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Sex-specific life-history trait expression in hybrids of a cave- and surface-dwelling fish (*Poecilia mexicana*, Poeciliidae)

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Abstract

Evaluating the fitness of hybrids can provide important insights into genetic differences between species or diverging populations. We focused on surface- and cave-ecotypes of the widespread Atlantic molly *Poecilia mexicana* and raised F1 hybrids of reciprocal crosses to sexual maturity in a common-garden experiment. Hybrids were reared in a fully factorial 2 × 2 design consisting of lighting (light vs. darkness) and resource availability (high vs. low food). We quantified survival, ability to realize their full reproductive potential (i.e., completed maturation for males and 3 consecutive births for females) and essential life-history traits. Compared to the performance of pure cave and surface fish from a previous experiment, F1s had the highest death rate and the lowest proportion of fish that reached their full reproductive potential. We also uncovered an intriguing pattern of sex-specific phenotype expression, because male hybrids expressed cave molly life histories, while female hybrids expressed surface molly life histories. Our results provide evidence for strong selection against hybrids in the cave molly system, but also suggest a complex pattern of sex-specific (opposing) dominance, with certain surface molly genes being dominant in female hybrids and certain cave molly genes being dominant in male hybrids.

Key words: dominance, life-history evolution, local adaptation, postzygotic isolation, selection against hybrids.

The act of lineage crossing, or hybridization, is an important event in evolutionary biology in general (Barton et al. 2007; Futuyma 2013), and the fate and fitness of hybrids is of particular interest in speciation research (Coyne and Orr 2004; Nosil 2012; Abbott et al. 2013). The most pronounced role of hybridization in speciation can arguably be found within the "hybrid speciation" model, in which the new species is the result of a hybridization event (which often also involves a change in ploidy levels; Mallet 2007). Alternatively, in both "mutation-order speciation" and "ecological speciation" models, selection against hybrids is one important factor for the emergence and maintenance of reproductive isolation (Schluter 2009; Nosil 2012), for example, because of genetic/genomic incompatibilities, such as Bateson-Dobzhansky-Muller incompatibilities (i.e., negative interactions between alleles at two or more loci: Orr 1996; Nosil 2012; Coyne 2016). In mutation-order speciation, this is the result of different and reciprocally incompatible mutations fixing in the two original populations that adapt to the same environmental conditions (Schluter 2009), while in ecological speciation, hybrids are selected against because they are maladapted to either of the different environments experienced by the original two populations (Nosil 2012). Therefore, evaluating the exact fate of hybrids is of central importance when investigating populations that are undergoing incipient speciation.

Here, we make use of a cave form of the Atlantic molly *Poecilia mexicana* (Plath and Tobler 2010; Tobler and Plath 2011), to further investigate the role of hybrids and aspects of reproductive isolation in this system. The name cave molly refers to subterranean populations of the Atlantic molly that have colonized two separate limestone caves (one with hydrogen-sulfide toxic water) in Tabasco, southern Mexico (Gordon and Rosen 1962). Previous work in this system has revealed incipient ecological speciation between cave and surface mollies, which are only few hundred meters apart and, because aquatic habitats are continuous, no physical barriers would prevent both ecotypes from mixing and interbreeding (Figure 1). Yet, we found evidence for pronounced genetic (e.g., Plath et al. 2007; Tobler et al. 2008, 2009) and heritable phenotypic differentiation (e.g., Parzefall 2001; Plath et al. 2003; Tobler et al. 2008; Riesch et al. 2010, 2011a,c) between cave and surface mollies; for example, cave mollies exhibit strongly reduced fecundity coupled with increased offspring size (Riesch et al. 2010). There is also good evidence of speciation being the complex interplay of different pre-zygotic isolating mechanisms in this system, ranging from sexual isolation (Tobler et al. 2009) to selection against immigrants (e.g., due to predation: Tobler 2009; due to permanent darkness: Riesch et al. 2011b, 2016; Torres-Dowdall et al. 2018; due to hydrogen-sulfide toxicity: Tobler et al. 2009).

Figure 1. Map indicating location of origin for the two *P. mexicana* ecotypes. (A) Location of our study area in Tabasco, Mexico. (B) Detail map of the location of the Cueva del Azufre (CdA; origin for cave mollies) and the Río Oxolotán (Oxo; origin for surface mollies) near the village of Tapijulapa. Reference cities and villages in black circles; A created with QGIS 3.2 (https://www.qgis.org/en/site/); B drawn by R. Riesch.

However, evidence for any role of postzygotic isolation, and more specifically, the fate of hybrids, is so far lacking; indeed, none of our previous studies revealed the existence of naturally occurring hybrids in this system.

We address this outstanding issue here, by conducting a reciprocal F1-hybrid crossing experiment, and then raising the resulting F1 in the same (life history-focused) common-garden setup previously established for the two pure ecotypes in an experiment with a slightly different focus (Riesch et al. 2011b, 2016; Torres-Dowdall et al. 2018). This common-garden setup follows a fully factorial 2 × 2 design involving two different treatments: resource availability (high vs. low food) and lighting (permanent darkness vs. 12:12 h light:dark cycle; Figure 2). On the extreme endpoints of those 4 treatment combinations, we have one condition resembling the cave environment (low food × permanent darkness) and one resembling the normal surface environment (high food × 12:12 h light:dark cycle). We then evaluated F1 fitness in two different ways: (1) we scored survival until the end of the experiment, and (2) we quantified a set of important life-history traits for those fish that did complete the experiment (including size and age at maturity, fecundity, and interbrood interval). In order to make meaningful comparisons, we then compared the performance of F1 hybrids in both aspects of fitness to the performance of the two pure ecotype lineages (i.e., surface and cave mollies), which we had obtained from the same experimental setup, but from a separate experiment that preceded the current experiment (Riesch et al. 2011b, 2016). We predicted that, (1) as further evidence for local adaptation and ecological speciation in this system, F1 survival should be worse than that of either parental ecotype; (2) life-history traits of F1 across experimental treatments would be largely intermediate to those of the parental ecotypes (which would make hybrids maladapted to either parental environment).

Materials and Methods

Populations and fish

Experimental subjects were hybrid *P. mexicana* derived from laboratory stocks of two populations originating from Tabasco, southern Mexico: a mix of surface populations (called Oxolotán; henceforth "surface") and a population from the toxic Cueva del Azufre, chamber V (henceforth "cave"; Figure 1). Both laboratory populations were established in 2005 and maintained separately in common garden conditions inside large (1000 L) flow-through stock tanks within a greenhouse at the Aquatic Research Facility of the University of Oklahoma, which is now the International Stock Center for Livebearing Fishes. Every few years, these stocks were genetically replenished by additional wild-caught fish. In the spring of 2012, virgin females and mature males from these stock populations were used to create F1 hybrid strains.

In order to make meaningful comparisons, we also re-analyzed data from our previous (but separate) experiment of the same kind, in which we had raised pure-bred F1 offspring of wild-caught individuals of the two parental (P) ecotypes (i.e., pure surface and cave mollies) in the same experimental setup and using the same 4 treatments (Riesch et al. 2011b, 2016; Figure 1). This means that we are not directly comparing parental to F1 performance but are rather comparing the performance of our F1 hybrids from the current experiment with the performance of the pure cave and surface molly ecotypes from the previous experiment (Supplementary Figure S1). A direct comparison of P to F1 phenotypes was logistically not possible due to the total length of such an experiment (i.e., given the time it takes *P. mexicana* to mature and reproduce, such an experiment would have run continuously for 3–4 years). However, the fish used for both experiments were derived from the same stock populations, the same experimental protocol was followed (see Supplementary Methods
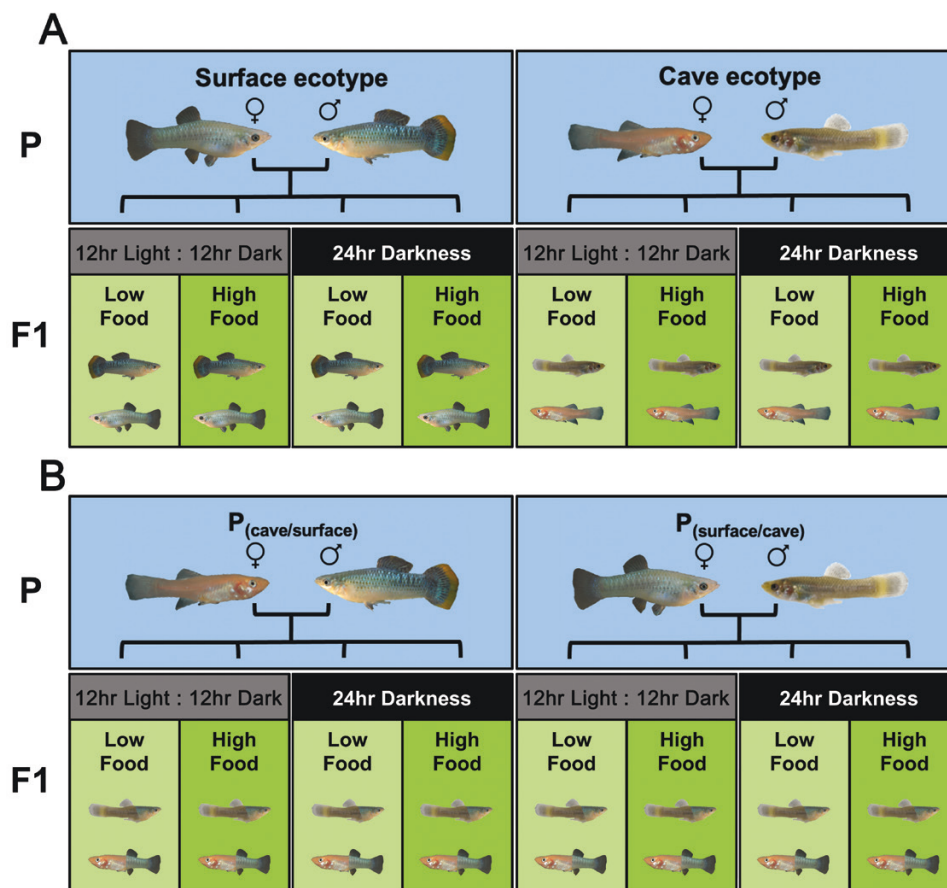
Figure 2. Graphical depiction of the experimental setup for (A) the original pure ecotype experiment (Riesch et al. 2016) and (B) the current F1 hybrid experiment. F1 offspring of different parental lines (P) were raised to maturity in either permanent darkness or under a natural 12:12 hr light:dark cycle. Within each light treatment, half the fish were raised on a high-food treatment, while the other half were raised under a low-food treatment. Pictures of hybrids are digitally merged and do not represent actual pictures of hybrids.

and Supplementary Table S1), and the equipment and setup used were the same for both experiments. This should minimize variance introduced by comparing data derived from two separate experiments.

Obtaining F1 hybrids

In a first step, we needed to produce parents (P) for our subsequent hybrids. For this purpose, and to partially control for maternal effects, virgin females of both stock populations were isolated in 5 L tanks, and a randomly selected male from another stock tank of the same population was added to this tank for one day each week. Each day, females were fed commercial flake food and the tanks were checked for offspring. To prevent cannibalism, a plastic mesh was used to provide the babies with a sheltered portion of the tank, which the female could not access. Once a female had given birth, the female was removed, and the babies were subsequently fed pulverized commercial flake food and *Artemia* nauplii daily. These babies were the eventual $P_{(cave)}$ and $P_{(surface)}$ used to create F1 hybrids. After the babies reached a length of approximately 20 mm, the tanks were checked every other day for maturing males. In this species, males develop a gonopodium, the copulatory organ characteristic for the family Poeciliidae, which is easily recognized as a transforming anal fin (Greven 2011). Males were immediately removed and placed in a separate tank.

Each virgin $P_{(cave)}$ and $P_{(surface)}$ female was transferred to separate 38 L tanks to prevent overcrowding. Once P females reached a minimum length of 30 mm, a single P male from the other ecotype (i.e., a $P_{(surface)}$ or $P_{(cave)}$) was introduced into the tank and they were fed twice daily with frozen blood worms and commercial flake food. Once pregnant, individual P females were isolated in 5 L tanks equipped with a plastic mesh to provide shelter for F1 newborns. The tanks were checked daily for newborn F1 hybrids. Once a P female had given birth, her weight, standard length, number of F1 offspring for that birth (i.e., fecundity), and the date of birth were recorded. She was then placed back in a 38 L tank. In total, 4 $P_{(surface/cave)}$ (i.e., surface molly mother and cave molly father) and 3 $P_{(cave/surface)}$ lineages were obtained. A maximum of 32 F1 hybrids were used from each cross (i.e., a single female x male parental combination).

2 × 2 Common-garden experiment

Experimental design followed previously published protocols (Riesch et al. 2011b, 2016; Torres-Dowdall et al. 2018). In short, once born, a maximum of 5 F1 hybrid babies were kept in 5 L tanks to prevent overcrowding and were fed ad libitum amounts of *Artemia* nauplii and pulverized flake food twice a day. Each tank received a partial water change every other day for 37 days. After this period of time, randomly selected F1 babies were transferred into new individual 5L tanks and

randomly assigned to one of the two light regime treatments and one of the two food regime treatments. These treatments were (1) 12:12 h light:dark vs. (2) permanent darkness and (3) high food vs. (4) low food (Figure 1). Fish were fed twice daily with a Hamilton micropipette containing measured amounts of *Artemia* nauplii in the morning and *Daphnia* in the afternoon; food treatments followed published protocols (Supplementary Table S1; Riesch et al. 2011b, 2016). Every 2 weeks, wet mass and standard length (SL; length from tip of snout to center of caudal peduncle) of each F1 fish were measured, and the tank then received a partial water change.

All tanks were checked daily for maturing F1 males following the procedure outlined above. Male F1 hybrids were deemed sexually mature and to have reached their "full reproductive potential" when gonopodium development had ended, i.e., the gonopodium had become largely translucent, the distal tip was pointed, the distal hook and gonopodial hood were fully developed and males had gained full muscular control of the gonopodium (see also Riesch et al. 2016). At this point, F1 males were removed from the experiment, sacrificed with an overdose of the anesthetic tricaine mesylate (MS-222), weighed and measured, and preserved in a 10% formalin solution.

Unfortunately, females lack an obvious external sign of sexual maturity. However, most males reach sexual maturity at much younger ages than females in this species (Riesch et al. 2016), so after the first F1 males of a given cross began to mature, all other fish from that lineage (i.e., putative F1 females) were assigned a parental population to be mated with (either surface or cave). Males from that parental population were then placed in their tanks overnight once every week. The males were placed in the tank after the afternoon feeding had concluded to prevent them from competing with the F1 hybrids for food; they were also removed before the F1 hybrids were fed the next morning. From this point forward, putative F1 females were carefully scanned for visual signs of pregnancy and tanks were carefully scanned for newborn fry twice a day. Some of these putative F1 females subsequently started to develop into males, at which point they were treated as outlined above. F1 females were only scored as "reproductively active" if they successfully produced a brood of offspring within their first year of life, and scored as having reached their "full reproductive potential" within the experimental setup if they successfully produced 3 broods within this experiment. All offspring from litters 1 through 3 were immediately sacrificed and preserved in formalin, while F1 females were measured for mass and length after each litter, and then sacrificed and preserved immediately after having given birth to their third litter. Total F1 sample sizes for each experimental group were as follows: $P_{(surface/cave)}$, light:dark cycle × low food = 25, light:dark cycle × high food = 28, darkness × low food = 23, darkness × high food = 22; $P_{(cave/surface)}$, light:dark cycle × low food = 13, light:dark cycle x high food = 12, darkness × low food = 14, darkness × high food = 13.

Quantifying life-history traits

Preserved F1 males and females were dissected following standard and well-established protocols (Riesch et al. 2010, 2011a, 2016). These dissections together with the data collected during the experiment provided us with the following male life-history traits: SL at maturity (mm), age at maturity (days), dry and lean mass at maturity (g), fat content at maturity (%), gonadosomatic index (GSI) at maturity (%)

(i.e., testis dry mass divided by the sum of testis dry mass plus somatic dry mass), and maturation time (days) (i.e., the time it took from the first indication of anal fin metamorphosis to the fully developed gonopodium). For F1 females, we quantified age at first parturition (days), standard length (SL) at first and third parturition (mm), as well as somatic dry and lean mass (g), and fat content (%). Furthermore, we measured fecundity (number of offspring), neonate dry and lean mass (mg), and neonate fat content (%) for broods 1–3, and quantified interbrood intervals (days) (between the first and second as well as between the second and third brood). Descriptive statistics for all fish from the hybrid (this study) and the pure ecotype experiment (Riesch et al. 2016) can be found in Supplementary Tables S2 and S3.

Prior to statistical analyses, we $\log_{10}$-transformed all time, length and mass measurements, square root-transformed fecundity, and arcsin(square root)-transformed all percentages. Subsequently, all variables were z-transformed to ensure all variables were on the same scale of measurement.

Statistical analyses

All statistical tests were conducted in IBM® SPSS® Statistics 25 (IBM® Corporation).

Ability to reproduce

We grouped all F1 fish into one of 3 different categories: (1) fish that had "successfully reproduced" (i.e., males that had successfully matured and females that produced at least one litter within the experiment), (2) those that "failed to reproduce" (i.e., these fish survived all 52 weeks of the experiment, but failed to mature as males or successfully produce at least one litter as females) and (3) those that "died" (to be conservative we counted only those fish as "died" that died before the end of the experiment and until that point had never produced a litter or fully matured to a male; females that produced at least one litter and then died were included in the "successful reproduction" category). When F1 hybrids were treated separately based on the direction of the cross (i.e., $F1_{(cave/surface)}$ versus $F1_{(surface/cave)}$), this resulted in extremely low sample sizes for some of the 3 categories, so we pooled all F1 hybrids for these analyses. We then conducted two Chi-Square tests to compare these counts between pooled F1 hybrids and the pure ecotypes from the original experiment (Riesch et al. 2011b). The first model tested for group-level differences between pooled F1 hybrids and pooled pure ecotypes. However, in the original experiment, surface mollies had performed worse than cave mollies (Riesch et al. 2011b), so our second model specifically tested for differences between pooled hybrids and surface mollies only. To account for multiple testing, we evaluated significance of both models at α = 0.025.

Full reproductive potential

We next tested for differences in the proportions of fish that reached their full reproductive potential within the experimental setup (see also Riesch et al. 2016). Since preliminary data analysis indicated that the two F1 hybrid lines ($F1_{(cave/surface)}$ and $F1_{(surface/cave)}$) did not differ significantly in the proportion of fish that had reached their full reproductive potential (*N* = 109; *Wald Chi-Square* = 0.016, df = 1, *P* = 0.900), we pooled all F1 hybrids also for this analysis. To test for differences between the sexes, we further excluded all fish (*N* = 51) that died without exhibiting any identifiable sex-specific characteristics. We conducted step-wise backwards

logistic regression based on Maximum Likelihood with "full reproductive potential" (binary: 1 = reached, 0 = not reached) as dependent variable and with the following covariates: "experimental line" (pooled F1 hybrids vs. pure surface mollies vs. pure cave mollies), "food treatment" (high food vs. low food), "light treatment" (light:dark cycle vs. permanent darkness), and sex (males vs. females). Since our main focus was on hybrid performance, we also coded all possible two-way interactions between "experimental line" and the other 3 covariates. We had initially planned to use a Generalized Linear Mixed Model for this, with "mother ID" as a random factor, but in this model the final Hessian matrix failed to be positive definite.

Ecotype assignment of hybrids

To our knowledge, running this in a single sex-specific model in SPSS is not possible, so we had to separate this analysis into two separate steps. We, thus, conducted 4 discriminant function analyses (DFAs) based on life-history traits. For males, we used 6 life-history traits as independent variables: SL at maturity (mm), age at maturity (days), lean mass at maturity (g), fat content at maturity (%), gonadosomatic index (GSI) at maturity (%), and maturation time (days). For females, we included 9 life-history traits: age at first parturition (days), SL at first parturition (mm), SL at third parturition (mm), lean mass (g) at third parturition, fat content (%) at third parturition, average fecundity (number of offspring) for broods 1–3, average neonate lean mass (mg) for broods 1–3, average neonate fat content (%) for broods 1–3, and average interbrood interval (days). To correct for the allometric effects of SL on male and female lean mass and fecundity, we used residuals of these traits, which were derived from preparatory sex-specific ANCOVAs that included SL as a covariate. Ecotype (cave vs. surface) served as the grouping variable for all 4 sex-specific DFAs.

First, to evaluate how well the pure ecotypes that made it through the original experiment (Riesch et al. 2011b, 2016) could be classified as either cave or surface mollies despite having been raised under different combinations of the experimental treatments, we conducted two sex-specific DFAs with a jackknife (leave-one-out) sampling scheme. This provided us with a baseline to which we could then subsequently compare F1-hybrid phenotypes to. For each of these two DFAs, a priori probabilities were calculated based on group sizes. Second, to test whether F1 hybrid life-history phenotypes matched those of either pure ecotype or were intermediate to them, we then commenced with two additional sex-specific DFAs. To be able to test classification success of F1 hybrids with respect to the pure ecotypes, we decided to use the maternal lineage as a proxy, and so for these DFAs, hybrids derived from crosses involving a surface molly female (i.e., F1$_{(surface/cave)}$) were designated as potential surface mollies, while hybrids derived from crosses involving a cave molly female (i.e., F1$_{(cave/surface)}$) were designated as potential cave mollies. Please note that this classification of hybrids into "surface" or "cave" mollies was arbitrary, and we could have easily done this the other way around; both approaches would have led to the same qualitative result. We then conducted DFA as follows: a discriminant function was again calculated based on the original ecotype data only (i.e., this step is identical to the same step in the first set of DFAs, so axis loadings were the same for both sets of models). We then inserted life-history data from the F1 hybrid samples into the discriminant functions and assigned each hybrid to the most parsimonious category (i.e., being more similar to a cave or a surface molly; Hair et al. 1995).

Results

Ability to reproduce

Thirteen F1 females (two from permanent darkness and 11 from the light:dark cycle) produced at least one brood but died before producing their third brood (i.e., did not reach their full reproductive potential). Additionally, one F1 male showed no signs of gonopodial development and was misclassified as female until a testis was found during subsequent dissection. This F1 male remained in the treatment for the full 52 weeks before it was euthanized; it was therefore also marked as not having reached its full reproductive potential.

The proportions of fish that successfully reproduced, failed to reproduce and those that died differed significantly between pooled F1 hybrids and the pure ecotypes (N = 295, Chi-*Square* = 25.294, *df* = 2, *P* < 0.001; Figure 3A). While both F1 hybrids and pure ecotypes had approximately the same proportion of fish that failed to reproduce, hybrids had a smaller proportion of fish that successfully reproduced and a higher proportion that died, compared to the original fish. These deaths occurred on a fairly consistent basis during the span of the experiment and affected the hybrids across all developmental stages. These patterns also held true when comparing pooled F1 hybrids only to pure surface mollies (N = 230, *Chi-Square* = 8.483, *df* = 2, *P* = 0.014; Figure 3A).

Full reproductive potential

When analyzing differences in the ability to reach the full reproductive potential within the two experiments (N = 244), the final model (–2 Log likelihood = 127.689; Nagelkerke R^2 = 0.678) indicated significant contributions from "line" (Wald χ^2 = 36.459, df = 1, P < 0.001), "food regime" (Wald χ^2 = 16.958, df = 1, P < 0.001), "line × light regime" (Wald χ^2 = 16.978, *df* = 1, P < 0.001), and "line × sex" (Wald χ^2 = 41.141, *df* = 1, P < 0.001), while the other covariates and interactions were removed during the stepwise backwards process. Pure cave mollies had the highest proportion of fish that successfully reached their full reproductive potential, followed by pure surface mollies, with pooled F1 hybrids performing the worst, and more fish reached their full reproductive potential in high than low food (Figure 3C). Moreover, while cave mollies had the highest proportion and hybrids the lowest proportion of fish that reached their full reproductive potential in either light regime, the degree to which light regime impacted performance differed between cave mollies, surface mollies and their hybrids. Similarly, compared to females, a higher proportion of males reached their full reproductive potential within the experiment, but the difference between sexes was dependent on the experimental line (Figure 3C).

Ecotype assignment

Jackknife DFA on males was 68.8% successful (relative to a random classification success of 51.2%). When investigating ecotype-specific classification success, 59.3% of pure cave molly males were correctly classified as cave mollies and 75.7% of pure surface molly males as surface mollies. However, for male F1 hybrids, DFA success was only 43.1%. When investigating group-specific classification success, it became apparent that this was due to almost all male F1 hybrids (84.3%) being classified as cave mollies based on
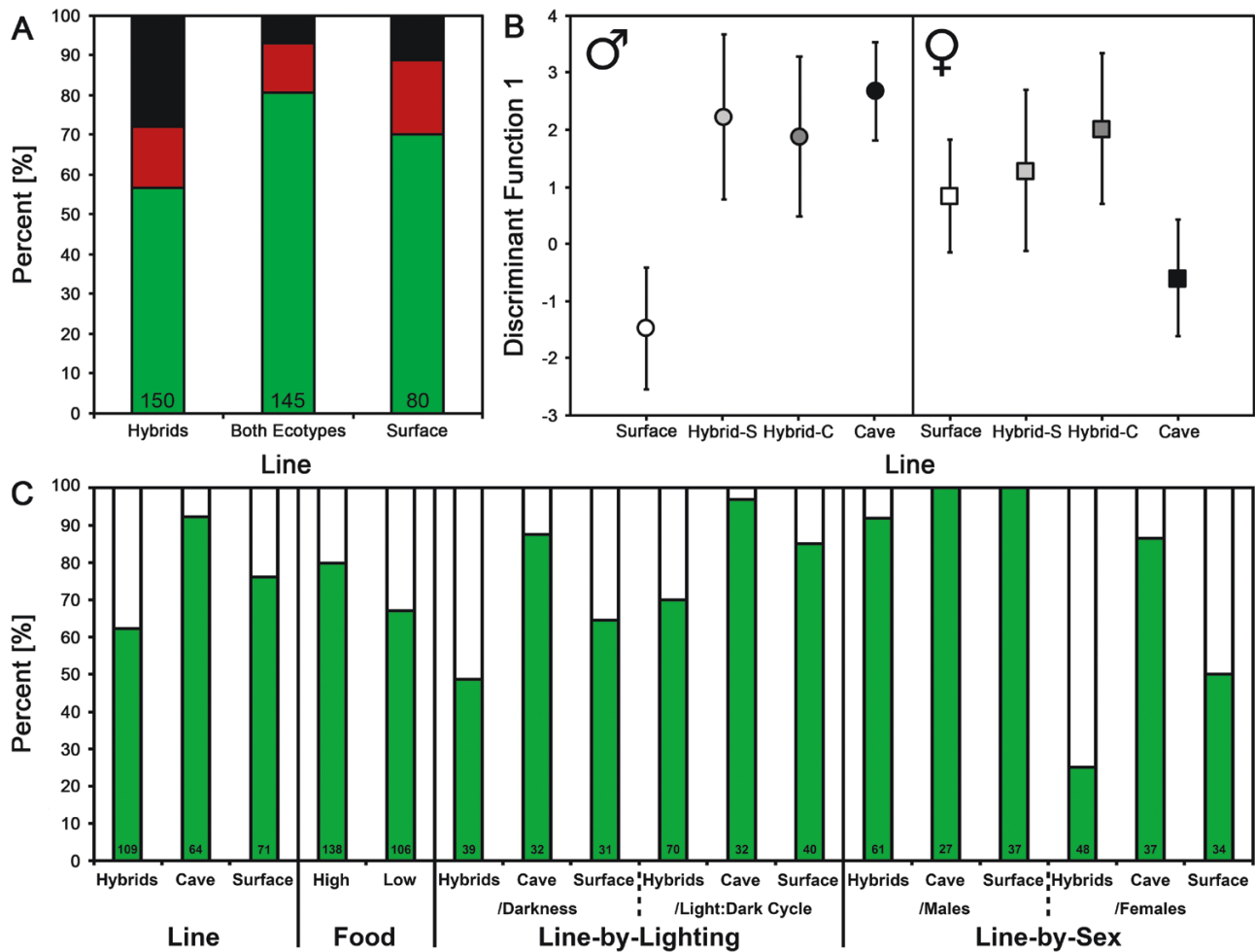
Figure 3. (A) Proportion of *Poecilia mexicana* (*N* = 295) that successfully reproduced (green or light gray), failed to reproduce (red or dark gray) or died (black) within two separate experiments. (B) Mean ± SD of discriminant function scores for males (circles) and females (squares), based on life histories. (C) Proportion of *P. mexicana* (*N* = 244) that reached their full reproductive potential (green; i.e., males that were fully mature or females that gave birth to 3 litters) within the limits of our experiments. Visualized are the non-significant effect of hybrid line (hybrid-S: F1$_{(surface/cave)}$ vs. hybrid-C: F1$_{(cave/surface)}$), as well as the significant effects of line (hybrids vs. cave mollies vs. surface mollies), food treatment, light treatment, and sex. Numbers within the bars represent sample sizes. Hybrids: all F1 hybrids pooled; both ecotypes: pooled surface and cave mollies; cave: cave mollies; surface: surface mollies; data on pure ecotypes were reanalyzed from Riesch et al. (2016).

their life-history traits (100% of F1$_{(cave/surface)}$ and 78.4% of F1$_{(surface/cave)}$ hybrids; Figure 3B). Classification success was predominantly based on age at maturity (discriminant function loading = 0.457), maturation time (0.380), SL at maturity (0.368), and fat content at maturity (–0.370).

Jackknife DFA on females had a success rate of 95.8% (relative to a random classification success of 54.3%). Ecotype-specific classification success was 96.8% for pure cave molly females and 94.1% for pure surface molly females. However, for female F1 hybrids, DFA was only to 56.3% successful. In stark contrast to F1 males, the majority of F1 females (87.5%) were classified as surface mollies based on their life-history traits (100% of F1$_{(cave/surface)}$ and 81.8% of F1$_{(surface/cave)}$ hybrids; Figure 3B). Specifically, classification success was mostly determined by offspring lean mass (discriminant function loading = –0.681), adult lean mass (0.454), and fecundity (0.381).

Discussion

We found evidence for strong selection against F1 hybrids in our experimental setup, which also included conditions

resembling the environments experienced by wild cave and surface mollies. We also uncovered an intriguing pattern of sex-specific life-history trait expression, whereby male F1 express cave molly life histories, while female F1 express surface molly life histories.

Congruent with our prediction 1, our experiment provided strong evidence for postzygotic isolation via selection against hybrids in the cave molly system, because a higher proportion of F1 hybrid fish died prematurely and a smaller proportion of F1 hybrid fish successfully reproduced, compared to pure cave and surface mollies. Similar patterns have been found in many other taxa, including sticklebacks, *Gasterosteus aculeatus* (e.g., Hatfield and Schluter 1999; Gow et al. 2007; Shurtliff 2011; Coughlan and Matute 2020). Indeed, our results, which showed a greater mortality of hybrids throughout development, closely matches the patterns Gow et al. (2007) found for hybrid sticklebacks, whose relative frequency in natural populations declined across their life-cycle.

For logistical reasons, transplanting hybrids into the natural environment of cave and surface mollies in southern

Mexico was not possible. Thus, based on our data, we cannot say with certainty how exactly this lower fitness of hybrids originates and how hybrid fitness would manifest under natural conditions (i.e., is this due to ecological selection, and thus a footprint of ecological speciation, or not? e.g., Rundle and Whitlock 2001). Yet, our current results suggest that hybrid performance was inferior to the performance of pure ecotypes in all 4 experimental treatments, and not just in the two treatments resembling the environments experienced by surface (12:12 h light:dark cycle and high food availability) and cave (permanent darkness and low food availability) mollies. Since fish were housed in individual tanks, this poor performance is not the result of inferior competitive abilities. Rather, this suggests intrinsic genetic incompatibilities between loci and not just ecologically dependent selection against hybrids (i.e., ecological mismatch between hybrid phenotype and the parental environments; Rundle and Whitlock 2001; Nosil 2012). These arise when two populations experience different substitutions at pleiotropically interacting loci that, when they come together in a hybrid, fail to interact harmoniously (Coyne and Orr 2004; Bolnick et al. 2006; Nosil 2012). In theory, intrinsic genetic incompatibilities could be either the result of ecologically based divergent selection (i.e., ecological speciation) or could be uncoupled from divergent selection (Coyne and Orr 2004; Nosil 2012). Nonetheless, intrinsic reproductive isolation (often via Bateson-Dobzhansky-Muller incompatibilities) has been demonstrated (or implicated) as a result of divergent selection in a range of taxa, including plants (*Mimulus* monkeyflowers: e.g., Martin and Willis 2010; Coughlan et al. 2020; *Helianthus* sunflowers: e.g., Sambatti et al. 2008), insects (e.g., Funk et al. 2006), and fishes (swordtails, *Xiphophorus* spp.: Powell et al. 2020; *Coregonus* whitefish: e.g., Rogers and Bernatchez 2006; Woods et al. 2009; *Gasterosteus* sticklebacks: e.g., Lackey and Boughman 2017; Centrarchidae: Bolnick et al. 2006). Current studies in the cave molly system will attempt to elucidate the genetic basis for the reduced hybrid fitness we uncovered here, and whether this is linked to loci under divergent selection or not.

In our experiment, we had fewer $P_{(cave/surface)}$-hybrids (i.e., cave molly mother and surface molly father) than $P_{(surface/cave)}$-hybrids. This discrepancy in sample sizes is likely due to a combination of reasons. First, this could simply be stochasticity. We set up similar number of pairings to produce F1 hybrids in both crossing directions, but fewer cave molly females got pregnant than surface molly females during the time we had available to produce these hybrids. After several weeks, and due to logistical constraints, we were forced to proceed with the sample sizes we had. Second, this could indicate that this particular direction of hybridization results in less viable offspring. While this would be a very intriguing result, unfortunately, we did not dissect the parental females afterwards. We therefore do not know if they might have carried broods of regressors (i.e., embryos that died during development). Lastly, cave molly females usually have a highly reduced fecundity (Riesch et al. 2009; Riesch et al. 2010) and thus, only produce a handful of offspring per clutch while similar-sized surface molly females usually produce several dozen offspring per clutch. Thus, the $P_{(cave/surface)}$ crosses should have only resulted in very few offspring per clutch, providing us with fewer F1 to work with in the experiment compared to $P_{(surface/cave)}$ crosses.

Among those fish that started reproduction within the experiment, we further uncovered a unique and interesting pattern of sex-specific life-history trait expression, which provides evidence against our prediction 2 that F1 phenotypes are intermediate (Figure 3B). Specifically, male hybrids expressed cave molly life histories, while female hybrids expressed surface molly life histories. To our knowledge, this type of sex-specific multivariate phenotype expression has not yet been reported; in fact, hybrids often exhibit maternal inheritance of male traits (e.g., Atsumi et al. 2021). While this could suggest that these phenotypes are sex-linked, that is, that the genes responsible for these phenotypes are located on the sex chromosomes (Barton et al. 2007; Futuyma 2013), we consider this to be highly unlikely for at least two reasons. First, previous analyses of the genetic basis for life-history traits in the closely related guppy *Poecilia reticulata* and other taxa has revealed that they tend to be polygenic and are often widespread across the genome (e.g., guppy: Whiting et al. 2022; Atlantic salmon *Salmo salar*: Vasemägi et al. 2010; the common frog *Rana temporaria*: Palomar et al. 2019). Second, we did not simply quantify a single trait but a set of traits that all act together to produce the cave and surface life-history phenotype (although some traits, like offspring mass and fecundity, seem to be more important than others). Given what we just outlined regarding the genetic architecture of individual life-history traits, this makes it even less likely that all genes involved are located only on or near the sex-determining loci. For example, there is only evidence for a single life-history trait being associated with the sex-determining region on Chromosome 12 in the guppy (Whiting et al. 2022).

Thus, if these traits are not sex-linked, how can the pattern we uncovered be explained? It's been well established that hybridization often has different effects on female progeny compared to male progeny, an effect that can be tied to sex chromosomes via Haldane's rule (e.g., Haldane 1922; Naisbit et al. 2002; Kenchington et al. 2020; Riddle et al. 2021; Sánchez-Ramírez et al. 2021), and sex-specific parent-of-origin gene expression has also been documented in several different systems (e.g., Gibson et al. 2004; Gregg et al. 2010). Yet we find no evidence in support of Haldane's rule; in fact, males generally performed better than females and not worse (as would be expected, given they are the heterogametic sex). Moreover, recent work has highlighted that F1 hybrids do not always exhibit fully intermediate phenotypes, but rather tend to be more similar to one of the two parental species (Atsumi et al. 2021; Thompson et al. 2021). In fact, the pattern we uncovered here best fits a scenario of mismatched combinations of divergent traits due to opposing dominance (Matsubayashi et al. 2010; Cooper et al. 2018; Thompson et al. 2021). This creates phenotypes in which hybrids resemble parent 1 for a certain set of traits and parent 2 for certain other sets of traits, which is possible when dominance acts in opposing directions, and can lead to intriguing barriers to gene flow (e.g., Matsubayashi et al. 2010). In the case of *P. mexicana*, however, rather than generally applying to F1 hybrids, mismatched trait combinations seem to be sex-specific. Specifically, it seems that surface molly genes (or quantitative trait loci) underlying patterns of offspring mass, fecundity, and adult lean mass, appear to be dominant in female F1, while cave molly genes underlying patterns of age at maturity, SL at maturity, maturation time and fat content at maturity appear to be dominant in male F1 (largely irrespective of the direction of the cross).

Here, we only investigated differences in life-history traits, but our results raise the intriguing question of how other traits, which are known to be divergent and heritable between the two ecotypes (including behavioral and morphological traits: e.g., Parzefall 2001; Plath et al. 2003; Tobler et al. 2008), manifest in F1 hybrids. Current and future studies will investigate this further, as well as the underlying genetic basis of the observed patterns.

Author Contributions

All authors contributed to study conception and design. Luis R. Arriaga conducted the hybrid experiment under supervision of Ingo Schlupp. Data analysis was performed by Rüdiger Riesch. The first manuscript version was drafted by Rüdiger Riesch and all authors contributed to revisions and further improvements. All authors read and approved the final manuscript.

Conflict of Interest

All authors declared no competing interests.

Ethical Approval

This work was approved by the University of Oklahoma IACUC (R11-020).

Data Availability

Data supporting this paper can be found on Figshare (doi: 10.17637/rh.21024196).

Supplementary Material

Supplementary material can be found at https://academic.oup.com/cz.

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