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

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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 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 and Arnold 1983). We have known that inheritance causes resemblance between parents and their offspring for 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 gives 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 between mothers and their developing young (Lindholm et al. 2006; Wolf and 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

55 responding to anthropogenic climate change (Schuler and Orrock 2012; Leonard and Lancaster
56 2020). Maladaptive maternal effects can also be related to stress, where physiological stress
57 responses in mothers have unintended negative consequences on offspring (MacLeod et al. 2021).
58 Regardless of whether maternal effects are adaptive, they are important biological phenomena that
59 warrant careful attention and explicit accounting in evolutionary analyses due to the non-genetic
60 effects on phenotypic expression.

61 Phenotypic variation in nature is common along environmental gradients, but it is often
62 unclear whether it is caused by genetic differentiation among populations or plastic effects that arise
63 from population-specific environmental exposure histories experienced by mothers or directly by
64 their offspring. For example, freshwater springs rich in toxic hydrogen sulfide (H_2S) in the Grijalva
65 River basin of southern Mexico are extreme environments that are connected to adjacent nontoxic
66 streams, and stark phenotypic gradients can be observed in fish occupying both habitats in as little as
67 a few meters. Sulfide springs are complex ecosystems with several correlated sources of selection
68 (Tobler et al. 2016b). H_2S is toxic because it disrupts aerobic ATP production (Reiffenstein et al.
69 1992; Cooper and Brown 2008), but habitats rich in H_2S also differ from nonsulfidic habitats in
70 other physical and chemical water parameters (dissolved oxygen concentrations, pH, and salinity),
71 resource availability, and the presence of competitors and predators (Riesch et al. 2010a; Tobler et al.
72 2011; Greenway et al. 2014; Tobler et al. 2015). Populations of Atlantic mollies (*Poecilia mexicana*), a
73 species of livebearing fish of the family Poeciliidae, have independently colonized and adapted to
74 sulfidic streams across multiple river drainages, and previous studies have documented that
75 colonization of sulfide springs has been associated with convergent changes in morphology,
76 locomotion, and respiration (Tobler and Hastings 2011; Camarillo et al. 2020), behavior (Plath et al.
77 2007; Doran et al. 2021; Lukas et al. 2021), physiology (Tobler et al. 2011; Barts et al. 2018;
78 Greenway et al. 2020), and life history traits (Riesch et al. 2011a,b, 2014). Phenotypic divergence

79 between sulfidic and nonsulfidic mollies likely has a significant genetic component, because it
80 coincides with strong genetic differentiation between populations, even though there are no physical
81 barriers separating populations in the different habitat types (Palacios et al. 2013; Plath et al. 2013;
82 Riesch et al. 2016b). However, trait variation between populations likely also has an environmental
83 component; while population differentiation persists in captive populations reared in common
84 garden conditions in the lab (Tobler et al. 2016a; Greenway et al. 2020), there is also evidence for
85 plasticity caused by short-term exposure to different environmental conditions (Bierbach et al. 2011;
86 Passow et al. 2017b). In contrast, the impact of maternal effects on offspring trait expression
87 remains to be investigated in these livebearing fish. Hence, we tested how genetic differentiation,
88 maternal effects, and their interactions shape phenotypic expression in *P. mexicana* populations from
89 sulfidic and nonsulfidic habitats. To induce maternal effects, we manipulated resource availability to
90 pregnant mothers, because natural populations vary substantially in nutritional state. Fish in sulfidic
91 habitats have significantly reduced body condition (both when inferred through length-weight
92 regression and body fat content analysis; Tobler et al. 2006; Tobler 2008) because of constraints
93 associated with resource acquisition. H_2S exacerbates hypoxia, and fish from sulfidic habitats have to
94 trade off performing aquatic surface respiration, a compensatory behavior to access better-
95 oxygenated surface waters, with benthic foraging (Tobler et al. 2009). In addition, the resource
96 environment experienced by mothers has been shown to impact offspring trait expression in other
97 species, including other members of the family Poeciliidae (Reznick et al. 1996a; Altmann and
98 Alberts 2005; Boots and Roberts 2012).

99 To quantify population differentiation and maternal effects in populations of *P. mexicana*, we
100 followed families of offspring from birth to the onset of maturation and repeatedly quantified a host
101 of complex phenotypic traits. Focal traits included brood size, size at birth, and age at maturity and
102 ontogenetic variation in growth rates, burst swimming, exploratory behavior, and feeding accuracy.

103 We chose these traits because they likely affect fitness, and we have prior knowledge for many of
104 them from natural populations, providing us with a framework to make *a priori* predictions. Our
105 experiments sought to address four specific questions: (1) Is there evidence for differences in
106 phenotypic traits between populations from sulfidic and nonsulfidic habitats that persist in fish
107 reared in a common-garden environment for multiple generations? Divergence in phenotypic traits
108 between populations regardless of maternal food treatments would indicate that trait differentiation
109 is due to genetic variation between populations. (2) Is there evidence for maternal effects in
110 response to resource availability? Significant differences in offspring traits between maternal food
111 treatments, irrespective of population of origin, would suggest resource-induced maternal effects. (3)
112 How do functional traits vary throughout ontogeny, and how do population differences and
113 maternal effects interact with ontogeny? Age is a major determinant of expression for many traits
114 (Hegyi et al. 2006; Zhang et al. 2015), but how population differences and maternal effects impact
115 trait expression through ontogeny is less clear. In other poeciliids, maternal effects tend to be
116 present at birth and decline with age (Lindholm et al. 2006). In contrast, population differences
117 between sulfidic and nonsulfidic *P. mexicana* are stark in adults, suggesting differences may emerge
118 early in life and even increase throughout ontogeny (Riesch et al. 2011b). Accordingly, we predicted
119 that age would impact most of the traits measured, that population differentiation would increase
120 with age, and that maternal effects would diminish with age. (4) How do population differences
121 interact with maternal effects? A difference in how each population responds to variation in
122 maternal resource availability would indicate genotype-by-environment interactions. Fish from
123 sulfidic habitats generally face constraints in resource levels, while those from nonsulfidic habitats
124 have relatively more access to resources (Tobler et al. 2006; Tobler 2008; Tobler et al. 2009). Hence,
125 we predicted that trait variation induced by the low-food treatment would occur in the same
126 direction as trait variation produced by differences between the nonsulfidic and sulfidic populations

127 (i.e., maternal effects would be "aligned" with population differences). In this case, maternal effects
128 would accentuate divergence between populations that resembles patterns of variation found in the
129 wild. Alternatively, low-resource traits may be canalized in the sulfidic population because sulfidic
130 individuals are constantly food stressed in nature. In this case, maternal effects may be weaker in the
131 sulfidic population.
132

133 **Methods**

134 *Experimental overview*

135 For our experiments, we used two lab-reared populations of *Poecilia mexicana* originating from wild-
136 caught relatives in the Tacotalpa River drainage. One population originated from a sulfide spring
137 complex called El Azufre I (according to Plath et al. 2013; hereafter referred to as the sulfidic
138 population or ecotype), and the other population was from a nonsulfidic stream connected to the
139 mainstem of the Tacotalpa River, called Arroyo Bonita (according to Plath et al. 2013; hereafter
140 referred to as the nonsulfidic population or ecotype). Both populations were reared in 680-liter stock
141 tanks filled with filtered tap water. Tanks were fed *ad libitum* twice daily with commercial dry fish
142 food (Purina), and ~50 % of the water was exchanged weekly. Fish used in this experiment were
143 raised under common-garden conditions for at least three generations (*i.e.*, they were at least great-
144 grandchildren from individuals originally collected in the wild).

145 From each tank, 30 females were caught with a dipnet and isolated in a 19-liter tank with an
146 aerating filter and a bundle of plastic mesh to help fry hide once they were born. Females were fed
147 twice daily, with aquatic gel diet for omnivorous fish (Mazuri) in the morning and freshly hatched
148 *Artemia* nauplii (Brine Shrimp Direct) in the evening. Females were randomly assigned to either a
149 "high-food" diet (0.32 ml per feeding) or a "low-food" diet (0.08 ml per feeding), with equal
150 numbers of females from each population being assigned to each group (Figure 1). Each tank was

151 checked daily for newborn fry. By the end of the experiment, 17 sulfidic females gave birth (11 from
152 the high food treatment and 6 from the low food treatment) and 22 nonsulfidic females produced a
153 brood (12 from the high food treatment and 10 from the low food treatment). Whenever a brood
154 was born, we recorded the brood size, date of birth for each family, the length of time that the
155 female was in the food treatment (days), and the mother's standard length (distance from the
156 anterior tip of the snout to the caudal peduncle, mm). Mothers were then removed from the tanks,
157 and we randomly selected 15 newborn fry (if available) from each family to remain in the
158 experimental tanks to minimize density-dependent effects.

159 From that point on, we followed the developing families through ontogeny. All fry—
160 irrespective of the mother's food treatment—received the same amount of food; they were fed *ad*
161 *libitum* twice daily with a mixture of decapsulated brine shrimp eggs (Brine Shrimp Direct) and dry
162 food. We assessed offspring phenotypes at approximately weekly intervals by measuring life history
163 traits (size at birth, weekly growth rate, and age at maturity) and behavioral traits (burst swimming,
164 exploratory behavior, and feeding accuracy; see Figure 1). We specifically tested whether there were
165 differences in these phenotypes between maternal food treatments (*i.e.*, maternal effects), between
166 populations (*i.e.*, sulfidic *vs.* nonsulfidic, indicating effects of evolved population differences), or
167 both. All analyses were conducted in R 4.0.5 (R Core Team 2021). Code and data to reproduce all
168 analyses can be found on GitHub (https://github.com/michitobler/common-garden).

169

170 *Size at birth and growth rate*

171 To measure size at birth, we photographed each family from above on the day of their birth with a
172 Nikon D90 digital camera fitted with an AF-S Micro NIKKOR 105mm f/2.8 lens. A ruler was
173 included in the background of each image. Images were imported to ImageJ v. 1.53 (Schneider et al.
174 2012) and calibrated by setting the scale. We measured the standard length (mm) of each offspring

175 in the family. These measurements were averaged across all individuals to obtain a single mean size
176 at birth for each family. This measurement was completed weekly, and the measurement for each
177 family was subtracted from that family's measurement from the week prior to obtain an average
178 growth rate [mm/week]. To account for allometric differences in growth rate, we converted the
179 average weekly growth rates to a proportional growth rate by dividing the average weekly growth
180 rate by the mean body size the week prior.
181
182 *Age at maturity*
183 We estimated the minimum age at maturity for each family using morphological characteristics of
184 male sexual maturity. While the sexes of juvenile livebearers are difficult to distinguish, at the onset
185 of sexual maturity, the male anal fin is modified into an intromittent organ called a gonopodium,
186 while it remains unmodified in females (Rosen and Gordon 1953; Chambers 1987). We therefore
187 measured minimum age at maturity [days] as the time it took to for the first male in a group to
188 development its gonopodium.
189
190 *Burst swimming*
191 Most fish avoid predation with a highly conserved, reflexive escape response that causes the head to
192 move away from the stimulus, bending the body into a 'C' shape (Eaton et al. 1977). Then, a strong
193 stroke of the caudal fin starts the movement away from the stimulus (Domenici and Blake 1997).
194 This process (bending into a 'C' and propelling away from the stimulus) is known as a 'C-start'
195 response and is frequently used as a metric of escape performance in fish (Walker 1997; Ghalambor
196 et al. 2004; Langerhans et al. 2004; Camarillo et al. 2020). To quantify this burst swimming behavior,
197 we adopted the methods and metrics used by prior studies (Langerhans et al. 2004; Ingley et al.
198 2016; Camarillo et al. 2020). We placed a haphazardly chosen individual from each family in a glass

199 petri dish (9 cm in diameter, containing 2 cm of water) with opaque sides suspended above an
200 angled mirror, providing a ventral view of each fish. After a 5 min acclimation, we struck the surface
201 of the water within a body length of the posterior of the fish with a probe and recorded the
202 movement of the fish from below with a Sony NEX-FS700R camcorder at 60 frames per second
203 with 1080p resolution. We converted the resulting .mts files into .mp4 files (to enhance compatibility
204 with downstream applications) with FFmpeg (Tomar 2006).

205 We used DLTdv8 (Hedrick 2008) to digitize the 2-D location of the isthmus (*i.e.*, the area on
206 the ventral surface of the head where the opercula converge) of the fish in each frame. Digitized
207 points were then used to calculate the maximum velocity (v_{max}, mm/s), maximum acceleration (a_{max},
208 mm/s^2), and net distance traveled (d_{net}, mm of displacement within 1/12th of a second after the C-
209 start). To calculate v_{max}, we calculated the straight-line distance between each pair of successive
210 digitized points, divided this distance by the inverse of the frame rate (60 fps), and found the
211 maximum value between any two points. a_{max} was calculated by subtracting the velocity value at each
212 point from the velocity value at the point immediately preceding it and finding the maximum value.
213 d_{net} was calculated by recording the 2-D position immediately after the fish ends the C-start with a
214 single stroke of the caudal fin, and then recording the 2-D position 1/12th of a second later (5 video
215 frames later) and calculating the straight-line distance between the two points. To reduce the
216 dimensionality of this dataset, we then conducted a principal component analysis (PCA) using the
217 prcomp function with a correlation matrix. There was one principal component with an eigenvalue
218 greater than 1 (explaining 85.6 % of the total variance), which was retained as a compound metric of
219 burst swimming performance. Positive scores along this principal component axis were associated
220 with higher velocity, acceleration, and distance traveled (Table S1A). All mathematical operations
221 were conducted using packages contained in the base distribution of R.
222

223 *Exploratory behavior*

224 We used an open field test to quantify the exploratory tendencies of fry. We filled a 9 cm Styrofoam
225 cup with 3 cm of water and covered the arena with a sheet of glass coated in anti-fog solution (rain-x
226 interior glass anti-fog liquid). We haphazardly selected one individual from each family and placed it
227 in the arena undisturbed for a 5-min acclimation period, after which time we recorded 5 min of
228 video from above with a GoPro Hero 4 (1080 px resolution, linear field of view, 30 fps).

229 We used idTracker (v. 2.1, bundled with 64-bit Matlab Compiler Runtime 8.3; Pérez-
230 Escudero et al. 2014) to track the 2-D location of the fish automatically for the entire 5-min
231 recording. We set the number of individuals to be 1 and manually determined the intensity threshold
232 (0.5-0.8) and minimum size (40-250 pixels) based on each video. We also imported a still frame from
233 each video into ImageJ to measure the centroid coordinates and arena radius.

234 Using the 2-D coordinates in each frame, we calculated several metrics of motion that we
235 used as proxies for exploratory behavior. We calculated distance traveled between each pair of
236 successive points, the velocity, the acceleration, and the total cumulative distance traveled (d_{total},
237 mm), as above. We then calculated average velocity (v_{avg}, mm/s), maximum velocity (v_{max}, mm/s),
238 and maximum acceleration (a_{max}, mm/s^2) by finding the means and maxima of all velocity and
239 acceleration values. Finally, we calculated the proportional average distance from the center of the
240 arena (d_{center}, dimensionless) by calculating the distance from the fish's location to the centroid of the
241 arena and dividing this value by the arena radius. Videos were excluded if the fish was completely
242 still in all frames. To reduce the dimensionality and observe the correlation structure within this
243 dataset, we ran a PCA on v_{avg}, v_{max}, a_{max}, d_{total}, and d_{center} with a correlation matrix. We retained scores
244 along the first PC axis (explaining 56.9 % of the total variance) as a composite exploratory behavior
245 score. As shown in Table S1B, higher PC1 scores were associated with more exploratory behavior,
246 with positive loadings for all input variables.

247

248 *Feeding accuracy*

249 To measure feeding accuracy, we withheld food from individuals of each tank for 24 h prior to the
250 experiment and placed one haphazardly selected fry in a viewing tank for a 5-min acclimation
251 period. The viewing tank was constructed by connecting two 10 cm × 10 cm sheets of glass (1/8th
252 inch thickness) to either side of 1 cm diameter glass rods with waterproof silicone. The rear wall of
253 the viewing tank was covered with a black sheet of plastic to enhance contrast between the
254 background and the fish in the tank. The feeding solution consisted of 1 g of freshly hatched, live
255 *Artemia* nauplii diluted into 100 ml of filtered tap water. After the 5-min acclimation, we added 0.08
256 ml of the feeding solution to the viewing tank and recorded 5 min of video with the camcorder. We
257 analyzed the video frame-by-frame using BORIS v. 7.13 (Friard and Gamba 2016) and recorded the
258 number of successful strikes (a feeding strike that ends in consumption of the food item) and
259 unsuccessful strikes (a feeding strike that either misses the food item or results in regurgitation of
260 the food item). We divided the number of successful strikes by the total number of strikes to get an
261 overall proportional estimate of feeding accuracy, which was arcsine-square-root transformed prior
262 to analysis.

263

264 *Statistical analyses of individual traits*

265 There were many potential sources of variation in our experiment. Other than the effects of interest
266 for our study (population, maternal food treatment, and their interaction), the observed variation in
267 traits could also have arisen from differences in fry age, maternal standard length, the duration of
268 her food treatment, and brood size. Consequently, we used a model selection approach to find the
269 model that was best supported by our data from each experiment separately. For each phenotype,
270 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 contained '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 AICc. Full model selection tables are available for each phenotype in Table S2. 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 (ANOVA) or Wald's chi-square tests (see Table 1). Quantification and visualization of effects was accomplished by calculating and plotting estimated marginal means for the effects of 'Population' and/or 'Food Treatment', depending on the best-supported model, using the Effect function from the effects package v. 4.2-2 (Fox and Weisberg 2018a,b). To aid in drawing inferences from our models, we generated 95 % confidence intervals (CI) for model coefficients in our top models using the confint function form the base R distribution. Effect sizes were calculated as partial eta-squared (η_p^2), which represents the proportion of variance explained by a particular variable after accounting for the variance explained by all other variables. We calculated η_p^2 with the etasq function (Fox et al. 2021) for general linear models or the eta_squared function (Ben-Shachar et al. 2020) for linear mixed models.

Multivariate analysis

Since selection ultimately acts on complex, multivariate phenotypes (Lande and Arnold 1983), we sought to understand how the traits measured for our analyses vary and covary to jointly shape multivariate phenotypes. To do so, we averaged each phenotype across all ages for each tank. We

295 used this approach rather than including age as a covariate and analyzing raw phenotypic scores
296 because of timing and logistical constraints that made it impossible to conduct each experiment on
297 offspring that were the same age. We analyzed the averaged phenotypes with a PCA (correlation
298 matrix) and used scores along the first two principal components—which had eigenvalues greater
299 than 1—as dependent variables. We analyzed principal component scores along each axis separately
300 because the axes were orthogonal. For each axis, we created a global model containing all possible
301 effects and selected the best-supported model based on AICc, as explained above.
302
303 **Results**
304 We measured seven functional traits in offspring from sulfidic and nonsulfidic populations
305 throughout ontogeny. Model selection tables for the analysis of each trait at birth and separately
306 across all ages can be found in Table S2, and the best-supported models are summarized in Table 1.
307 For brevity, we will present results in the context of our hypotheses outlined in the Introduction,
308 focusing on how all traits vary through ontogeny in terms of population differentiation, maternal
309 effects, and their interaction, rather than presenting the results for each trait separately.
310
311 *Is there evidence for population differentiation?*
312 We found evidence for significant population differences in five of the seven traits measured: size at
313 birth, age at maturity, burst swimming, exploratory behavior, and feeding accuracy (but not brood
314 size and growth rate). Age at maturity, burst swimming, and feeding accuracy were significantly
315 different between populations, but not between food treatments, whereas there were significant
316 effects of 'Population' and 'Food Treatment' (but no interactions) on size at birth and exploratory
317 behavior (see Table 1 and below). Sulfidic individuals were born 9.2 % larger [$F_{1,35}$ = 20.032; P <
318 0.001; estimated marginal mean for sulfidic fish (EMM_S) = 10.03 mm, estimated marginal mean for

319 nonsulfidic fish (EMM_{NS}) = 9.18 mm; η_p^2 = 0.36; Figure 2A], matured an average of 10.5 days later

320 ($F_{1,26}$ = 4.420; P = 0.045; EMM_S = 54.2 days, EMM_{NS} = 43.7 days; η_p^2 = 0.15), had significantly

321 lower burst swimming performance (χ^2_1= 4.308; P = 0.038; CI: -0.94, -0.03; η_p^2 = 0.03), were more

322 exploratory (χ^2_1 = 7.513; P = 0.006; CI: 0.29, 1.70; η_p^2 = 0.28), and were 7.2 % less accurate during

323 feeding (χ^2_1 = 4.645; P = 0.031; EMM_S = 0.72, EMM_{NS} = 0.80; η_p^2 = 0.04; Figure 3).

324

325 *3.2 Is there evidence for maternal effects in response to resource availability?*

326 To test whether maternal effects induced by resource availability during pregnancy impact functional

327 traits in offspring, we compared each phenotype between offspring born to mothers who

328 experienced a high-food environment and mothers who experienced a low-food environment.

329 Mothers in low-food treatments produced offspring that were 3.4 % larger at birth [$F_{1,35}$ = 4.354; P

330 = 0.044; estimated marginal mean for fish in the low-food treatment (EMM_{low}) = 9.8 mm, estimated

331 marginal mean for fish in the high-food treatment (EMM_{high}) = 9.4 mm; η_p^2 = 0.11; Figure 2A] and

332 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

333 remaining traits (brood size, age at maturity, growth rate, burst swimming, and feeding accuracy),

334 'Food Treatment' was not included in the best-supported model (see Table 1), suggesting that

335 maternal effects did not affect the expression of those traits.

336

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

338 To determine how traits changed through offspring development, we compared each phenotype

339 across age groups. 'Age' was a significant predictor of growth rate and burst swimming, indicating

340 that these traits changed throughout ontogeny, while the other phenotypes were not affected by age.

341 Across populations and food treatments, fry grew at a slower relative rate (χ^2_1 = 147.91; P < 0.001,

342 CI: -0.010, -0.007; $\eta_p^2 = 0.50$) and performed better in burst swimming trials ($\chi^2_1 = 30.178$; $P <$
343 0.001; CI: 0.024, 0.049; $\eta_p^2 = 0.16$) as they got older (Table 1). Burst swimming scores were also
344 lower in sulfidic individuals (see above), but the interaction term 'Population × Age' was not
345 significant, demonstrating that both populations exhibited similar changes in burst swimming
346 throughout ontogeny.

347 To ensure that signals of population differentiation and maternal effects occurring at birth
348 were not obscured by measurements later in life, we also subset our dataset for each phenotype to
349 obtain only the earliest data point for each family. As mentioned above, fry from the sulfidic
350 population and the low-food maternal treatment had significantly larger size at birth (Figure 2; Table
351 1). However, there were no population differences or maternal effects on brood size, which was
352 higher in larger mothers (Mother SL; $F_{1,37} = 23.342$; $P < 0.001$; CI: 0.73, 1.79; $\eta_p^2 = 0.38$) and
353 mothers who spent longer in the food treatment (Treatment Length; $F_{1,37} = 8.926$; $P = 0.005$; CI:
354 0.07, 0.40; $\eta_p^2 = 0.19$). Growth rate at birth was significantly lower in sulfidic fry ($F_{1,37} = 5.025$; $P =$
355 0.032; CI: -0.12, -0.006; $\eta_p^2 = 0.12$; Table 1; Figure 3A), but there was no evidence for maternal
356 effects on growth rate at birth ('Food Treatment' was not included in best-supported model). Note
357 that the population difference in growth rate at birth disappeared as fry developed (Figure 3B). All
358 other traits that were measured throughout ontogeny (burst swimming, exploratory behavior, and
359 feeding accuracy) did not exhibit significant population differences or maternal effects at birth.
360 Interestingly, the maternal effect that we detected on exploratory behavior across all ages (see above)
361 was not evident at birth ($F_{1,31} = 2.727$; $P = 0.108$; CI: -0.29, 2.81). These results collectively
362 demonstrate that—contrary to our hypothesis regarding ontogenetic variation of functional traits—
363 population differentiation did not increase with age, and maternal effects were not always observable
364 at birth, nor did they decline with age.

365

366 *How do population differences interact with maternal effects?*

367 We hypothesized that variation from maternal effects would be aligned with population differences,
368 but that there would be a significant interaction between population differences and maternal effects
369 (*i.e.*, different magnitudes of maternal effects in each population). Contrary to our predictions, no
370 'Population × Food Treatment' interactions were included in the best-supported model for any of
371 the traits we measured (Table 1), suggesting a general lack of support for significant interactions
372 between population differences and maternal effects. Therefore, maternal effects—if present—were
373 similar in direction and magnitude between populations.

374 To further address our hypothesis, we asked whether population differences and maternal
375 effects were, in fact, aligned, and whether they explained a similar proportion of phenotypic variance
376 when they acted in unison. We compared the signs (positive *vs.* negative coefficient estimates) and
377 effect sizes (η_p^2) of population differences and maternal effects for the two traits with evidence of
378 both effects simultaneously impacting trait expression—size at birth and exploratory behavior. For
379 both traits, the difference between the nonsulfidic and sulfidic population occurred in the same
380 direction as the trait shifts between the high- and low-food treatments, indicating that the effects
381 were aligned (Figures 2A and 3E). Effect size estimates for 'Population' and 'Food Treatment' for
382 both traits indicated that 'Population' had a larger effect on trait expression than 'Food Treatment'
383 (η_p^2 = 0.36 *vs.* 0.11 for size at birth and 0.28 *vs.* 018 for exploratory behavior).

384 In addition to the univariate analyses of trait variation, we were also interested in
385 understanding how the traits covaried with one another, and whether and how multivariate
386 phenotypes were impacted by population differences and maternal effects. We averaged each
387 phenotype across ages for each family and conducted a PCA with a correlation matrix. We then
388 analyzed principal component scores along the first two principal component axes. The first

389 principal component accounted for 28.5 % of the variance in multivariate phenotypes, and scores
390 along the first principal component were positively correlated with brood size, growth rate, and
391 feeding accuracy, and they were negatively correlated with size at birth, age at maturity, burst
392 swimming, and exploratory behavior (see Table S1C). The second principal component explained
393 21.1 % of the variance and was positively correlated with burst swimming and size at birth and
394 negatively correlated with brood size, growth rate, feeding accuracy, exploratory behavior, and age at
395 maturity (Table S1). After conducting model selection, the best-supported model for PC1 contained
396 the main effects of 'Population', 'Food Treatment', and 'Treatment Length', and the best-supported
397 model for PC2 contained the main effects of 'Population', 'Mother SL', and 'Treatment Length'.
398 Sulfidic offspring had significantly lower scores along PC1 ($F_{1,24}$ = 16.266; P < 0.001; CI: -
399 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, -
400 0.04), indicating population differences and maternal effects on PC1 scores. As for the univariate
401 analyses, maternal effects were aligned with population differences in PC1 scores (Figure 4),
402 'Population' had a larger effect than 'Food Treatment' (η_p^2 for 'Population' = 0.40; η_p^2 for 'Food
403 Treatment' = 0.17), and there was no evidence for a significant 'Population × Food Treatment'
404 interaction (the term was not included in best-supported model). We also found that sulfidic
405 individuals exhibited marginally lower PC2 scores ($F_{1,24}$ = 3.321; P = 0.081; CI: -1.45, 0.09; η_p^2 =
406 0.12) but detected no evidence for maternal effects (the 'Food Treatment' term was not included in
407 the best-supported model) on PC2.
408

409 **Discussion**

410 Phenotypic variation can be shaped by multiple genetic and nongenetic factors, but the interplay of
411 genes and environmental effects is rarely disentangled in natural systems even though it
412 fundamentally impacts our inference of adaptation. Therefore, we examined how genetic variation

413 (*i.e.*, population differences), phenotypic plasticity mediated by maternal effects, and their
414 interactions shape trait expression in two populations of *P. mexicana* that are exposed to strong
415 divergent selection in nature. We found significant trait differences between populations even
416 though fish were housed under common-garden conditions in the laboratory for at least three
417 generations. In contrast, exposure of mothers to different food treatments impacted relatively few
418 traits in their offspring and had weaker effects than the population differences. In addition, maternal
419 effects tended to be aligned with population differences and act in the same way across populations.
420 Principal component analysis supported these overall conclusions in multivariate trait space. We also
421 found no evidence for interactions between populations and food treatments, suggesting that while
422 populations have diverged in phenotypic traits, they have retained similar maternal influences on
423 those same traits. Overall, we found that the stark phenotypic differences between populations of *P.*
424 *mexicana* that are evident in nature are largely a consequence of genetic divergence, likely
425 representing local adaptation to the distinct ecological conditions of their habitats. Maternal effects
426 in response to resource availability, while present in some traits, appear to accentuate population
427 differences, but not cause them.
428
429 *Trait variation across populations and maternal food treatments*
430 There is a rich history integrating field-based studies that quantify trait variation of poeciliid fishes in
431 nature with laboratory-based studies that isolate causative environmental factors (Endler 1980, 1995;
432 Reznick and Bryga 1987; Reznick et al. 1990; Langerhans et al. 2007; Tobler et al. 2008; Ghalambor
433 et al. 2015; Ingley and Johnson 2016). While most studies focus on single or a few related traits and
434 take a snapshot at a single ontogenetic stage, our study demonstrated that multiple complex trait
435 differences quantified in common garden conditions, across food treatments and across ontogeny,
436 closely mirror trait differences between populations in nature.

437 First, our study corroborates a genetic basis for population divergence in reproductive life
438 history traits. We documented significant population divergence in size at birth, with sulfidic mollies
439 giving birth to larger offspring (see Figures 2A and 4). This finding is consistent with life history
440 studies of sulfide spring populations in *P. mexicana* and other poeciliid species (Riesch et al. 2010b,
441 2014), suggesting that divergence in offspring size between sulfidic and nonsulfidic populations is a
442 repeatable evolutionary consequence of sulfide spring colonization that is driven by natural selection
443 for larger offspring size. Among-population variation in size at birth has also been documented in
444 other poeciliids that are exposed to other sources of divergent selection (Weeks and Gaggiotti 1993;
445 Reznick et al. 1996b; Schrader and Travis 2012; Belk et al. 2020). Our multivariate results (Figure 4)
446 also indicated that, like other poeciliids, the nonsulfidic population of *P. mexicana* closely resembles
447 an opportunistic life history strategy (Winemiller and Rose 1992), which places a premium on earlier
448 maturity and higher fecundity, at the expense of lower juvenile survivorship. The population
449 differences we found in the sulfidic population may represent a shift in the direction of the
450 equilibrium strategy, which is energetically costly to parents but should ultimately benefit offspring
451 competitive ability. In natural populations, maternal effects induced by variation in resource
452 availability likely accentuate genetic differences in offspring size across sulfidic and nonsulfidic
453 habitats. This pattern was also evident across a plethora of life history traits in guppies; in all ten life
454 history traits with evidence for significant genetic divergence and maternal effects, these effects
455 occurred in the same direction (Felmy et al. 2022).
456 Second, we found patterns of population differentiation in age at maturity that match with
457 previous work in livebearing fishes. Prior studies have demonstrated that guppies in streams with
458 high predation pressure on adults mature earlier than conspecifics from low-predation populations
459 (Reznick and Endler 1982). Several studies have also documented genetic differences in age at
460 maturity between populations of *P. mexicana*. For example, cave mollies mature significantly later

461 than surface populations, but unlike our study, there was also significant plasticity in age at maturity
462 due to resource availability (Riesch et al. 2016a). Another study of surface mollies that compared size
463 at maturity in sulfidic and nonsulfidic populations found that sulfidic individuals reached maturity at
464 a significantly smaller size than nonsulfidic individuals (Riesch et al. 2011a), likely because energy
465 limitation caused by reduced resource availability in sulfidic habitats selects for reduced growth rates
466 and smaller size at maturity (Passow et al. 2017a). Because poeciliid males exhibit determinate
467 growth that ends at maturity (Stearns and Sage 1980), size at maturity should closely mirror age at
468 maturity, assuming growth rates are the same as we found in our study. However, we found that
469 sulfidic mollies matured at a significantly later age than nonsulfidic individuals, despite identical
470 growth rates across ontogeny (Figure 3B). This discrepancy between our findings and those of
471 previous studies suggests that plasticity in age at maturity may be strong and that variation in
472 experimental design and rearing conditions matters.

473 Third, we found significant behavioral differences between populations from sulfidic and
474 nonsulfidic habitats, including exploratory behavior, feeding accuracy, and burst swimming. Previous
475 work has shown that nonsulfidic mollies—and ones that were better fed—were bolder in their
476 natural habitat, but behavioral differences also disappeared in the laboratory (Riesch et al. 2009).
477 While we did not measure boldness *per se* in our experiments, exploratory behavior as measured in
478 our experiment (*i.e.*, activity levels in a novel arena) is often characterized as part of a behavioral
479 syndrome that correlates with boldness (Conrad et al. 2011). Unlike previous work, our study found
480 that individuals from the sulfidic population and the low-food treatment were more exploratory
481 (Table 1 and Figure 3E). These results support that maternal resource environment affects
482 exploratory behavior, but also imply heritable differences between populations. Similar heritable
483 population differences were also found for feeding accuracy and burst swimming but without any

484 evidence for maternal effects. At least for burst swimming, the pattern of population differentiation
485 in our experiment again mirrors findings from adult fish in natural habitats (Camarillo et al. 2020).
486
487 *Trait variation and adaptive function*
488 Sulfide springs and adjacent nonsulfidic habitats do not only differ in the presence and absence of
489 H_2S, but they also vary in numerous abiotic and biotic factors that are often unaddressed in studies
490 of adaptation. Sulfidic habitats tend to have lower dissolved oxygen concentrations, higher
491 temperature, higher specific conductivity, and lower pH (Tobler et al. 2011), which in turn affect the
492 biotic communities (Greenway et al. 2014) and selection associated with resource exploitation,
493 competition, predation, and parasites (Riesch et al. 2010a; Tobler et al. 2014, 2015). Adaptation in
494 sulfide springs is therefore not solely in response to selection from H_2S but is instead in response to
495 a multifarious selective regime that has caused multivariate phenotypic differentiation between
496 populations (Tobler et al. 2018). The complexity of selective regimes and evolutionary responses
497 make disentangling cause-and-effect relationships difficult, especially because theoretical predictions
498 for the effects of different, covarying sources of selection are non-mutually exclusive. For example,
499 the evolution of large offspring size at birth could be explained by (1) selection from H_2S, which
500 should favor larger offspring with a lower surface-to-volume ratio to reduce the influx of toxic H_2S
501 (Reznick et al. 1996a); (2) selection from resource constraints, which favors larger offspring with
502 higher energy stores (Reznick et al. 1996a); or (3) relaxation of selection from predation, which also
503 favors larger offspring (Reznick and Endler 1982; Johnson and Belk 2001; Jennions et al. 2006).
504 Similarly, low resource and low predation environments also favor more exploratory individuals that
505 are better able to locate and exploit resources under those conditions (Teska et al. 1990; Kaun et al.
506 2007; Huang et al. 2012). Assessing the adaptive value of trait differences between sulfidic and
507 nonsulfidic population is consequently nontrivial and a work in progress.

508 Variation in some traits investigated in our study may also be the consequence of genetic,
509 developmental, or functional tradeoffs with other traits. Such tradeoffs are common in organisms
510 inhabiting contrasting environments because divergent selection acting to optimize one trait can
511 inadvertently influence other traits due to constraints (Ghalambor et al. 2004; Kawecki and Ebert
512 2004; Garland et al. 2022). For example, reductions in burst swimming performance—as
513 documented in our study for fish from the sulfidic population—may arise as a consequence of
514 selection for increased steady swimming efficiency, as different body shapes are associated with
515 optimization of steady *vs.* unsteady swimming (Langerhans 2007, 2009; Tokić and Yue 2012).
516 Indeed, a tradeoff between burst speed and sustained swimming performance has been documented
517 in adult fish from the same populations we studied here. The tradeoff is likely balanced by the need
518 for energy-efficient swimming in sulfidic habitats with resource constraints (but low predation) and
519 selection for efficient predator avoidance in nonsulfidic habitats with high abundances of natural
520 enemies (but abundant resources) (Camarillo et al. 2020). Our study found a similar reduction in
521 burst swimming performance in the sulfidic population, even among individuals that had not yet
522 reached maturity, suggesting population differences in burst swimming arise early in ontogeny. Our
523 results also matched *a priori* predictions regarding tradeoffs between respiration and feeding.
524 Habitats rich in H_2S also experience rampant hypoxia, which has selected for the evolution of
525 craniofacial traits (larger heads and jaws and longer gill filaments) that increase ventilation efficiency
526 (Camarillo et al. 2020). In addition to changes in morphology, sulfidic individuals also exhibit
527 decreased foraging efficiency compared to nonsulfidic individuals (Tobler et al. 2009), which was
528 supported by our findings related to feeding accuracy (Figure 3F). The decreases in feeding
529 efficiency noted in this and other studies may therefore be a consequence of the craniofacial
530 modifications that accompany colonization of sulfidic habitats.
531

532 *Plasticity accentuates genetic trait differentiation in natural populations*

533 Phenotypes in nature are the sum of genetic and environmental effects, but surprisingly, we found
534 no evidence for canalization or the evolution of plasticity by genotype × environment interactions.
535 Our work demonstrated that maternal effects were aligned with population differences, accentuating
536 trait divergence between populations, and that the trait shifts induced by maternal effects were of a
537 similar magnitude in both populations. For both traits in which we observed significant population
538 differentiation and maternal effects (size at birth and exploratory behavior), the lowest phenotypic
539 scores were found in nonsulfidic fry from high-food mothers, and the highest scores were found in
540 sulfidic fry from low-food mothers (Figures 2A and 3E). Because sulfidic mollies exhibit reduced
541 foraging efficiency and body condition as a consequence of hypoxia (Tobler 2008; Tobler et al.
542 2009), sulfidic habitats are naturally analogous to our low-food treatment, and nonsulfidic habitats
543 are similar to the high-food treatment. If maternal effects enhance population differences in natural
544 populations similarly as in our experiments, then it could explain why stronger trait divergence is
545 typically observed in nature than in common garden reared fish (Tobler et al. 2008; Passow et al.
546 2015). Likewise, it is important to note that our experiments only captured a small aspect of
547 plasticity, the portion controlled by the mother. Future work needs to address genetic effects,
548 maternal effects, and plasticity in response to variation in environmental factors directly experienced
549 by the offspring to better understand the forces driving trait variation in nature. We also caution that
550 the shifts found in our study are only from comparisons of a single population pair. Our
551 understanding of the relative roles of genetic and plastic effects on phenotypic variation would
552 benefit greatly from studies that expand to other sulfidic and nonsulfidic population pairs in *P.*
553 *mexicana* and other species.

554

555 *Conclusions*

Natural selection can act on phenotypes to cause evolutionary change in populations within a single generation (Grant and Grant 1993). However, selection rarely impacts a single trait, since many traits have numerous correlated characters (Lande and Arnold 1983). Likewise, selection can act differently throughout an organism's life (Freda et al. 2019), so ontogenetic patterns of trait variation are crucial for our understanding of how traits evolve. It is therefore necessary to analyze variation in multiple fitness-relevant traits across ontogeny to truly understand adaptation. By doing so, our study provided strong evidence that the patterns of phenotypic variation found in wild populations of *P. mexicana* inhabiting divergent habitats are primarily driven by population differences indicative of local adaptation. Maternal effects, on the other hand, were present but generally weak, so they likely accentuate population differences, rather than causing them.

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Author Contributions

JLC and MT designed the study. JLC conducted the experiments and analyzed the data. MT provided logistical support. JLC drafted the manuscript; both authors contributed text and worked on the final version of this manuscript.

Conflict of Interest Statement

The authors declare no conflict of interest.

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		$F_{1,24} = 7.722$ $P = 0.010$	$F_{1,24} = 5.292$ $P = 0.030$		$F_{1,24} = 3.321$ $P = 0.081$	

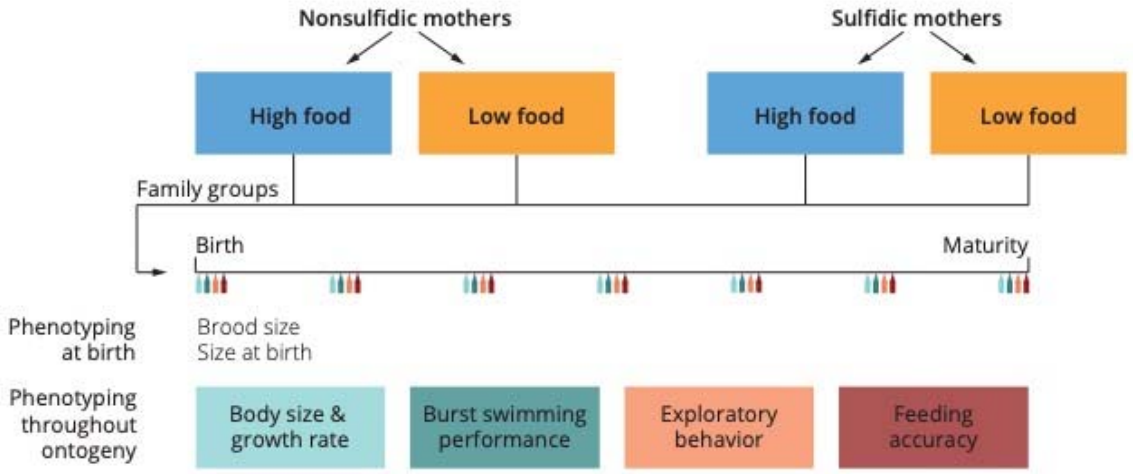

Figure 1. Overview of our experimental design. We subjected pregnant sulfidic and nonsulfidic mothers to either a high- or a low-food treatment and measured seven traits in their offspring throughout their development. These traits include four life history traits (birth size, brood size, growth rate,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

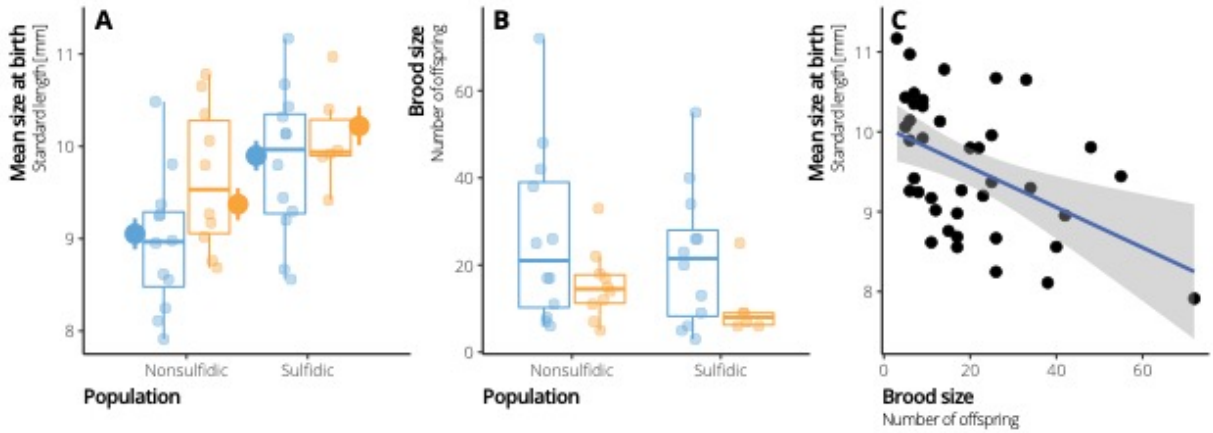

Figure 2. Plots of average standard length (A), brood size (B), and their relationship (C) for each family. Data is summarized in boxplots with raw data points overlaid. In both populations, the high-food treatment is shown in blue, and the low-food treatment is shown in orange. When the best-supported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

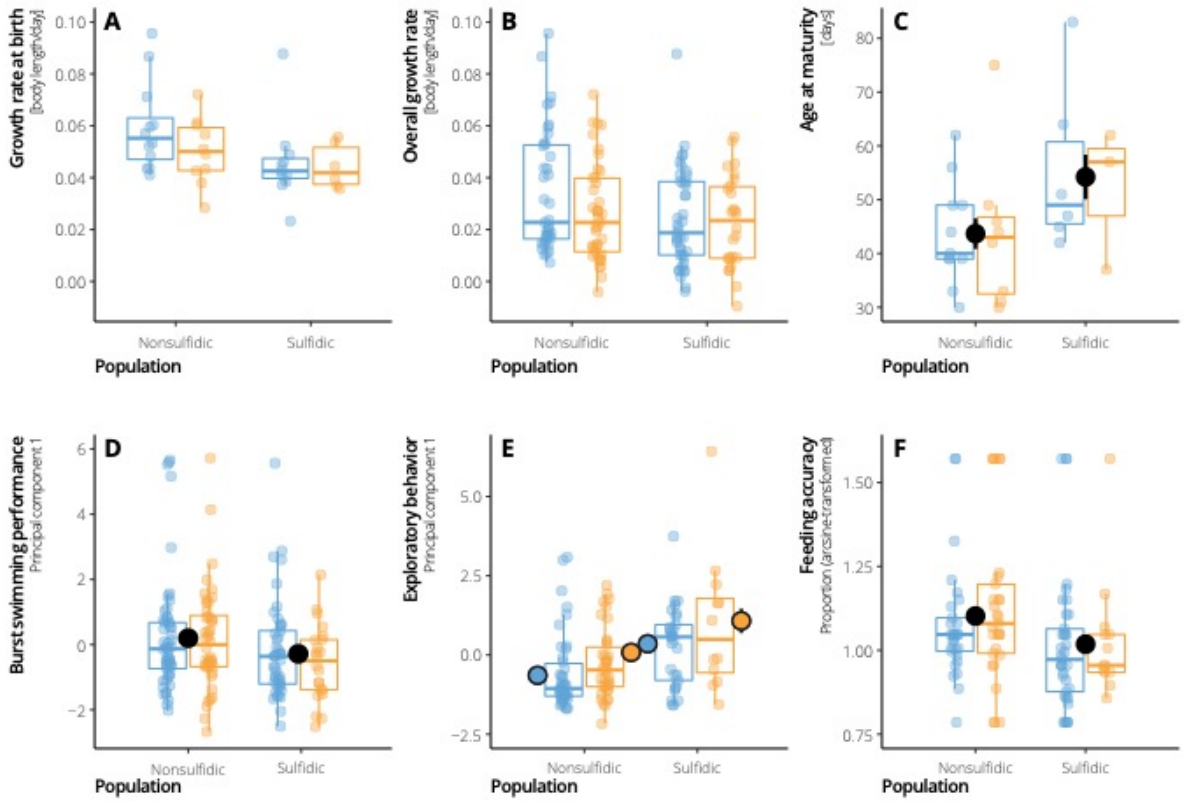

Figure 3. Data for each individual phenotype, including (A) growth rate at birth, (B) overall growth rate, (C) age at maturity, (D) burst swimming, (E) exploratory behavior, and (F) feeding accuracy. Data is summarized in boxplots with raw data points overlaid. In both populations, the high-food treatment 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.

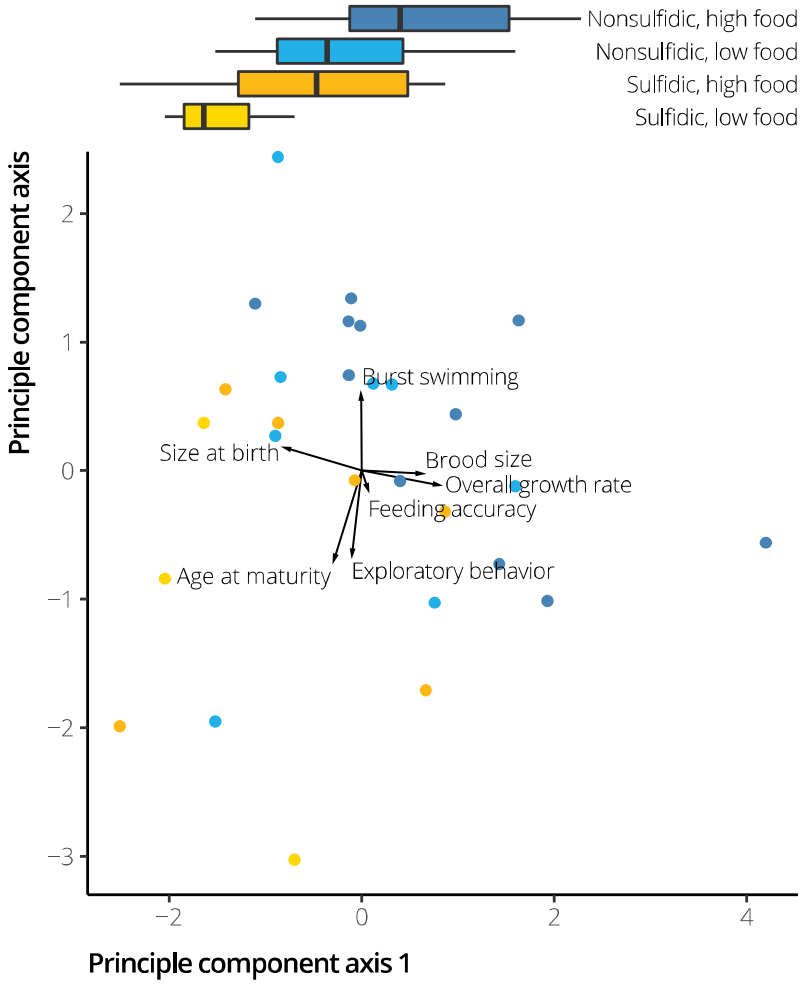

Figure 4. Plot of principal component scores representing linear combinations of all phenotypes in multivariate space. Scores were plotted along the first two principal components. Nonsulfidic families are shown in shades of blue, while sulfidic individuals are shown in shades of yellow. The high-food 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'.