ $P = 0.384$				
Overall Feeding Accuracy					$\chi^2_1 = 4.645$ $P = 0.031$	
Multivariate Trait Variation PC1		$F_{1,24} = 15.817$ $P < 0.001$			$F_{1,24} = 14.984$ $P < 0.001$	$F_{1,24} = 4.746$ $P = 0.039$
Multivariate Trait Variation PC2		$F_{1,24} = 7.722$ $P = 0.010$	$F_{1,24} = 5.292$ $P = 0.030$		$F_{1,24} = 3.321$ $P = 0.081$	

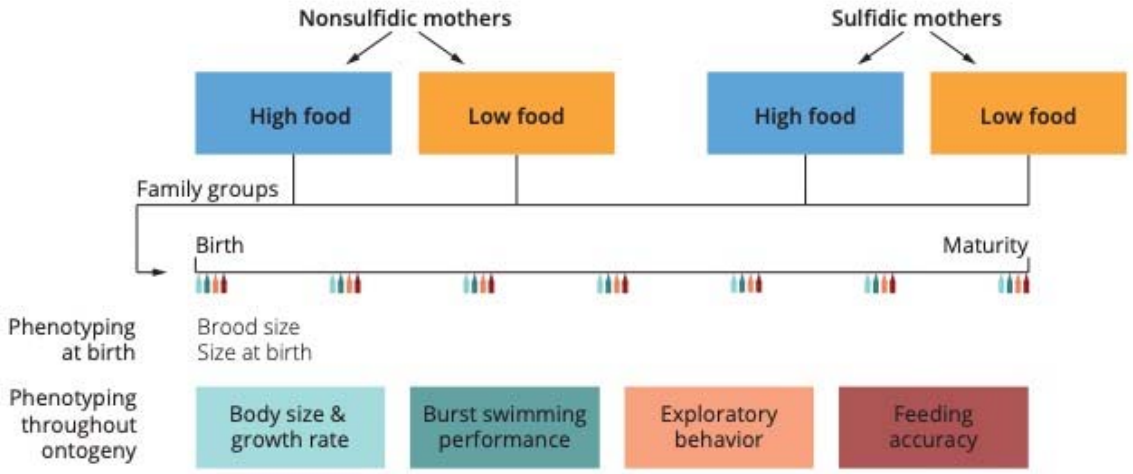

Figure 1. Overview of our experimental design. We subjected pregnant sulfidic and nonsulfidic mothers to either a high- or a low-food treatment and measured seven traits in their offspring throughout their development. These traits include four life history traits (birth size, brood size, growth rate, and age at maturity) and three behavioral traits (burst swimming, exploratory behavior, and feeding accuracy).

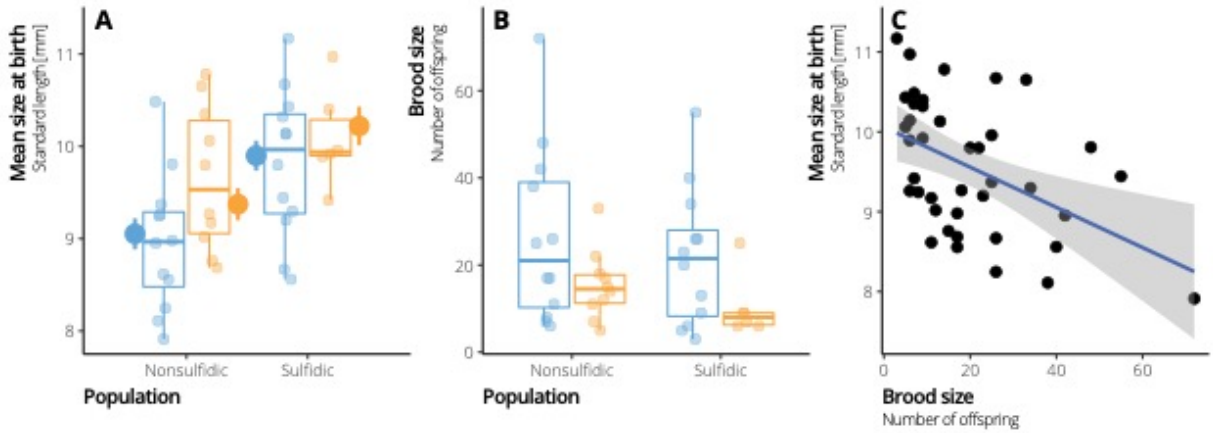

Figure 2. Plots of average standard length (A), brood size (B), and their relationship (C) for each family. Data is summarized in boxplots with raw data points overlaid. In both populations, the high-food treatment is shown in blue, and the low-food treatment is shown in orange. When the best-supported model for a phenotype contained the terms 'Population' and 'Food Treatment', the estimated marginal means for those effects were visualized as large points.

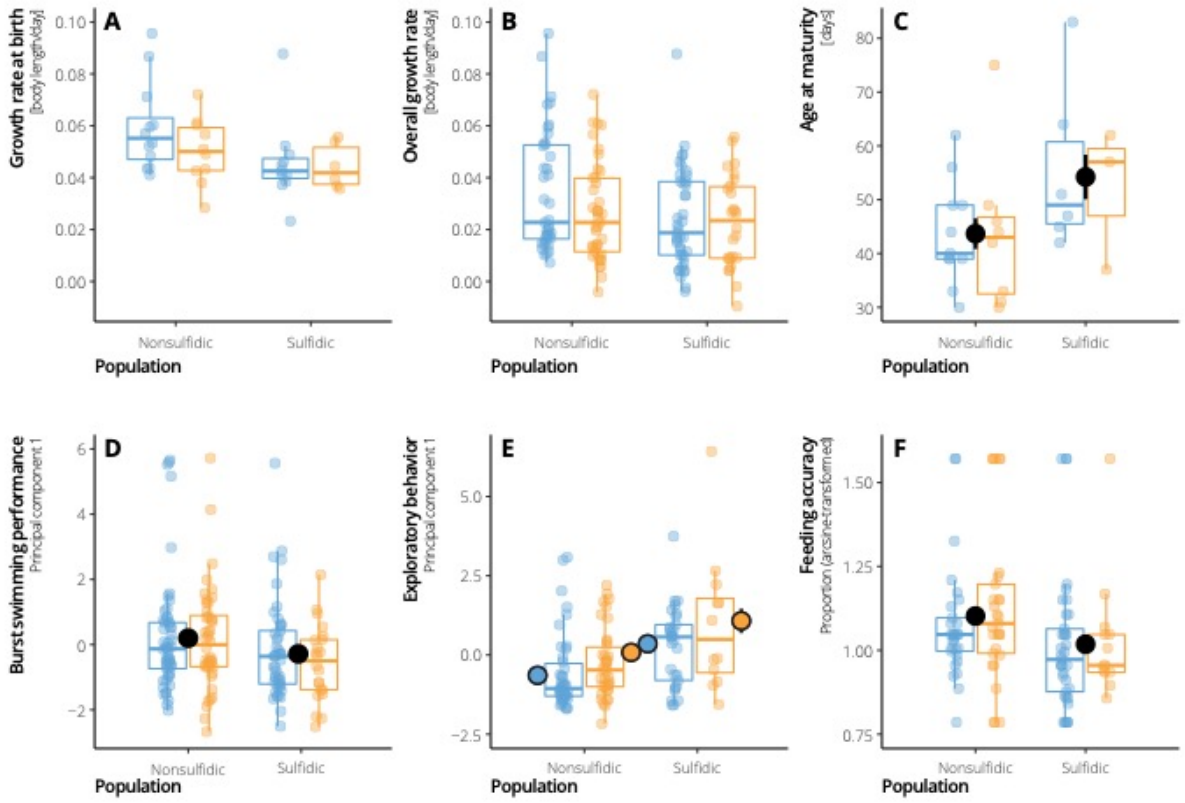

Figure 3. Data for each individual phenotype, including (A) growth rate at birth, (B) overall growth rate, (C) age at maturity, (D) burst swimming, (E) exploratory behavior, and (F) feeding accuracy. Data is summarized in boxplots with raw data points overlaid. In both populations, the high-food treatment is shown in blue, and the low-food treatment is shown in orange. When the best-supported model for a phenotype contained the terms 'Population' and/or 'Food Treatment', the estimated marginal means for those effects were visualized as large points.

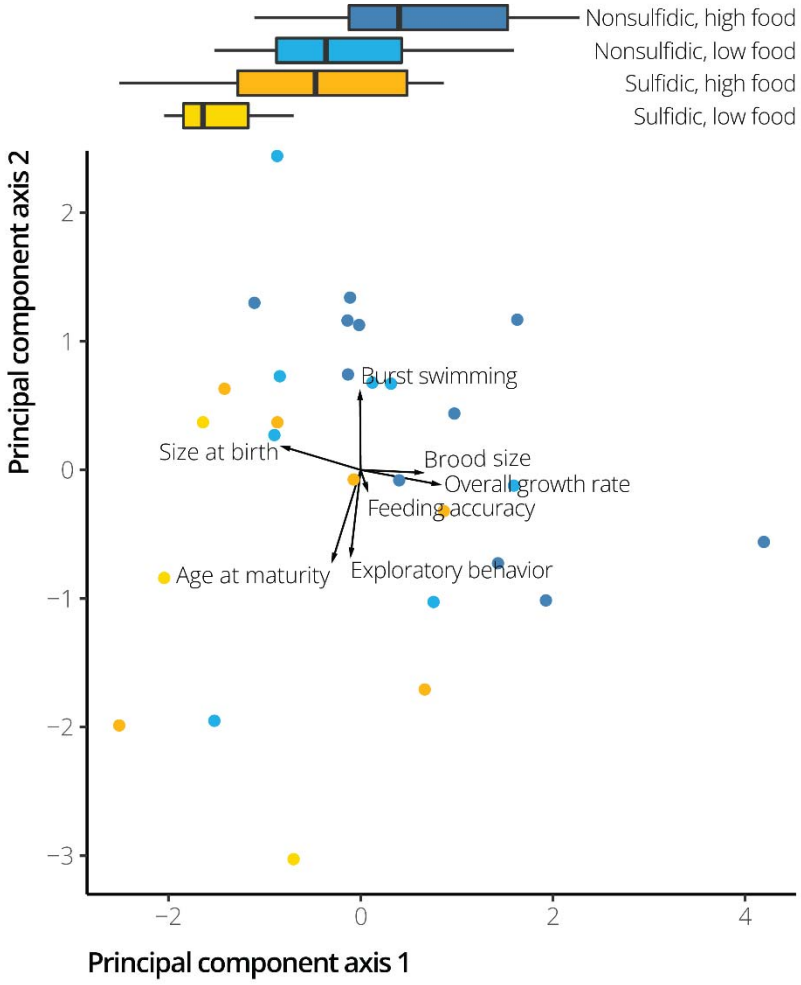

Figure 4. Plot of principal component scores representing linear combinations of all phenotypes in multivariate space. Scores were plotted along the first two principal components. Nonsulfidic families are shown in shades of blue, while sulfidic families are shown in shades of yellow. The high-food treatment within each population is shown in the darker shade (*i.e.*, dark blue or dark yellow). Radiating from the origin are arrows that represent the correlation between the principal component scores and each input variable (shown as text at the end of each arrow). Loadings were calculated by multiplying the eigenvector for each input variable by the square-root of the eigenvalue for that principal component axis. At the top of the figure are boxplots that show the distribution of principal component scores along the first principal component axis, which exhibited significant effects of 'Population' and 'Food Treatment'.