From genomes to genitalia: ecological speciation in sulfide spring fishes

by

Ryan Greenway

B.S., Oklahoma State University, 2014

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Division of Biology
College of Arts and Sciences

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Abstract

As closely related lineages adapt to habitats characterized by divergent sources of natural selection, the accumulation of different adaptations can lead to reproductive isolation and a reduction in gene flow between those lineages, facilitating the speciation process. Organisms living in extreme environments – habitats characterized by physiochemical stressors lethal to most forms of life – provide ideal systems for the study of adaptive divergence and ecological speciation. Extreme environments exhibit clearly defined selective regimes, enabling hypothesis-driven tests of the effects of physiochemical stressors on trait evolution across levels of biological organization. Additionally, lineages closely related to those found in extreme environments often occur in adjacent, benign habitats, facilitating studies of divergence between habitats. I used a unique system in which evolutionarily independent populations of livebearing fishes (family Poeciliidae) have colonized extreme environments in the form of freshwater springs rich in the naturally occurring toxicant hydrogen sulfide (H_2S) to address three major objectives. (1) I determined whether female fish use adaptive trait differences between populations in different habitats as signals for making mating decisions that contribute to reproductive isolation between populations from different habitat types. (2) I measured variation in female and male genitalia among populations to determine if divergence in genital traits between habitat types could facilitate the evolution of reproductive isolation at early stages of ecological speciation. (3) I characterized adaptive divergence and reproductive isolation between population pairs of three distantly related species that occur in the same sulfide spring and adjacent non-sulfidic stream to identify common patterns of adaptive divergence across levels of biological organization. Using a combination of field and laboratory experiments in conjunction with genomic tools, I found that (1) female fish prefer aspects of male body shape indicative of

local adaptation, (2) female and male genitalia coevolve within populations and diverge among populations in different habitat types during the early stages of speciation, potentially contributing to reproductive isolation between populations, and (3) convergent patterns of adaptive divergence and ecological speciation can be the result of unique genomic mechanisms in distantly related species. Overall, my dissertation research links evolutionary changes across multiple levels of biological organization in order to understand the formation of new species and the predictability of evolution driven by natural selection.

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Approved by:

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Dedication

I would like to dedicate this dissertation to my friends Eric Dreasher, Skylar Brannon Buettner, Brandon Buettner, Alex Garner, and Sophie Higgs, as well as Chase, my four-legged best friend and adventure companion. I am lucky to have had each of you in my life.

Preface

Background

Understanding the processes and mechanisms responsible for generating and maintaining biological diversity is fundamental to evolutionary ecology. Divergent natural selection is an evolutionary force capable of driving both intra- and interspecific diversification along ecological gradients and between contrasting environments (Kawecki and Ebert 2004). Moreover, divergent selective pressures within and among populations can lead to the generation of biodiversity when adaptation causes the evolution of reproductive isolation between populations in different environments, a process known as ecological speciation (Schluter 2001; Nosil 2012).

Extreme environments, those characterized by conditions lethal or detrimental to most organisms, provide ideal systems for the study of organismal evolution (Bell 2012). Extreme environments exhibit clearly defined selective regimes, enabling hypothesis-driven tests of their effects on trait evolution at all levels of organismal organization (Riesch et al. 2015a). As a result, extremophiles capable of withstanding the physicochemical stressors characteristic of many extreme environments are uniquely suited for quantitative analyses of adaptive evolution and ecological speciation (Bell 2012). Another advantageous characteristic of extreme environments is the frequent occurrence of adjacent benign habitats harboring non-extremophile lineages closely related to those found living in extreme conditions (Riesch et al. 2015a). These benign/extreme habitat pairs often occur in a replicated fashion across large geographic scales, producing "natural experiments" that facilitate comparative analyses among evolutionarily independent lineages occurring in both geographically similar and disparate locations (Riesch et al. 2015a).

Among the most extreme freshwater ecosystems are freshwater springs rich in toxic hydrogen sulfide (H_2S) (Tobler et al. 2016b). Due to its lipid solubility, H_2S freely penetrates biological membranes and readily invades organisms (Bagarinao 1992; Reiffenstein et al. 1992). Like cyanide, it inhibits cytochrome c oxidase and blocks electron transport in aerobic respiration, thereby hampering the function of mitochondria and the production of ATP (Evans 1967; Cooper and Brown 2008). H_2S is also able to modify oxygen transport proteins (Park et al. 1986) and inhibit about 20 other enzymes (Reiffenstein et al. 1992). Consequently, H_2S is highly toxic for aerobic organisms, even in micromolar concentrations (Beauchamp et al. 1984; Bagarinao 1992). In addition, H_2S spontaneously oxidizes in water, creating hypoxic conditions in aquatic systems (Cline and Richards 1969; Chen and Morris 1972). Due to the extreme nature of these habitats, few metazoans have successfully colonized sulfide springs, even though such springs occur worldwide (Greenway et al. 2014). In order to overcome the challenges of life in sulfide springs, organisms colonizing these habitats require some combination of adaptations for detoxifying H_2S, reducing the absorption of H_2S, preventing the inhibition of cytochrome c oxidase and blockage of the electron transport chain, as well as increasing the amount of obtained dissolved oxygen to be used for respiration (Tobler et al. 2016b).

Fish of the family Poeciliidae are among the most prominent inhabitants of sulfide springs, as several species have colonized and adapted to sulfidic conditions throughout the Americas and Caribbean (Greenway et al. 2014; Tobler et al. 2018). Over the past decade, a plethora of research has been conducted on sulfide spring poeciliids, particularly on the *Poecilia mexicana*-species complex, which has a wide distribution throughout Mexico and Central America (Miller et al. 2005). In southern Mexico, four evolutionarily independent lineages – two within *P. mexicana mexicana,* as well as two derived from *P. mexicana limantouri* – have

colonized sulfidic springs in the Río Grijalva drainage (Palacios et al. 2013). For this system, there is a wealth of existing information, including characterizations of environmental variation (Tobler et al. 2006; Tobler et al. 2008), phenotypic variation (Tobler et al. 2011a; Riesch et al. 2014), genetic differentiation (Palacios et al. 2013; Plath et al. 2013), and different reproductive isolating barriers (Tobler et al. 2009a; Plath et al. 2013) between ecotypes from adjacent sulfidic and non-sulfidic habitats in multiple river drainages. There have been studies of survival strategies in these toxic habitats (Tobler et al. 2009b), and the ecology and behavior of sulfide spring fish (Tobler 2008; Tobler et al. 2009a; Tobler et al. 2015; Culumber et al. 2016; Tobler et al. 2016a). More recently, the molecular and physiological mechanisms of adaptation to H_2S are being revealed (Pfenninger et al. 2014; Tobler et al. 2014; Passow et al. 2015; Pfenninger et al. 2015; Kelley et al. 2016), and genomic resources for *P. mexicana* have been produced (Kelley et al. 2012; Warren et al. 2018). Despite these studies, many questions have remained unaddressed in sulfide spring poeciliids, particularly surrounding the mechanisms of ecological speciation between sulfidic and non-sulfidic fishes.

The aim of this dissertation is to provide novel insight into how different reproductive isolating barriers evolve and interact to facilitate the ecological speciation process, as well as to investigate to what extent adaptive evolution is predictable and repeatable in organisms adapting to similar environmental conditions. Employing replicated sulfidic and non-sulfidic population pairs of poeciliid species to address these aims, this dissertation research focuses on three primary objectives:

Chapter 1. Assortative mating preferences based on traits indicative of local adaptation can help drive the evolution of reproductive isolation during ecological speciation. I sought to determine

the role of two different mechanisms that could generate previously observed assortative mating preferences between sulfidic and non-sulfidic populations of *P. mexicana*. Body shape variation between sulfidic and non-sulfidic *P. mexicana* consists of aspects potentially influencing assortative mating through magic trait and condition-dependent trait mechanisms. I quantified male body shape variation in order to produce computer-generated animations of male fish used in behavioral trials investigating female preferences for different aspects of male body shape.

Chapter 2. The divergence of genital traits between closely related lineages has been thought play a role in reproductive isolation via genital incompatibilities between female and male genital traits. However, we know little about how genitalia diverge among lineages and coevolve among the sexes during early stages of speciation. I characterized variation in female and male genitalia among populations of sulfidic and non-sulfidic *P. mexicana* to determine if genital traits diverge among populations in divergent habitats and coevolve between females and males within populations during the early stages of ecological speciation.

Chapter 3. Many studies of the repeatability of adaptive divergence focus on parallel speciation events, where geographically distinct but closely related population pairs undergo ecological speciation as the result of adaptation to similar selective regimes. Understanding how predictable and repeatable adaptive divergence is has been biased by focusing on speciation events that share a common genomic background and which occur in the presence of unquantified variation in selective regimes. Eliminating the potential effects of environmental variation experienced by different population pairs and shared genomic backgrounds provides a means to study the role of shared selective pressures in the evolution of reproductive isolation between populations and to

test whether similar patterns of adaptive divergence play a role in replicated speciation events. I used a combination of genomic and transcriptomic analyses, field experiments, and physiological tolerance trials to investigate shared patterns of adaptive divergence between sulfidic and non-sulfidic population pairs of three distantly related poeciliid species occurring in a single sulfide spring and an adjacent non-sulfidic stream.

Synthesis and future research

Assortative mating is a critical component of reproductive isolation during speciation, yet the mechanisms underlying mating preferences are often unknown (Schluter 2001; Servedio et al. 2011; Nosil 2012). In Chapter 1, I found that assortative mating preferences contributing to sexual selection against migrants between sulfidic and non-sulfidic populations of *P. mexicana* are the result of female preferences for aspects of male body shape indicative of local adaptation. Furthermore, I found that preferences for this trait likely evolved as a response to natural selection against hybridization between populations in the contrasting habitat types, demonstrating how traits evolving in response to natural selection can be coopted by sexual selection to reinforce reproductive isolation during ecological speciation.

Divergence of genital traits between lineages has been hypothesized to serve as an effective reproductive isolating barrier when successful copulation, insemination, or fertilization are inhibited or prevented due to incompatibilities between the genitalia of males and females from different lineages (Masly 2012; Brennan and Prum 2015; Langerhans et al. 2016). However, the timing and role of genital divergence as a barrier to gene flow during speciation remains unclear (Langerhans et al. 2016; Yassin 2016). Results from Chapter 2 provide evidence that genitalia rapidly diverge between populations during the early stages of speciation. In

addition, there is evidence for coevolution between the sexes within populations as well as convergence among populations experiencing similar sources of natural selection. These results provide evidence that genital divergence has the potential to contribute to the early stages of speciation with ongoing gene flow, rather than only as a result of reproductive character displacement upon contact between highly divergent lineages at later stages of speciation.

As demonstrated in Chapters 1 and 2, replicated cases of ecological speciation along similar environmental gradients provide the chance to link convergent aspects of adaptation to the emergence of reproductive isolation (Rundle et al. 2000; Rosenblum and Harmon 2011; Nosil 2012). While convergent evolution at both the phenotypic and genomic level has been documented during replicated speciation events in multiple systems (Conte et al. 2012; Jones et al. 2012; Nosil et al. 2018), under what circumstances adaptive divergence and ecological speciation should occur in a repeatable manner among lineages experiencing similar selective regimes remains unresolved. With Chapter 3, I documented how striking convergence at higher levels of biological organization can come about through unique genomic mechanisms acting in different species experiencing the exact same selective regime. Results from this chapter indicate that the recent focus on cases of parallel speciation in nature may have biased the field's interpretations of the predictability and repeatability of adaptation at the molecular level, suggesting that the shared genomic backgrounds of closely related lineages are particularly important for driving convergent evolutionary outcomes.

Overall, my dissertation demonstrates that adaptive divergence and ecological speciation can proceed through similar mechanisms at higher levels of organismal organization, both across several populations of the same species and among distantly related species. I documented evidence supporting the occurrence of mechanisms of reproductive isolation (assortative mating

preferences for a “magic trait” and genital divergence) for which empirical support of their role during ecological speciation had previously been scarce. My research also highlights the importance of using novel natural systems to address the generality of particular hypotheses in evolutionary ecology that have only been studied in a relatively small group of taxa. Based on the results of my research, several avenues of research have opened to be addressed within the sulfide spring system. It remains to be seen how genital trait divergence influences reproductive success during interpopulation matings. Studies are needed to determine the functional relevance of genital trait variation for both females and males. Without an understanding of the functional role of these traits, it remains to be seen whether genital trait divergence actually play a role in facilitating reproductive isolation. Additionally, the results of Chapters 1 and 2 provide tangential evidence that hybrid inviability may exist between sulfidic and non-sulfidic populations and cause the divergence of mating-associated traits, *i.e.* reinforcement. Future studies are required to determine whether hybrid offspring from sulfidic and non-sulfidic crosses indeed exhibit lower fitness than the parental lineages. Of particular interest is whether the loci involved in adaptation to sulfide springs are the same loci as those causing incompatibilities among populations, and whether any reduced hybrid fitness is intrinsic or dependent upon environmental conditions. Addressing these questions will be a fruitful means to further the field’s understand of the mechanisms of reproductive isolation driving ecological speciation.

Chapter 1 - Adaptive, but not condition-dependent, body shape differences contribute to assortative mating preferences during ecological speciation*

Ryan Greenway, Shannon Drexler, Lenin Arias-Rodriguez, and Michael Tobler

Abstract

Assortative mating is critical for reproductive isolation during speciation, however, the mechanisms underlying mating preferences are often unknown. Assortative mating can be mediated through preferences for condition-dependent and adaptive ("magic") traits, but rigorously testing these hypotheses has been impeded by trait covariation in living organisms. We used computer-generated models to examine the role of body shape in producing association preferences between fish populations undergoing ecological speciation in different habitat types. We demonstrate that body shape can serve as an adaptive trait (variation in head size between populations) and a condition-dependent signal (variation in abdominal distention among individuals). Female preferences for stimuli varying in only one aspect of body shape uncovered evidence for body shape as a magic trait across population pairs, but no evidence for body shape serving as a condition-dependent signal. Evolution of preferences only in females from one habitat type as well as stronger preferences in sympatric non-sulfidic as opposed to allopatric non-sulfidic populations suggests that reinforcement may have played a role in producing the observed patterns.

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Introduction

Ecological speciation occurs when reproductive isolation evolves as a byproduct of adaptation in populations exposed to divergent selection (Schluter 2001; Rundle and Nosil 2005; Schluter 2009; Nosil 2012). Although divergent selection is required for ecological speciation, theoretical models indicate that it is rarely sufficient to drive speciation to completion, and some degree of assortative mating is required to counteract the homogenizing effects of gene flow between incipient species (van Doorn et al. 2009). Assortative mating has been widely documented in systems undergoing ecological speciation (Elmer et al. 2009; Schluter 2009; Schluter and Conte 2009; Bolnick and Kirkpatrick 2012), and the mechanisms leading to assortative mating preferences have been studied using theoretical models (Weissing et al. 2011; Thibert-Plante and Gavrilets 2013). Nonetheless, it remains largely unclear what mechanisms underlie the evolution of assortative mating preferences, and how different mechanisms contribute to total reproductive isolation (Schluter 2001; Servedio et al. 2011; Nosil 2012).

In theory, the emergence of assortative mating could be mediated by a number of mechanisms, including condition-dependent traits, magic traits, and reinforcement (Nosil 2012). Condition-dependent mechanisms assume that the expression of signals used during mate choice is indicative of an individual's degree of local adaptation (van Doorn et al. 2009; Thibert‐Plante and Gavrilets 2013). Condition-dependent mechanisms do not require the divergence of mating preferences among populations, as assortative mating results from the reduced expression of traits indicative of genetic quality in maladapted migrants and hybrids with lower body condition (van Doorn et al. 2009; Weissing et al. 2011). Magic trait mechanisms hinge on mating preferences that are based on a trait under divergent ecological selection. In this scenario, adaptive traits function pleiotropically as signals during mate choice, and mating preferences

diverge among populations undergoing speciation (Gavrilets 2004; Servedio et al. 2011). Magic trait mechanisms assume that mating preferences evolve concomitantly as a byproduct of divergent selection on ecological traits that serve as cues (Servedio et al. 2011; Maan and Seehausen 2012), and assortative mating should therefore evolve early during the speciation process (Thibert‐Plante and Gavrilets 2013). Divergence in preferences can also arise later in the speciation process via reinforcement, where assortative mating evolves through direct selection on female mating preferences to reduce costs associated with hybridization (Kirkpatrick 2001; Servedio and Noor 2003). During reinforcement, divergent ecological traits may be co-opted as mating signals (Maan and Seehausen 2012), producing patterns of assortative mating similar to magic trait mechanisms. However, the strength of assortative mating during reinforcement should be correlated with the opportunity and strength of selection on mating preferences. Hence, reinforcement mechanisms should lead to pronounced patterns of assortative mating in contact zones between incipient species, but not in allopatric populations (Rundle and Schluter 1998; Noor 1999; Higgie et al. 2000; Coyne and Orr 2004).

While few empirical studies have explicitly tested for the role of condition-dependent traits in generating assortative mating during ecological speciation (van Doorn et al. 2009; Nosil 2012), magic trait mechanisms are frequently invoked (Maan and Seehausen 2011; Servedio et al. 2011; Servedio and Kopp 2012). Servedio et al. (2011) outlined two criteria for providing conclusive evidence for a magic trait: (1) the magic trait must be subject to divergent selection, and (2) the magic trait must generate non-random mating. Particularly strong evidence for a single trait subject to divergent ecological selection also generating non-random mating has been found for mimetic wing color-patterns in *Heliconius* butterflies (Jiggins 2008). In many other cases, however, manipulative experiments isolating the effects of individual adaptive traits on

assortative mating preferences are often lacking and alternative hypotheses – such as reinforcement – often ignored (Maan and Seehausen 2011; Servedio et al. 2011; Maan and Seehausen 2012; Nosil 2012). In fact, disentangling the role of different traits in mediating reproductive isolation has been a major challenge in testing hypotheses about mechanisms underlying assortative mating, because behavioral, morphological, and even physiological traits co-vary in living organisms. Such trait co-variation among incipient species arises from multivariate trait divergence during adaptation to divergent ecological conditions (*e.g.,* Rosenblum and Harmon 2011; Lee and Mitchell-Olds 2013). Therefore, failures to link specific adaptive traits to mating decisions in live organisms potentially differing in entire suites of traits has frequently prevented unequivocal inference about the role of any one mechanism in generating assortative mating.

Recent technological advances have enabled researchers to begin manipulating mating signals and control for trait co-variation through audio (Maan and Cummings 2009; Akre et al. 2011) and video playback (Oliveira et al. 2000; Fisher et al. 2009; Schlupp et al. 2010). The advent of computer generated model creation is allowing researchers to construct animated stimuli for behavioral experiments using video playback (Woo and Rieucau 2011; Veen et al. 2013; Ingley et al. 2015). Animated models provide the benefit of testing organismal preferences for stimuli differing in virtually any trait, while holding all other traits constant (Woo and Rieucau 2011; Culumber and Rosenthal 2013). Consequently, technological advances in combination with behavioral experiments provide a powerful tool to test for magic traits and potential alternatives. We deployed such an approach in a system where independent lineages along replicated ecological gradients are undergoing ecological speciation.

Poeciliid fishes in the *Poecilia mexicana* species complex have independently colonized toxic hydrogen sulfide-rich springs in multiple tributaries of the Río Grijalva in southern Mexico (Tobler et al. 2011a; Palacios et al. 2013). Sulfide spring fishes are locally adapted and differ from ancestral populations in adjacent non-sulfidic habitats in physiological, morphological, behavioral, and life-history traits (Tobler et al. 2011a; Riesch et al. 2014; Passow et al. 2015). Sulfidic populations are also genetically differentiated from neighboring non-sulfidic populations despite a lack of physical barriers (Palacios et al. 2013; Plath et al. 2013). Reproductive isolation is in part mediated by natural selection against migrants, and there is also evidence for assortative mating preferences between sulfidic and non-sulfidic populations (Plath et al. 2010; Plath et al. 2013). However, it remains unknown what mechanisms have contributed to the emergence of these assortative mating preferences.

We focused on aspects of body shape as potential signals mediating assortative mating, because body shape variation has the potential to act both as a magic trait and as a condition-dependent signal. Features of body shape are under divergent ecological selection along the gradient between sulfidic and non-sulfidic habitats, with sulfide spring fishes having evolved larger heads in convergence (Tobler and Hastings 2011, Tobler et al. 2011a, Palacios et al. 2013). Increased head size is correlated with larger gill surface area, which – in combination – facilitates efficient oxygen acquisition necessary for survival in hypoxic sulfide spring environments (Plath et al. 2007b; Tobler et al. 2009b; Tobler and Hastings 2011). Other aspects of body shape also exhibit condition-dependence, as abdominal distension reflects the nutritional state (condition) of individual fish (Plath et al. 2005; Tobler et al. 2011b). Prior studies have documented female *P. mexicana* preferring well-fed males over nutritionally deprived individuals (Plath et al. 2005; Tobler et al. 2011b). Using computer generated fish models, we

tested for female association preferences as a function of body shape differences among populations (a putative magic trait) and body shape variation in relation to condition in three sulfidic and non-sulfidic population pairs of the *P. mexicana* complex. This approach allowed us to break up natural trait co-variation and test how specific traits (aspects of body shape) potentially contribute to assortative mating preferences. We also compared female association preferences in sympatric non-sulfidic and allopatric non-sulfidic populations to distinguish between classic magic trait and reinforcement mechanisms driving preference evolution. Specifically, we asked the following questions: (1) How does male body shape vary among populations from different habitat types and among individuals in different nutritional states? Based on previous studies, fish from sulfidic environments are expected to have larger heads and low condition individuals less abdominal distention. (2) Does body shape act as a condition-dependent signal? If condition-dependent mechanisms mediate assortative mating, females – irrespective of habitat type of origin – are predicted to associate with male models exhibiting a high condition body shape. (3) Does body shape act as a magic trait mediating assortative mating? If magic trait mechanisms mediate assortative mating in this system, females are predicted to associate with male models exhibiting a body shape representative of the same habitat type. (4) What is the potential role of reinforcement in shaping preference evolution? If body shape has been co-opted as a signal via reinforcement, we expect to see preferences for models with a body shape representative of the same habitat type in sympatric, but not allopatric, populations.

Materials and Methods

Study system

We examined male body shape and tested for female mating preferences in three pairs of sulfidic and non-sulfidic populations from major tributaries of the Río Grijalva: the Ríos Tacotalpa, Puyacatengo, and Pichucalco (Figure A1, Table A1; see Palacios et al. (2013) for detailed descriptions of these sites). Note that all populations nominally belong to *P. mexicana*, except for the sulfide spring population from the Pichucalco drainage, which represents a phylogenetically older lineage that has been described as a distinct species (*P. sulphuraria*; Palacios et al. 2013). Sulfide spring complexes are located in the upper reaches of each of the river drainages in the foothills of the Sierra Madre de Chiapas. The most prominent difference between sulfidic and non-sulfidic habitats is the presence of hydrogen sulfide (H_2S), a naturally occurring toxicant, in the water (Greenway et al. 2014; Riesch et al. 2015b). Even in micromolar amounts, H_2S is highly toxic to aerobic organisms due to its ability to freely penetrate biological membranes and block electron transport during aerobic metabolism (Cooper and Brown 2008; Tobler et al. 2016b). Each sulfide spring eventually discharges into a non-sulfidic stream or river, such that physical barriers preventing organismal dispersal between sulfidic and non-sulfidic habitats are mostly lacking (Tobler et al. 2011a). Proximate sympatric non-sulfidic reference populations were selected for each sulfide spring within the same drainage. In addition, we quantified female association preferences in two allopatric non-sulfidic populations from the Río Tulija drainage (Figure A1, Table A1), which has no documented evidence for sulfide springs and is located at least 60 km straight distance from the nearest sulfidic population in the Río Grijalva drainage. There are no known allopatric sulfidic populations to have included in this study due to the wide distribution and high abundance of *P. mexicana* in freshwater streams of this region.

Testing for body shape variation among habitat types and nutritional states

In order to ground truth body shape's potential value as a mating signal in this system, we first quantified variation in body shape between individuals subjected to variation in resource availability as well as between populations from sulfidic and non-sulfidic habitats. To test for morphological variation associated with nutritional state (condition), adult males from laboratory-reared stocks of a sulfidic and a non-sulfidic population (derived from wild-caught Río Tacotalpa populations) were subjected to feeding treatments to induce variation in body condition. Adult males were divided into population specific groups of three to five individuals, placed into 20L aquaria, and assigned to one of three different food treatments ($n \geq 8$ males per population in each treatment; Table A1). Food treatments followed Passow et al. (2015), where fish in the normal food treatment received commercially available fish flake food according to the following formula: $0.0124 \times (\text{total fish body mass})^{0.65}$. Fish in the high food treatment received twice that amount; fish in the low food treatment received half that amount. At the end of two weeks under these feeding regimes, all fish were euthanized with buffered MS222 and fixed in a 10% formaldehyde solution. To test for among-population variation, adult male *P. mexicana* were collected from our study sites using seines, euthanized with buffered MS222 immediately after capture, and fixed in a 10% formaldehyde solution ($n = 10$ individuals for each of the six sites; Table A1).

For all specimens, lateral photographs were taken with a Canon EOS 400D digital camera (Canon USA Inc., Lake Success, NY, USA) mounted on a copy stand. We digitized 14 morphological landmarks (Figure A2) using the software program tpsDig (Rohlf 2004). The coordinates from these digitized landmarks were extracted and used to conduct geometric morphometric analyses following Zelditch et al. (2012). Landmark coordinates were aligned

using least-squares superimposition as implemented in the program tpsRelw (Rohlf 2007) to remove effects of translation, rotation, and scale. We then calculated centroid size and relative warp scores plus uniform components (weight matrix) for each individual based on these aligned coordinates. The weight matrix was subjected to a principal component analysis based on a covariance matrix to reduce data dimensionality, and principal axes with eigenvalues greater than the average eigenvalue were retained as shape variables (for laboratory-reared individuals: 13 axes explaining 95.5% of observed variance; for wild-caught individuals: 10 axes accounting for 95.0 % of observed variance). We used multivariate analyses of covariance (MANCOVA) on these shape variables to test for morphological variation between individuals of different nutritional states and sulfidic and non-sulfidic populations. For the analysis of effects of body condition, we used "food treatment" (high/normal/low) and "ecotype" (sulfidic/non-sulfidic) as factors, and centroid size as a covariate to control for multivariate allometry. For the analysis of body shape variation in natural populations, we included "ecotype" (sulfidic/non-sulfidic) and "drainage" (Tacotalpa, Puyacatengo, Pichucalco) as factors, and centroid size as a covariate. *F*-values were approximated using Wilks' lambda and effect strengths by use of partial eta squared (η_p^2). Interaction terms with $P > 0.05$ were excluded from the final models. To isolate the effects of specific predictor variables and visualize body shape variation between sulfidic and non-sulfidic habitats and among food treatments, we calculated the divergence vector score (*sensu* Langerhans 2009) of each individual fish for the first principle component of the among-group covariance matrix for the corresponding term in the MANCOVA (Klingenberg and Spence 1993). This allows for the visualization of body shape variation in response to a particular factor, while correcting for all other effects in the model.

Designing animations for video playback

The *anyFish* program suite, an open-source software tool for generating animated fish models, was used for the creation of model stimuli for behavioral experiments (Veen et al. 2013; Ingley et al. 2015). *anyFish* allows for the manipulation of morphological, behavioral, and visual inputs during the creation of animated stimuli. We utilized these functions to create animated *P. mexicana* model pairs differing only in aspects of body shape related to our questions and morphological analyses.

Using the same photographs of *P. mexicana* males used in the analyses of body shape variation, we digitized 42 morphological landmarks and semi-landmarks following Ingley et al. (2015) using the software program tpsDig (Rohlf 2004). Note that we were required to use different sets of landmarks for the analysis of shape (see above) and the generation of models, both to fulfill the assumptions of geometric morphometric analyses (a smaller set of homologous landmarks) and to match the morphological inputs necessary to generate a poeciliid rig (*i.e.*, the model "skeleton") within *anyFish* (Ingley et al. 2015). For each set of specimens, tpsRelw (Rohlf 2007) was used to calculate the consensus morphology as well as phenotypic extremes based on size-corrected landmark data. This process allowed us to generate two model pair inputs for the creation of fish animations in *anyFish*: one pair reflecting body shape variation as a function of condition, and the other differences between habitat types (Figure A3). Note that a *post hoc* analysis of the body shape of our stimulus fish using the reduced set of landmarks indicated that they adequately captured the shape differences among habitat types and food treatments (Figure 1.1). A single digital image of a wild male *P. mexicana* representing typical coloration was then warped in tpsTransformer (Veen et al. 2013) in order to fit the models generated by the *anyFish* software suite. Animations consisted of a male swimming toward the left side of the screen,

performing a courting display (raising the dorsal fin and swimming slowly), turning and performing the display in the center of the screen, swimming and displaying on the right of the screen, again displaying in the center, then returning to the starting location. This animation looped continuously, with each loop lasting 24 seconds. The final size of the animation on the playback monitors was adjusted to match the mean size of wild-caught males used in our morphological analyses (standard length = 28 mm). The background color was gray-blue and the substrate beige in all animations (Culumber and Rosenthal 2013). Thus, the animations of all different models were identical in every aspect except for variation in the specific trait of interest. We also conducted control experiments to determine whether females actually respond to the animations. To do so, we created an animation pair that showed a high condition male animation versus the standard animation background without an animated fish.

Behavioral testing

Adult female *P. mexicana* were collected from each focal site (Table A1) using seines and transported in insulated coolers to a nearby field station. Fish were kept in population specific holding tanks and allowed to acclimate for 48 hours before testing. Preference testing closely followed published methods (Fisher et al. 2009; Verzijden and Rosenthal 2011; Culumber and Rosenthal 2013). Preference tests were conducted in aquaria (60×30×45cm) filled to a depth of 20 cm with aerated water. Apple iPad displays (Apple Inc., Cupertino, CA, USA) were placed against the glass at each end of the aquaria to play video animations to females. At the start of a trial, a single female was introduced into each testing tank and allowed to acclimate for 10 minutes prior to video playback. The playback procedure consisted of 300 seconds of video playback of animated stimulus pairs, 180 seconds of black screen, and an additional 300 seconds

of video playback with the stimuli switched to the opposite displays. This allowed controlling for side biases (see below). The side on which a stimulus animation was first shown was randomized across trials.

All preference trials were recorded from above with GoPro digital cameras (GoPro, Inc., San Mateo, CA, USA). The resulting videos were used to score association times of females with each animated stimulus using Behavioral Observation Research Interactive Software (BORIS), an open-source event logging software (Friard and Gamba 2016). Association time is a standard metric of mating preference in poeciliid fishes and has been shown to be a repeatable predictor of mate choice and reproductive outcomes (Cummings and Mollaghan 2006; Walling et al. 2010; Plath et al. 2013; but see Gabor 1999). All videos were blindly scored without the scorer knowing what animation was on which side of the aquarium. To determine association times with each stimulus, tanks were divided into 3 zones (15 cm left zone, 30 cm center zone, and 15 cm right zone) by placing marks above the waterline of the test aquaria that were visible only to the human observer. Females were considered to be associating with a stimulus anytime they were in the zone nearest that stimulus (preference zones). If a female's body spanned the boundary between zones, she was only considered to be associating with the stimulus if she was facing that stimulus. This approach was used to test females of each population with each of the animation pairs (high *vs*. low condition, sulfidic *vs*. non-sulfidic) as well as controls (animated male *vs*. background). The exceptions were the two allopatric populations, which were not evaluated for preferences associated with body condition. Each female was tested only once, and all fish were released at their original collection site after testing.

Analysis of behavioral data

Individuals exhibiting side biases or being unresponsive were excluded from data analyses. Side bias was defined as females spending more than 85% of the total time in one particular preference zone (Rosenthal and Evans 1998; Korner et al. 1999; Landmann et al. 1999; Wong and Rosenthal 2006; Fisher et al. 2009). Unresponsiveness was defined as females failing to spend more than 25% of the total observation time outside of the neutral zone (Schlupp and Ryan 1996; Korner et al. 1999; Landmann et al. 1999; Verzijden et al. 2012). Association times for all individuals tested (both responsive and unresponsive) can be found on Dryad Digital Repository (doi:10.5061/dryad.2dn85).

Our general analytical approach was to calculate the strength of female association preference (SOP) for a stimulus *A* relative to stimulus *B* combined across the two playback periods: $SOP = \frac{time_A - time_B}{time_A + time_B}$. SOP-values from each experiment were then used as the dependent variable in separate analyses of covariance (ANCOVA). Analyses of sympatric populations included "ecotype" (sulfidic/non-sulfidic) and "drainage" (Tacotalpa, Puyacatengo, Pichucalco) as factors. Standard length (SL) of focal females served as a covariate, because poeciliid females frequently exhibit size-dependent association preferences (Morris et al. 2006; Rios-Cardenas et al. 2007; Wong et al. 2011). Analyses comparing association preferences among sympatric non-sulfidic to allopatric non-sulfidic populations included "geographic context" (allopatric/sympatric) and "population" (nested within "geographic context") as factors, with SL as a covariate. Note that the standard length of each female was *z*-transformed relative to the length of other females from the same population. This was necessary because female size distributions consistently differ between sulfidic and non-sulfidic habitats, as well as among collections sites of the same habitat type (Passow et al., unpublished data).

SOP calculations differed for each animation pair, which is critical for the interpretation of results: (1) For the control animation pair, $SOP_{Control} = \frac{time_{fish} - time_{no\ fish}}{time_{fish} + time_{no\ fish}}$. Hence, positive values represent a preference to associate with the stimulus, and negative values avoidance of the stimulus. It is expected that females from all populations have a strong preference for associating with the stimulus animation. (2) For the animation pair depicting condition differences in body shape, $SOP_{Condition} = \frac{time_{high\ condition} - time_{low\ condition}}{time_{high\ condition} + time_{low\ condition}}$. Hence, positive values represent preference for the high condition model, and negative values preference for the low condition model. If condition-dependent mate choice is important, all females – irrespective of origin – should exhibit positive $SOP_{\text{Condition}}$ values. (3) For the animation pair depicting habitat type differences in body shape, $SOP_{Habitat} = \frac{time_{sulfidic} - time_{nonsulfidic}}{time_{sulfidic} + time_{nonsulfidic}}$. Hence, positive values represent preference for the sulfidic model, and negative values preference for the non-sulfidic model. If magic trait mechanisms are important in shaping assortative mating, females from sulfidic populations are expected to exhibit positive SOP_{Habitat} values, and those from non-sulfidic habitats negative ones. Additionally, if reinforcement contributed to the evolution of female preferences, females from sympatric non-sulfidic populations should exhibit negative SOP_{Habitat} values, whereas those from allopatric non-sulfidic populations should have no significant preference in either direction.

Results

Body shape variation among habitat types and nutritional states

Analyzing body shape variation in wild-caught males of *Poecilia* from sulfidic and non-sulfidic populations indicated significant effects of centroid size, drainage, and ecotype, as well as the

interaction between drainage and ecotype (Table 1.1). Even though all of these effects were statistically significant, ecotype exhibited the largest effect size, indicating shared differences in body shape between sulfidic and non-sulfidic populations from different drainages. Thin-plate spline transformation grids visualizing shape differences indicated that males from sulfidic populations consistently exhibited larger heads than those from non-sulfidic populations (Figure 1.1A).

Body shape also varied significantly among individuals subjected to different feeding treatments in the laboratory (Table 1.1). Note body shape differences between ecotypes were maintained in the laboratory, and population differences along with centroid size exhibited the largest effect sizes in the model (Table 1.1). Assessment of thin-plate spline transformation grids visualizing shape differences associated with feeding treatments revealed that fish in the high food treatment had greater abdominal distension than those in the low food treatment (Figure 1.1B).

Female preferences for male body shape

For sulfidic and sympatric non-sulfidic populations, we tested the preferences of 15 different females from each population per animation pair (n = 270). 87 individuals were excluded from analyses because of side biases or low responsiveness (38 sulfidic, 49 non-sulfidic); hence, analyses include association times for 183 females (n = 97 sulfidic, 86 non-sulfidic; Table A1). For allopatric non-sulfidic populations, preferences for the control animation were tested for a total of 18 females, and preferences for the potential magic trait were tested for a total of 39 different females; analyses included times for 37 responsive females (Table A1). Females from all populations responded positively to the control animation pair, consistently showing a

preference for the fish animation over the empty background animation (Figure A4). There were no differences in responsiveness between fish from different habitat types, drainages, or geographic contexts, or in fish with different body sizes (Table A2).

Testing female association preferences in response to condition-dependent body shape variation, we found no net preferences for or against high condition animations in any of the populations (Figure 1.2A), and responses did not differ among females from different habitat types or drainages (Table 1.2). However, ANCOVA indicated a significant – albeit weak – effect of body size (Table 1.2), with smaller fish tending to associate more with high condition stimuli and larger fish more with low condition stimuli (Figure A5).

Testing female association preferences in response to a potential magic trait (sulfidic *vs*. non-sulfidic body shape), we found significant differences in responses between females from sulfidic and sympatric non-sulfidic habitats irrespective of drainage or body size (Table 1.2). As predicted, females from sympatric non-sulfidic habitats exhibited negative SOP_{Habitat} values, indicating a preference for non-sulfidic over sulfidic stimulus animations (Figure 1.2B). In contrast, females from sulfidic habitats did not exhibit any association preference associated with habitat differences in body shape, with SOP_{Habitat} values that were not significantly different from zero.

Analyses of association preferences in response to sulfidic *vs.* non-sulfidic body shape animations in allopatric non-sulfidic populations compared to sympatric non-sulfidic populations revealed significant differences between the geographic contexts (Table 1.2). As predicted under the reinforcement hypothesis, females from sympatric non-sulfidic populations exhibited a preference for non-sulfidic animations, while females from allopatric non-sulfidic populations showed no significant preference in either direction (Figure 1.3).

Discussion

Different aspects of body shape have the potential to serve both as condition-dependent signals and magic traits mediating assortative mating among locally adapted populations in the *Poecilia mexicana* system. Our analyses corroborated the results of previous studies indicating that some aspects of body shape (primarily head size) are under divergent selection and have evolved in convergence in sulfide spring populations (Tobler and Hastings 2011; Tobler et al. 2011a; Palacios et al. 2013). Other aspects of body shape (primarily abdominal distension) reflect the nutritional condition of individuals (Plath et al. 2005; Tobler et al. 2011b). Behavioral experiments did not reveal any female association preferences for male models exhibiting a high condition body shape. However, females from sympatric non-sulfidic environments – but neither those from sulfidic nor allopatric non-sulfidic ones – consistently exhibited an association preference for males with a body shape of their own ecotype. Overall, these results suggest that aspects of body shape under ecological selection act as signals contributing to assortative mating patterns in a system undergoing ecological speciation. The asymmetrical preferences in adjacent population pairs and the lack of preferences in allopatric non-sulfidic populations suggest that reinforcement rather than classic magic trait mechanisms are likely contributors to the emergence of assortative mating in the system.

Body shape as a condition-dependent signal

Evidence for condition-dependent signals influencing mating decisions in organisms has been widely documented (Andersson 1986; Hill 1990; Houle and Kondrashov 2002; Cotton et al. 2004; Cotton et al. 2006), and theoretical models have implicated a potentially important role for condition-dependent signals in the ecological speciation process (Lorch et al. 2003; van Doorn et

al. 2009; Weissing et al. 2011; Thibert-Plante and Gavrilets 2013; Veen and Otto 2015). Nonetheless, there is – as of yet – no empirical evidence for condition-dependent traits driving the evolution of assortative mating between populations during ecological speciation, and our results found no support for females responding to condition-dependent variation in male body shape. Prior studies have demonstrated the impact of male nutritional state on female preferences in *P. mexicana* (Plath et al. 2005; Tobler et al. 2011b) and other poeciliid fishes (Fisher and Rosenthal 2006). Hence, our results do not imply that condition-dependent mechanisms are not important, but merely that body shape does not serve as a cue for females to discriminate between high and low condition mates. Male condition may cause variation in a suite of other traits, including coloration, olfactory cues, courtship intensity, and mating strategies (Kennedy et al. 1987; Kodric-Brown 1993; Kodric-Brown and Nicoletto 2001; Fisher and Rosenthal 2006; Kolluru et al. 2009), which all could serve to discriminate maladapted immigrants with low body condition and mediate female preferences for male condition documented in other studies. Hence, the potential role for condition-dependent signals during ecological speciation warrants further empirical investigation both in the sulfide spring fish system and additional cases of ecological speciation, and manipulative experiments with artificial stimuli will be critical to tease apart how different condition-dependent traits interact to influence female preferences during speciation.

Body shape as a magic trait

Magic traits have the potential to play an important role during ecological speciation. Magic traits should facilitate speciation even in the presence of gene flow and recombination, as a magic trait pleiotropically influences both adaptation and mate choice, circumventing a need for

linkage disequilibrium to arise and be maintained between traits encoded by separate genes (Gavrilets 2004; Servedio et al. 2011; Maan and Seehausen 2012; Servedio and Kopp 2012). The theoretical effectiveness of magic traits in driving speciation might lead to the expectation of magic traits being commonly documented during the speciation process, but few studies have found strong evidence for a single trait being subject to both divergent natural selection and generating assortative mating patterns (reviewed in Servedio et al. 2011). Many studies fall short of providing conclusive evidence for the role of magic traits during speciation by not appropriately controlling for trait covariation. By using computer generated animations, we isolated the effects of body shape variation between sulfidic and non-sulfidic ecotypes of *Poecilia* and found evidence for characteristics of body shape serving as a magic trait contributing to the emergence of divergent assortative mating preferences and reproductive isolation. Body shape has previously been hypothesized as having the potential to serve as a magic trait during ecological speciation (Langerhans et al. 2007; Head et al. 2013; Langerhans and Makowicz 2013; Martin 2013), both because it strongly affects swimming performance under different ecological settings (Douglas and Matthews 1992; Koehl 1996), and it can serve as a visual cue influencing mating decisions. In fact, our results indicate that the convergent evolution of similar body shapes in evolutionarily independent sulfide spring fish populations may be contributing to replicated speciation events between sulfidic and non-sulfidic ecotypes of *P. mexicana* (Tobler et al. 2011a; Plath et al. 2013). These cases of parallel speciation could be a consequence of body shape being a readily available signal serving as a magic trait.

Asymmetric preferences, magic traits, and reinforcement

While our results initially suggest that body shape variation between ecotypes of *P. mexicana* meets both commonly held criteria for classification as a magic trait (Gavrilets 2004; Servedio et al. 2011), the data are not consistent with the classic scenario assuming mating preferences for adaptive traits arise through byproduct mechanisms. Preferences for males with a matching body shape were only evident in females from sympatric non-sulfidic habitats, a result that is consistent with a previous study that measured female association preferences in multiple sulfidic and non-sulfidic *Poecilia* populations using live stimuli (Plath et al. 2013). In general, such asymmetrical behavioral isolation can arise as a by-product of adaptive and non-adaptive evolution in allopatry as well as selection on mating preferences during secondary contact between populations with postzygotic isolation (Kaneshiro 1980).

The lack of significant preferences in sulfidic fishes may arise as a result of regressive evolution in species recognition mechanisms. Sulfide springs generally harbor depauperate fish assemblages (Greenway et al. 2014) that may negate the necessity for distinguishing between conspecific and heterospecific individuals. Indeed, regressive evolution of species recognition has been documented in a cavernicolous population of *P. mexicana* (Riesch et al. 2006). However, one of the sulfidic populations tested here (La Gloria) co-occurs with two other sulfide adapted poeciliid species (Greenway et al. 2014), yet still shows no significant preferences. The lack of preferences in sulfidic populations could also be an artifact of our experimental design. For example, differences in water properties between sulfidic and non-sulfidic habitats (*e.g.* turbidity; Tobler et al. 2006) cause substantial variation in the ambient light environment of the two habitat types, which may be driving divergence in the visual systems between populations (Rennison, unpublished data). Since all animations were created under a uniform animated light

environment and all tests conducted under uniform non-sulfidic conditions, adaptations in the visual systems of sulfidic females may have affected their association behavior in our experiment. In addition, we created stimulus animations based on shared (convergent) body shape variation among all pairs of sulfidic and non-sulfidic populations, because convergence is indicative of adaptive evolution (Losos 2011). Ignoring drainage specific population differences, however, may have obscured aspects of body shape that are relevant for assortative mating in specific populations.

The documented patterns of assortative mating are also consistent with reinforcement (Kirkpatrick 2001; Servedio and Noor 2003). Divergence in mating preferences during reinforcement hinges on a high probability for encountering non-adapted mating partners, where selection acts to reduce costs from producing hybrid offspring with low fitness (Coyne and Orr 1997; Servedio and Noor 2003; Ortiz-Barrientos et al. 2009). The absence of assortative mating preferences in allopatric non-sulfidic females as well as females from sulfidic environments may be a consequence of a lack of selection on mating preferences. Fish from allopatric non-sulfidic populations and sulfidic populations are far less likely to encounter non-adapted mating partners in their native habitat. Reciprocal translocation experiments have demonstrated strong natural selection against non-sulfidic fish migrating between habitat types, because H_2S-toxicity causes high mortality within less than 24 hours (Plath et al. 2013). In contrast, natural selection against migrants is much weaker for sulfidic fishes moving into non-sulfidic habitats, resulting in the potential for higher encounter rates of non-adapted individuals in non-sulfidic habitats. Differences in encounter rates ultimately may have caused variation in natural selection on assortative mating preferences leading to the evolution of asymmetric mating preferences during reinforcement. Hence, it appears body shape may not have served as a classic magic trait

contributing to reproductive isolation during incipient ecological speciation. Instead, the combined results indicate that reproductive isolation may initially have evolved by other mechanisms (*e.g.* hybrid inviability), and reinforcement co-opted adaptive differences in body shape as an accessible signal for mate choice in later stages of speciation.

These nuances in differentiating between alternative hypotheses highlight a broader conceptual problem with documenting evidence for and uncovering the role of magic traits during the speciation process. While linking the role of specific traits in adaptation to specific environmental conditions and in serving as signals during mate choice is necessary to establish a potential role of magic traits, it is not sufficient. It is equally important to elucidate the forces that give rise to divergent mating preferences that ultimately mediate assortative mating (see Maan and Seehausen 2011, 2012), both in terms of evolutionary patterns that shape heritable differences in association behavior as well as environmental factors that can modulate preferences plastically during development or particular social contexts (Plath et al. 2008; Royle et al. 2008; Verzijden and Rosenthal 2011; Verzijden et al. 2012). Understanding the forces that generate variation in mating preferences and species recognition mechanisms has potential to provide a more robust understanding of how assortative mating contributes to the speciation process. In addition, it will allow for testing whether assortative mating is primarily involved in driving divergence in early stages or reinforcing isolation in later stages of speciation.

Conclusions

Our study established a role for body shape characteristics mediating assortative mating preferences in three evolutionarily independent population pairs of sulfidic and non-sulfidic fish. While this provides evidence for body shape meeting the criteria of a magic trait, asymmetrical

mating preferences in sympatric populations and the absence of preferences in allopatric non-sulfidic populations indicate that body shape-dependent association preferences may have arisen through reinforcement. Additionally, we demonstrated the utility of computer generated model creation and video playback in controlling for trait covariation and isolating traits of interest for rigorously testing hypotheses about the mechanisms underlying assortative mating. Using more manipulative experiments to study assortative mating is necessary to gain a more complete understanding of the importance of divergent mating preferences in the speciation process, and *anyFish* (Ingley et al. 2015) and other platforms for the generation of artificial stimuli will be instrumental in this endeavor. Though our findings highlight the role of assortative mating between sulfidic and non-sulfidic populations of *Poecilia*, it remains unclear how postzygotic isolating mechanisms and additional prezygotic mechanisms act to generate observed levels of reproductive isolation in this system. These represent necessary areas for future studies to more fully evaluate how ecological speciation operates in this system.

Figures

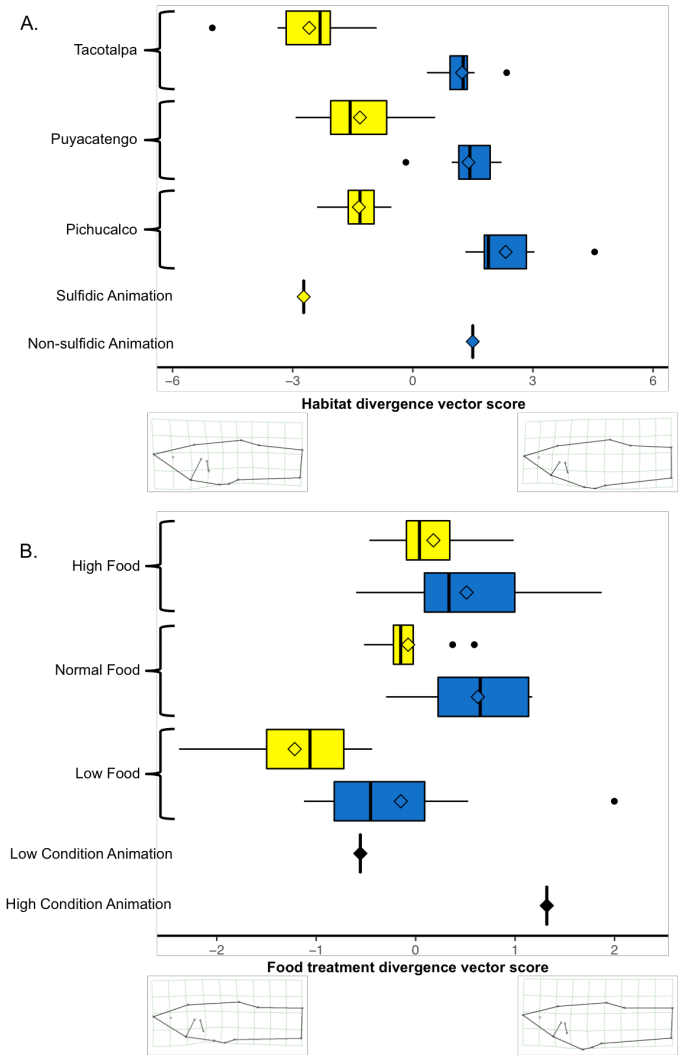

Figure 1.1 Morphological variation in male *Poecilia mexicana*

Visualization of morphological variation in male *Poecilia*. (A) Shared body shape differences between wild-caught specimens from sulfidic (yellow) and non-sulfidic (blue) populations from different river drainages, as well as the *post hoc* divergence vector scores for the animations used to test association preferences. Transformation grids depict the shared body shape divergence between sulfidic populations (negative scores) and non-sulfidic populations (positive scores), each relative to the overall average body shape (divergence vector score = 0). (B) Body shape differences among laboratory-raised specimens subjected to different food treatments for males from a sulfidic (yellow) and a non-sulfidic (blue) population, as well as the *post hoc* divergence vector scores for the animations used to test association preferences. Transformation grids depict the shared body shape differences between low food (negative scores) and high food (positive scores) males, each relative to the overall average body shape (divergence vector score = 0). Boxes cover the first through third quartile of the data; vertical black lines indicate the median. Closed dots indicate outliers, and open diamonds indicate the mean for each group.

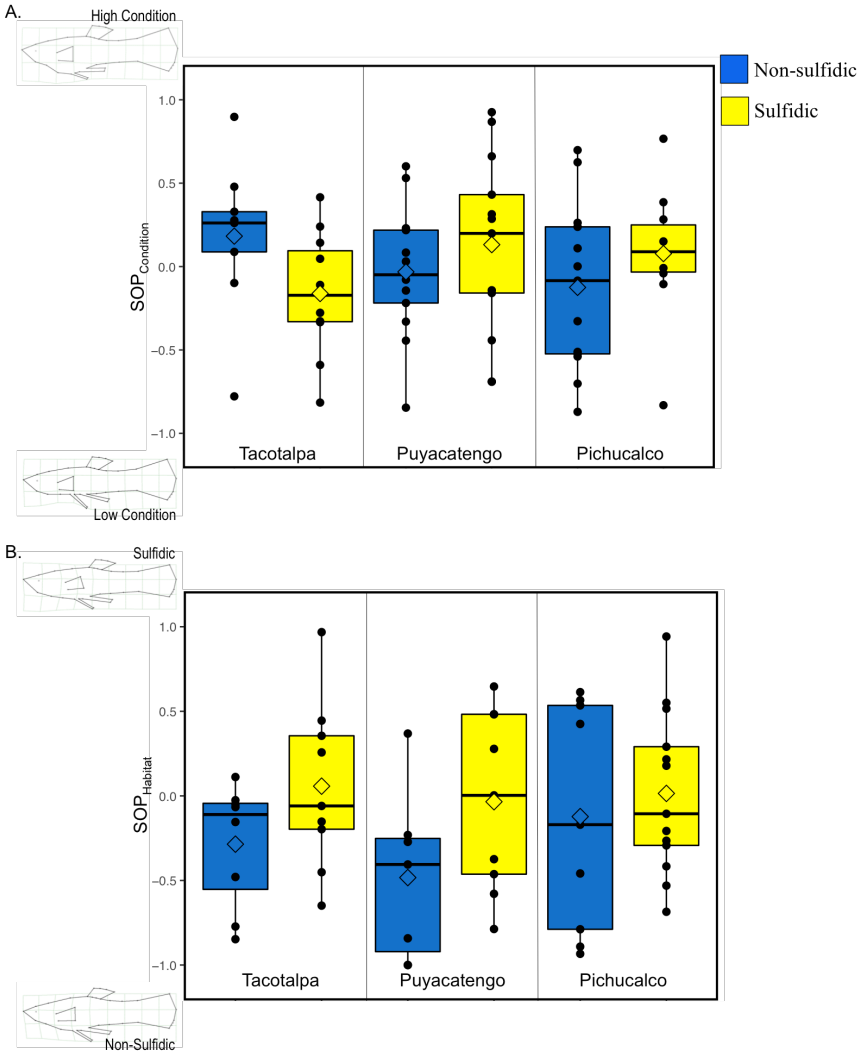

Figure 1.2 Female strength of preference for male animations

Box plots of female *Poecilia mexicana* strength of preference (SOP) for animated stimulus males. Focal fish could choose between two stimulus fish animations: (A) high condition or low condition males, where positive SOP indicate preference for high condition males, (B) head shapes representative of sulfidic and non-sulfidic morphologies, where positive SOP indicate preference for animations with sulfidic shape heads. Boxes cover the first through third quartile of the data; horizontal black lines indicate the median. Closed dots indicate a single female's preference, whereas open diamonds indicate the mean for each group.

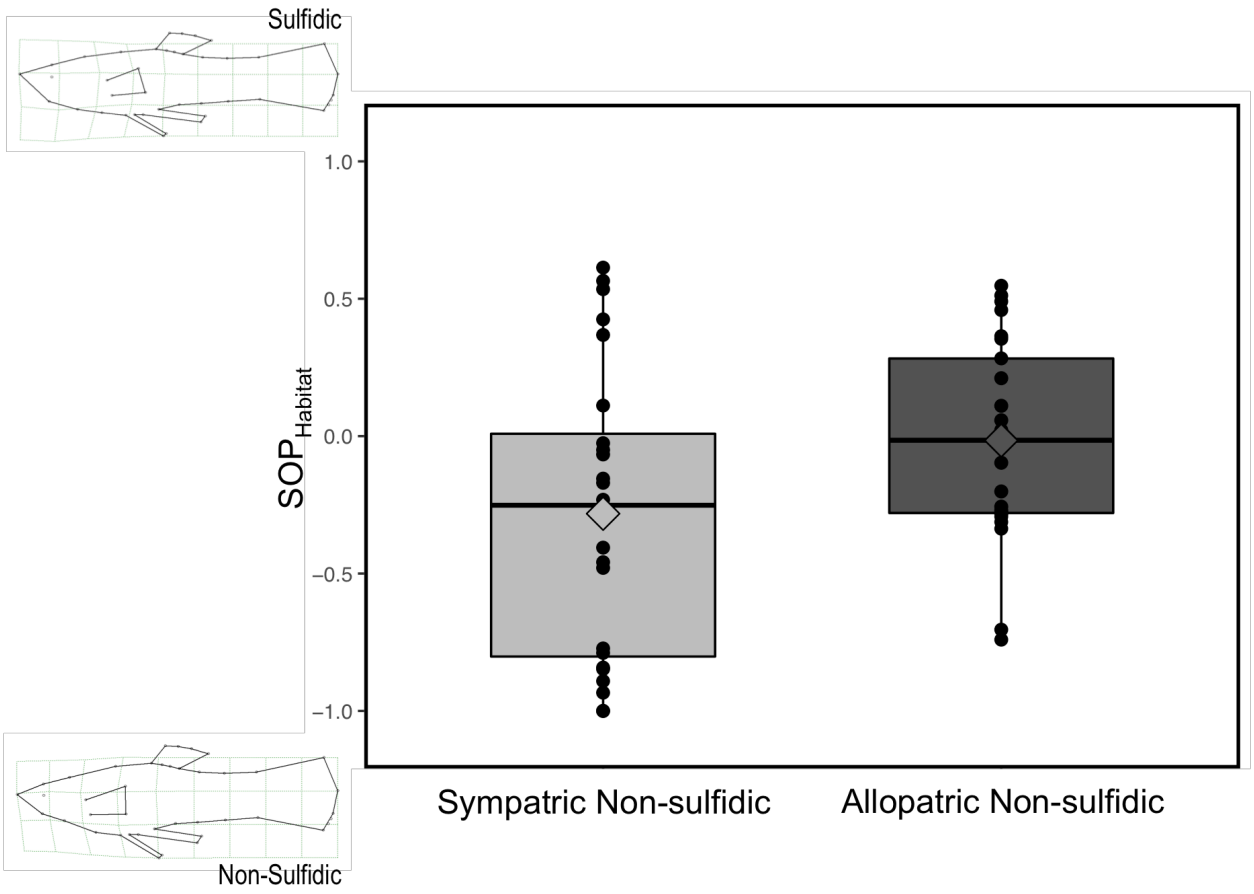

Figure 1.3 Strength of preference in sympatry and allopatry

Strength of preference (SOP) data from non-sulfidic *Poecilia mexicana* females collected from populations either sympatric or allopatric with sulfidic *P. mexicana* populations when presented animations with head shapes representative of sulfidic or non-sulfidic morphologies. Positive SOP indicates preference for animations with sulfidic type heads. Boxes cover the first through third quartile of the data; horizontal black lines indicate the median. Closed dots indicate one individual's preference, whereas open diamonds indicate the mean for each group.

Tables

Table 1.1 MANCOVA for male body shape

Results of multivariate analyses of covariance examining body shape variation in male *Poecilia mexicana* from sulfidic and non-sulfidic habitats, as well as those subjected to different food treatments.

Effect	Hypothesis *df*	Error *df*	*F*	*P*	η_p^2
Wild-caught specimens					
Centroid size	10	44	11.099	<0.001	0.716
Drainage	20	88	9.553	<0.001	0.685
Ecotype	10	44	28.750	<0.001	0.867
Drainage × Ecotype	20	88	7.091	<0.001	0.617
Laboratory experiment					
Centroid Size	13	38	7.606	<0.001	0.722
Food treatment	26	76	2.486	0.001	0.460
Ecotype	13	38	5.798	<0.001	0.665

Table 1.2 ANCOVA for female association preferences

Comparisons of the strength of preference (SOP) for different animation pairs in females across drainages and habitats. Significant effects are highlighted in bold typeface.

Effect	*df*	*Mean Square*	*F*	*P*	$\eta_p 2$
Condition-dependence ($SOP_{Condition}$)					
SL (*z*-transformed)	**1**	**1.103**	**5.595**	**0.021**	**0.080**
Drainage	2	0.046	0.234	0.792	0.007
Ecotype	1	0.015	0.075	0.785	0.001
Magic trait ($SOP_{Habitat}$)					
SL (*z*-transformed)	1	0.684	2.736	0.104	0.052
Drainage	2	0.176	0.704	0.500	0.027
Ecotype	**1**	**1.157**	**4.626**	**0.036**	**0.085**
Reinforcement ($SOP_{Habitat}$)					
SL (*z*-transformed)	1	0.023	0.117	0.734	0.003
Geographic Context	**1**	**0.942**	**4.718**	**0.035**	**0.099**
Population (Geographic Context)	3	0.329	1.647	0.193	0.103

Chapter 2 - Correlated divergence of female and male genitalia in replicated lineages with ongoing ecological speciation*

Ryan Greenway, Rachel McNemee, Alexander Okamoto, Martin Plath, Lenin Arias-Rodriguez, and Michael Tobler

Abstract

Divergence of genital traits among lineages has the potential to serve as a reproductive isolating barrier when copulation, insemination, or fertilization are inhibited by incompatibilities between female and male genitalia. Despite widespread evidence for genital trait diversity among closely related lineages and coevolution of female and male genitalia within lineages, few studies have investigated genital evolution during the early stages of speciation. We quantified genital variation in replicated population pairs of *Poecilia mexicana* with ongoing ecological speciation between sulfidic (H_2S-containing) and nearby non-sulfidic habitats. These analyses revealed rapid and correlated divergence of female and male genitalia across evolutionarily independent population pairs exposed to divergent selection regimes. Both sexes exhibited convergent evolution of genital traits among populations inhabiting similar habitat types. Our results demonstrate that genital evolution can occur during the early stages of speciation-with-gene-flow, potentially as a result of variation in the intensity of sexual conflict among populations. Our results suggest genitalia may contribute to early stages of divergence, and challenge the generality of previously suggested mechanisms of genital evolution in poeciliids.

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Introduction

The evolution of reproductive isolation between lineages early in the speciation process can be facilitated by the divergence of traits directly associated with reproduction (Coyne and Orr 2004; Hoskin and Higgie 2010), including divergence and incompatibilities of gametes and gamete recognition proteins (Van Doorn et al. 2001; Howard et al. 2009; Svensson et al. 2017), sexual behaviors (Fricke and Arnqvist 2004; Rodriguez and Cocroft 2006; Iliadi et al. 2009; Uy et al. 2009; Rudh et al. 2011), and morphological structures involved in mate recognition and breeding (Bergsten et al. 2001; Masly 2012; Perry and Rowe 2012; Barnard et al. 2017). Divergence of genital traits has been hypothesized to serve as an effective reproductive isolating barrier when successful copulation, insemination, or fertilization are inhibited or prevented due to incompatibilities between the genitalia of males and females from different lineages (Masly 2012; Brennan and Prum 2015; Langerhans et al. 2016). Even though genitalia are among the most rapidly evolving morphological traits (Eberhard 1985), with concomitant implications for the evolution of reproductive isolation (Shapiro and Porter 1989; Masly 2012), the timing and role of mechanical isolation via genital divergence as a barrier to gene flow during speciation remains unclear (Langerhans et al. 2016; Yassin 2016). Most studies quantify macroevolutionary patterns of genital variation across divergent species (McPeek et al. 2009; Arbuthnott et al. 2010; Kubota et al. 2013), though some have investigated intraspecific variation (Evans et al. 2011; Wojcieszek and Simmons 2012; Soto et al. 2013; Heinen‐Kay et al. 2014). Few studies have explicitly investigated patterns of genital evolution during early stages of the speciation process (Heinen‐Kay and Langerhans 2013; Anderson and Langerhans 2015; Barnard et al. 2017). As a result, the question remains whether genital divergence can contribute to reproductive isolation early in the speciation process, or if genital divergence occurs only at later stages of speciation,

after the accumulation of other reproductive isolating barriers (e.g., as a consequence of reinforcement upon secondary contact).

The generation of mechanical incompatibilities requires genital divergence among lineages in only one of the sexes (Masly 2012). However, the correlated evolution of female and male genitalia within lineages, combined with divergence among lineages, is especially effective for strengthening reproductive isolation (Masly 2012; Brennan and Prum 2015; Langerhans et al. 2016; Yassin 2016). Enhanced reproductive isolation as a result of genital divergence between lineages coinciding with genital coevolution within lineages can result from several (not mutually exclusive) processes. The lock-and-key hypothesis assumes reinforcement mechanisms (Masly 2012; Langerhans et al. 2016), predicting that natural selection should favor the evolution of genital incompatibilities between lineages and the evolution of complementary female (lock) and male (key) genitalia within lineages when inter-lineage matings have negative fitness consequences (e.g., hybrid inferiority, physical damage; Dufour 1844; Masly 2012; Langerhans et al. 2016). Alternatively, postcopulatory sexual selection via cryptic female choice or sexual conflict—both of which may be modulated by ecological factors—can drive genital coevolution within lineages, which can incidentally lead to genital divergence between lineages (Martin and Hosken 2003; Arnqvist and Rowe 2005; Masly 2012; Evans et al. 2013; Langerhans et al. 2016). Regardless of the mechanism of sexual selection driving genital divergence, reproductive isolation may then arise as a by-product if genital incompatibilities among lineages decrease fertilization success.

Male fishes of the family Poeciliidae possess a modified anal fin—the gonopodium—used as a copulatory organ to transfer sperm to the female genital tract (Rosen 1953; Greven 2011). Substantial variation in gonopodial morphology is well documented among poeciliid

genera and species (Rosen and Tucker 1961; Chambers 1987), and gonopodial traits are frequently used as diagnostic characters for delineating species (e.g., Rosen and Bailey 1963). Comparatively little is known about structural variation of female genitalia in poeciliids, although the position and size of female genital traits have been shown to vary among species (in *Gambusia*; Peden 1972a) and with reproductive status (Parzefall 1973). The morphologies of the female urogenital aperture and the complex tip of the male gonopodium are hypothesized to influence insemination success, as copulation consists of males contacting the female urogenital aperture with the tip of the gonopodium (Rosen 1953; Peden 1972b). Consequently, coevolution of female and male genital traits, as documented across populations (Evans et al. 2011; Evans et al. 2013; Anderson and Langerhans 2015) and among species (Peden 1972a; Langerhans 2011), has long prompted speculation that genital trait variation might enhance reproductive isolation between lineages of poeciliid fishes via genital incompatibilities (Langerhans 2011; Anderson and Langerhans 2015). Nonetheless, only one study (Anderson and Langerhans 2015) tested for the correlated evolution of female and male genitalia during early stages of speciation.

Poeciliid fishes in the *Poecilia mexicana* species complex offer an opportunity to study the patterns of genital evolution during the early stages of ecological speciation. Populations of *P. mexicana* have independently colonized toxic, hydrogen sulfide (H_2S)-rich springs in multiple tributaries of the Río Grijalva in southern Mexico (Greenway et al. 2014). Sulfide spring fishes are locally adapted and differ from ancestral populations in adjacent non-sulfidic habitats in physiological, morphological, behavioral, and life-history traits (Tobler et al. 2018). Trait divergence includes changes in sexual behaviors (less coercive mating attempts in sulfidic populations; Plath et al. 2003; Plath 2008) as well as aggression and boldness (both reduced in sulfidic populations; Riesch et al. 2009; Bierbach et al. 2017), which could influence genital

evolution. Populations in sulfide springs are also genetically differentiated from neighboring populations in non-sulfidic habitats despite a lack of physical barriers (Plath et al. 2013). Reproductive isolation between populations in different habitat types is in part facilitated by natural selection against migrants, as reciprocal translocation experiments revealed strong selection against migrants from non-sulfidic habitats into sulfide springs, as well as varying levels of selection against sulfidic individuals moving into non-sulfidic habitats (Plath et al. 2013). Additionally, mate choice experiments have revealed significant association preferences for individuals of the same ecotype in non-sulfidic females from populations adjacent to sulfide springs (Plath et al. 2013), which are linked to adaptive differences in body shape that serve as cues (Greenway et al. 2016). Importantly, neither sulfidic females nor females from non-sulfidic populations in river drainages lacking sulfide spring populations exhibit significant association preferences, suggesting that reinforcement (*i.e.* direct selection for assortative mating) may have shaped female association preferences (Greenway et al. 2016). However, the observed strengths of natural selection against immigrants and assortative mating preferences alone are not strong enough to explain the low observed levels of gene flow (Plath et al. 2013), indicating that other reproductive isolating barriers, such as genital incompatibilities, likely contribute to the strong reproductive isolation observed between populations (Bierbach et al. 2017).

We used this system with replicated and ongoing ecological speciation events to test whether genitals diverge during the early stages of speciation, potentially functioning as a barrier to gene flow. Environmental differences between sulfidic and non-sulfidic habitats are complex but highly replicable across river drainages (Tobler et al. 2018), and environmental similarity (e.g., in relation to the social structure, predation risk, competition, and resource availability) might drive genital trait evolution in a repeatable manner among different sulfide spring

populations. If genital evolution plays a role in reproductive isolation in this system, we would minimally predict consistent divergence in female and/or male genital traits between populations that are adapting to different environments. Consequently, we quantified shape variation in both female and male genitalia within and between populations of *P. mexicana* in sulfidic and non-sulfidic habitats to test (1) whether female and male genital morphology diverged between populations, (2) whether female and male genital morphologies coevolve, (3) whether aspects of genital morphology exhibit patterns of convergent evolution across population pairs exposed to similar environmental gradients, and (4) what mechanisms potentially explain genital divergence among population pairs.

Methods

We included four populations pairs of the *P. mexicana* species complex inhabiting sulfidic and non-sulfidic habitats in major tributaries of the Río Grijalva: the Ríos Tacotalpa, Puyacatengo, Ixtapangajoya, and Pichucalco (Table B1; see Palacios et al. (2013) for detailed descriptions of focal populations). Adult fish (N = 391; 187 females, 204 males) were collected from focal streams using seines, euthanized with buffered MS222 immediately after capture, and fixed in a 10% formaldehyde solution. Additionally, we investigated genital morphology in laboratory-reared adult fish from colonies that were originally seeded from the population pair in the Tacotalpa river drainage (N = 93; 39 females, 54 males). We measured each specimen's body mass to the closest 0.01 g, and standard length (SL) to the nearest 1 mm.

Quantifying variation in female and male genitalia

Photography and measurement of female genital traits closely followed procedures previously used for poeciliid fishes (Anderson and Langerhans 2015). We photographed female genitalia and size standards using a Nikon SMZ1000 stereomicroscope (Nikon Instruments Inc., Melville, NY, USA) equipped with a Canon EOS Rebel T5i digital camera (Canon USA Inc., Lake Success, NY, USA). For each specimen, 4-8 photographs were taken across a range of focal depths of the ventral side of the urogenital aperture (20-40-fold magnification depending on specimen size). Images were stacked into a single composite image for each specimen using the Helicon Focus software package (http://www.heliconsoft.com/). We then measured five genital traits using ImageJ version 1.50 (https://imagej.nih.gov/ij/): urogenital aperture area, apertural opening area, apertural papilla area, apertural opening width, and apertural opening length (see Figure 2.1; (Anderson and Langerhans 2015)). Three additional traits were calculated from these measurements: proportional apertural opening area (apertural opening area/urogenital apertural area), apertural elongateness (apertural opening width/length), and apertural opening aspect ratio (apertural opening width^2/apertural opening area) (Anderson and Langerhans 2015). We calculated the relative size of each genital variable by dividing the raw measurement by SL and arcsine (square-root)-transformed relative trait values to assure normal residual distributions in subsequent analyses. To reduce data dimensionality, we subjected the eight metrics of female genital morphology to a principle component analysis based on a covariance matrix as implemented in the R package stats (R Core Team 2017). For wild-caught fish, we retained three principal axes with eigenvalues greater than the mean eigenvalue and explaining >95.0 % of the total variance for further analysis (Table B2; Denis 2015).

To quantify male genital morphology, we isolated gonopodia by cutting through the gonopodial base (where the anal fin rays meet the body). We stained isolated gonopodia using a solution of 1 mg Alizarin Red dye per 100 mL of 1% KOH solution for six hours. Gonopodia were rinsed overnight in 1% KOH solution before storage in 70% isopropyl solution. Stained gonopodia and size standards were then photographed with the same stereomicroscope used for female genitalia. The left lateral side of each gonopodium was photographed 3-7 times across a range of focal depths (at 40-60-fold magnification depending on specimen size), and individual photographs were stacked into a single composite image as described before. Using the program tpsDig (Rohlf 2004), we digitized 29 morphological landmarks characterizing the shape of gonopodial distal tip elements expected to closely interact with the female urogenital aperture (Figures 2.1 & B1, Table B3) using geometric morphometric techniques (Zelditch et al. 2012; Heinen‐Kay and Langerhans 2013). Geometric morphometric techniques were used to quantify male genital variation because gonopodia consist of bony elements that facilitate the identification of homologous landmarks necessary for geometric morphometric analysis (Zelditch et al. 2012), as opposed to the soft tissue of female genital traits. Landmark coordinates were aligned by least-squares superimposition to remove the effects of translation, rotation, and scale using the program tpsRelw (Rohlf 2007). We calculated relative warp scores plus uniform components (weight matrix) for each gonopodium based on the aligned coordinates. To reduce data dimensionality, we subjected the weight matrix to a principle component analysis based on a covariance matrix and retained 11 principal axes with an above-average eigenvalue and explaining > 95.2% of the total variance as shape variables for further analysis of wild-caught specimens (Denis 2015). All data for these analyses can be found in the Supplemental Information.

To investigate whether genital variation in *P. mexicana* potentially has a genetic basis, we compared genital morphologies in individuals from sulfidic and non-sulfidic populations of the Tacotalpa river drainage (El Azufre I and Arroyo Bonita) that were born in the laboratory to captive born parents and reared under standardized non-sulfidic environmental conditions. Laboratory colonies consisted of large, outbred populations, initially stocked with individuals from multiple families of either population. Methods employed exactly matched those described for females and males above. For females, we retained three principal axis with eigenvalues greater than the mean eigenvalue and explaining >99.5% of the total variance. For males, we retained eleven principal axes with eigenvalues greater than the mean eigenvalue and explaining >96.1% of the total variance.

Data analysis

All statistical analyses were performed in R version 3.4.1 (R Core Team 2017). To test for repeatable shifts in genital morphology along ecological gradients in wild-caught specimens, we conducted separate multivariate analyses of covariance (MANCOVA) for females and males, using PC scores from axes with eigenvalues greater than the mean eigenvalue as dependent variables (3 axes for females; 11 axes for males) with the lm-function from the "stats" package (R Core Team 2017). We included "habitat type" (sulfidic/non-sulfidic) and "drainage" (*i.e.*, the specific river system where each population pair occurs) as factors, body size ($\log_{10}$-transformed body mass) as a covariate, as well as interactions among these terms. We excluded interaction terms with $P > 0.05$ from the final model. *F*-values were approximated using Wilks' lambda and effect sizes by use of partial eta squared (η_p^2). To examine the extent of shared (i.e., convergent) aspects of genital shape variation associated with sulfidic and non-sulfidic habitats, while

accounting for all other effects in the model, we calculated a divergence vector score (Langerhans 2009) for each individual based on the first principle component of the among-group covariance matrix for the habitat term in the MANCOVA (Klingenberg and Spence 1993). Divergence vector scores essentially summarize the linear combination of shape variables that contribute to the greatest difference in genital shape for a given term after controlling for other effects in the model without distorting morphological space (Langerhans 2009).

To analyze patterns of genital variation in laboratory-reared populations from the Tacotalpa river drainage, we used a similar analytical approach as described for wild-caught specimens above. However, we only included "habitat type" (sulfidic/non-sulfidic) as a factor and body size ($\log_{10}$-transformed body mass) as a covariate for both females and males; interactions among these terms were non-significant and excluded from the final models.

Most models of genital evolution (lock-and-key, sexual selection, and sexual conflict) predict correlated evolution between female and male genitalia (Masly 2012; Anderson and Langerhans 2015; Brennan and Prum 2015). We used a model selection approach to test whether among population variation in female urogenital aperture morphology was correlated with male gonopodial tip shape for wild-caught specimens. This approach allowed us to identify specific aspects of male morphology associated with variation in female genital morphology from a large number of potential models. We extracted the estimated marginal means (EMMs) for the "habitat × drainage" term for each of the female and male genital shape variables in the MANCOVA models described above to control for the effect of body size, yielding population-specific mean values for each trait. For each female trait axis, we tested for correlations with male genital traits using general linear models, as implemented in the R package glmulti (Calcagno and de Mazancourt 2010). The population-specific EMMs for the female genital variable of interest

served as a response variable, and the population-specific EMMs for the 11 gonopodial shape variables as independent variables. Models were evaluated based on Akaike Information Criteria with finite sample correction (AIC_C) (Johnson and Omland 2004), and we present alternative models with $\Delta AIC_C < 2$ (Burnham and Anderson 2003).

Correlated evolution between female and male genital traits was also explored using partial Mantel tests, which allowed us to control for potentially confounding effects of population genetic relationships among populations (Anderson and Langerhans 2015). To do so, we used previously published data of 17 nuclear microsatellites (Palacios et al. 2013; Plath et al. 2013) and calculated pairwise F_{ST}-values among all populations using GenAlEx v. 6.503 (Peakall and Smouse 2012). For females and males, divergence in genital traits was calculated as the Euclidian distance between population-specific EMMs derived from the MANCOVA models, as described above. Genital trait distance metrics were calculated in two ways. (1) Following Anderson and Langerhans (Anderson and Langerhans 2015), we only included traits that showed significant evidence for coevolution in the model selection approach; hence, pairwise population divergence in genital traits was calculated based on the EMMs of the second and third PC axes for females, and the second and ninth PC axes for males (see Results). (2) Pairwise population divergence in genital traits was calculated based on the EMMs of all PC axes for both females (three axes) and males (11 axes). Partial Mantel tests were then used to test for a correlation between female and male trait divergence, while accounting for population genetic differentiation based on F_{ST}. All tests were run with 9999 randomizations, as implemented in R's vegan package (Oksanen et al. 2013).

Finally, the lock-and-key model of genital evolution predicts that reproductive character displacement among populations subject to divergent selection should be more pronounced when

there is a high probability of interpopulation matings (Anderson and Langerhans 2015), leading to a negative correlation between metrics of pairwise population connectivity and trait divergence. Consequently, we used partial Mantel tests to explore correlations between population divergence in genital traits and F_{ST}. We accounted for habitat type for each pairwise comparison (i.e., same habitat type or different habitat type), because reinforcement driving lock-and-key is primarily expected between populations subject to divergent selection but not those occurring in the same habitat. Partial Mantel tests were conducted separately for males and females.

Results

Female genital morphology

Female genitalia exhibited variation in apertural shape (elongateness and aspect ratio) along PC1, apertural size (opening width and proportional opening area) along PC2, and additional variation in papilla area and proportional opening area along PC3 (see Table B2). MANCOVA revealed significant effects of habitat type, body size, and drainage, as well as interactions among these terms (Table 2.1). Effect sizes revealed that female genital morphology primarily varied among habitat types. The significant habitat term indicates convergent evolution of female genitalia across replicated population pairs, with females from populations in sulfidic habitats exhibiting similar patterns of divergence from proximate populations in non-sulfidic habitats (Figure 2.2A, B). Eigenvector coefficients of the female habitat divergence vector also revealed that variation along PC2 and PC3 strongly correlated with differences in female genitalia among populations in different habitat types (Table B4). Inspection of PC axes revealed consistent divergence primarily along PC2, and females from sulfidic habitats showed larger apertural

openings than females from non-sulfidic habitats (Figure 2.2A; Table B2). Besides the habitat term, body size exhibited the highest effect size, indicating pronounced allometric effects. Furthermore, substantive effect sizes of drainage and the drainage × habitat interaction indicate some drainage- and population-specific variation in female genitalia.

Male genital morphology

Analysis of male genital morphology indicated significant effects of body size, habitat, and drainage, as well as the interaction between habitat and drainage (Table 2.1). As observed for female genitalia, habitat type exhibited the largest effect size, indicating shared aspects of divergence in gonopodial morphology among populations inhabiting similar habitats across river drainages (Figure 2.2C, D). Eigenvector coefficients of the male habitat divergence vector indicated a strong influence of variation along PC2, PC3, and PC4 on male genital divergence associated with the different habitat types (Table B5). Inspection of PC axes and thin-plate spline transformation grids visualizing shape differences along the habitat divergence vector revealed that—relative to males from non-sulfidic habitats—males from sulfidic habitats exhibit more elongate and narrower distal tips with reduced hook and serrae angles (Figure 2.2C, D). Additionally, the interaction between habitat and drainage exhibited a modest effect size (Table 2.1), revealing population-specific aspects of gonopodial tip shape. Body size also served as an important predictor of gonopodial morphology based on estimates of effect size, suggesting allometric effects of gonopodial traits.

Genital morphology in laboratory-reared populations

We found that genital divergence for both females and males was maintained in laboratory-reared populations. MANCOVA indicated significant effects of body size ($F_{3,34}$ = 10.022, P < 0.001, η_p^2 = 0.469) as well as a comparatively weak – albeit significant – effect of habitat ($F_{3,34}$ = 3.015, P = 0.043, η_p^2 = 0.210) on female genital variation (Figure B2A). For males, MANCOVA indicated highly significant differences in gonopodium shape between laboratory-reared individuals from different habitat types ($F_{11,41}$ = 6.535, P < 0.001, η_p^2 = 0.637; Figure B2B), as well as significant effects of body size ($F_{11,41}$ = 2.342, P = 0.024, η_p^2 = 0.386).

Correlation between female and male genital morphologies

Model selection revealed significant among-population correlations between female and male genital morphologies in wild-caught fish. For all female genital variables, the top models uncovered a relationship with at least one axis of gonopodial distal-tip shape (Table 2.2). *Post-hoc* analyses indicated significant correlations with male genital shape variables for two of the female genital variables (female PC2 and male PC2; female PC3 and male PC9; Table 2.2), and a marginally non-significant trend for the remaining female variable (female PC1 and male PC1; Table 2.2). Of particular interest is the correlation of female PC2 and male PC2, as both females and males exhibited consistent divergence among habitat types along these axes.

Partial Mantel tests using distance metrics based on traits identified during model selection corroborated correlated evolution between female and male genitalia when controlling for population genetic relationships (r = 0.511, P = 0.024). However, this correlation disappeared when all aspects of genital trait variation were included in the analysis (r = 0.167, P = 0.243). This finding indicates that only specific elements of genital variation exhibit signatures of

coevolution between females and males, while other aspects of genital morphology vary in ways unrelated to the other sex (*i.e.*, the signal of coevolution is lost in analyses of all traits due to noise introduced from non-coevolving traits).

Population genetic divergence and genital evolution

Contrary to the predictions of the lock-and-key hypothesis as posited by Anderson and Langerhans (2015), partial Mantel tests did not indicate a negative relationship between population genetic divergence and divergence in genital traits. When conducted on distance metrics based on traits identified during model selection, we in fact found a positive correlation between F_{ST} and genital divergence in males (r = 0.429, P = 0.046), while there was no correlation in females (r = 0.392, P = 0.102). When conducted on all axes of genital trait variation, we detected a significant positive correlation for females (r = 0.459, P = 0.032) and a marginally non-significant correlation for males (r = 0.409, P = 0.063).

Discussion

Our study found evidence for rapid and correlated divergence of female and male genital morphologies across evolutionarily independent *Poecilia mexicana* population pairs exposed to divergent selection. The genitalia of both sexes exhibited convergent evolution along replicated environmental gradients, and differences in genital morphologies were maintained in laboratory-raised males and females from one population pair. Overall, our results add to a growing body of work suggesting that divergence in genital traits has the potential to contribute to reproductive isolation at early stages of speciation (Langerhans 2011; Anderson and Langerhans 2015). Assessing the importance of mechanical isolation during ecological speciation in the future

requires an extension of the pattern-based approaches employed to date, explicitly identifying the mechanisms driving genital evolution and exploring the functional significance of variation in genital traits for insemination and fertilization in both intra- and interpopulation matings.

Genital variation in sulfide spring fishes

The immediate sources of selection contributing to genital divergence remain unclear, because sulfidic and non-sulfidic habitats differ in a variety of relevant variables, including predation risk, resource availability, and social environment (Tobler and Plath 2011). Female *P. mexicana* in sulfidic habitats repeatedly evolved larger apertural openings and males evolved more elongate gonopodial tips with less pronounced hooks and serrae (Figure 2.2). The direction of these population differences resembles patterns observed in other poeciliids exposed to different predation risk (Jennions and Kelly 2002; Langerhans et al. 2005; Evans et al. 2011; Heinen‐Kay and Langerhans 2013; Heinen‐Kay et al. 2014; Anderson and Langerhans 2015). Ecological sources of selection—like predation—can directly impact genital traits, especially when there are functional trade-offs that affect other aspects of organismal performance or fitness (Langerhans et al. 2005). In sulfide spring fishes, selection might act directly on female genitalia as a result of life history shifts. Offspring size has repeatedly diverged between sulfidic and non-sulfidic populations of poeciliid fishes (Riesch et al. 2014), potentially driving the evolution of larger apertural openings to facilitate delivery of larger offspring in sulfidic populations. Likewise, male genitalia in sulfidic habitats may be under selection to avoid exposure of sperm to H_2S toxicity, driving the evolution of a more elongate and narrow gonopodium to facilitate rapid sperm transfer further into the female reproductive tract (van Lieshout and Elgar 2011).

While ecological selection can directly drive genital evolution, the effects of divergent ecological conditions might instead be indirect by altering the context of sexual selection. In other species, differences in male mating tactics between high-predation (higher frequency of rapid, forced copulations) and low predation populations (higher frequency of longer copulations and higher degrees of female cooperation) have been associated with variation in gonopodium length, distal tip morphology, and female genital size (Evans et al. 2011; Heinen‐Kay and Langerhans 2013). Populations in sulfidic and non-sulfidic environments differ in aspects of their social environments, including population densities (higher in sulfide populations), sex ratios (more even in sulfide populations), and male mating behaviors (less coercive mating in sulfide populations; Tobler and Plath 2011). The repeated patterns of genital trait divergence suggest that shared aspects of altered selective landscapes in sulfidic habitats drive genital evolution. Alternatively, we cannot entirely rule out that population differences in genitalia are the result of phenotypic plasticity, as exposure to differences in environmental conditions can shape the expression of morphological traits in fishes (e.g., Chapman et al. 2000; Johnson 2001; Wund et al. 2008). However, several studies have documented a heritable genetic basis to both female and male genital morphology in poeciliids (Evans et al. 2013; Heinen‐Kay and Langerhans 2013; Booksmythe et al. 2016), and differences in genital morphologies are maintained in laboratory populations of at least one population pair for both males and females (Figure B2).

Elucidating the drivers of genital evolution

The ecological hypothesis of genital evolution (Langerhans et al. 2016) posits that variation in abiotic or biotic environmental conditions can alter the context of sexual selection, which in turn

exerts selection on genitalia. While this hypothesis has spurred research documenting patterns of female and male genital variation among populations, it is far less understood how changes in the context of sexual selection affect variation in genitalia. Ultimately, variation in ecology can impact the social context of reproduction by creating opportunities to mate with maladapted migrants from different habitats (lock-and-key hypothesis; Masly 2012; Langerhans et al. 2016), changing opportunities for cryptic female choice and sperm competition (postcopulatory sexual selection hypothesis; Smith 1984; Eberhard 1996), or changing opportunities for sexually antagonistic selection (sexual conflict hypothesis; Hosken and Stockley 2004). All three mechanistic hypotheses are non-mutually exclusive with the ecological hypothesis, and they all predict correlated genital evolution between the sexes, which appears to be widespread in poeciliids and other taxa (Langerhans 2011; Evans et al. 2013; Anderson and Langerhans 2015; Wang et al. 2015). A consensus on the importance of specific mechanisms underlying genital diversification in poeciliids has been prevented by limited evidence for and against each hypothesis.

Previous studies on poeciliids provided some evidence of lock-and-key mechanisms. Evidence for lock-and-key mechanisms was primarily inferred from the presence of reproductive character displacement in sympatric *vs*. allopatric populations (Langerhans 2011), as well as correlations between interpopulation mating opportunities and genital divergence (Anderson and Langerhans 2015). However, emulating an analytical approach by Anderson and Langerhans (2015), we found no evidence for lock-and-key and detected positive—not the predicted negative—correlations between population genetic and genital divergence in most analyses, which could be explained if morphology and genetic similarity both diverge due to environmental sources of selection. Considering that previous studies only recovered weak

correlations (one-tailed tests) and failed to account for variation in the context of sexual selection (habitat type) in pairwise comparisons, support for lock-and-key mechanisms driving population divergence in poeciliid genitalia remains equivocal.

Studies on poeciliid fishes have uncovered evidence for pre-copulatory intersexual selection on male genital traits (Brooks and Caithness 1995; Langerhans et al. 2005; Kahn et al. 2009; Langerhans 2011), cryptic female choice (Pilastro et al. 2004; Gasparini and Pilastro 2011), and sperm competition (Constantz 1984; Gasparini et al. 2010). However, no studies have investigated whether variation in female genitalia might facilitate cryptic female choice or impact sperm competition in a manner that affects the correlated evolution of male genital traits. Evidence for genital coevolution as a result of cryptic female choice has been documented in other taxa, particularly arthropods (Huber et al. 2005; Simmons and Garcia-Gonzalez 2011; Kotrba et al. 2014; Brennan and Prum 2015).

Finally, there is clear evidence for sexual conflict in poeciliid fishes (Magurran and Seghers 1994; Plath et al. 2007a). Kwan et al. (2013) showed that male genital variation impacts fertilization success in the context of intersexual conflict, with distal gonopodial elements increasing male fertilization success during interactions with uncooperative—but not cooperative—females. Ecologically-dependent variation in the degree of sexual conflict (Magurran and Seghers 1994; Plath et al. 2003; Heinen et al. 2013) could therefore drive divergence of male genital traits. No studies have explored how variation in female genital morphology impacts male fertilization success when copulations are coercive *vs*. cooperative, even though it is tempting to speculate that reduced male sexual activity and less coercive mating in sulfide populations (Plath et al. 2003; Plath 2008) underlie larger apertural openings in females. This line of argument would imply that smaller apertural openings in non-sulfidic

habitats allow females to control male mating success (*i.e.*, to exert cryptic female choice) despite strong coercive mating frequencies.

Genital variation and reproductive isolation

Patterns of genital variation in *P. mexicana* indicate that differences among populations undergoing ecological speciation have the potential to impact reproductive isolation. The basic conditions for mechanical isolation (correlated divergence of female and male genitalia) are fulfilled, even though these populations span a range of divergence times (from <500 to ~ 10,000 years; Brown and Kelley, unpublished data), vary in the degree of total reproductive isolation, and differ in the relative importance of different reproductive isolating barriers (Tobler et al. 2018). The consistent divergence of genitalia in all population pairs suggests that these traits evolve rapidly and potentially cause mechanical isolation even at early stages of speciation. The documentation of similar patterns in other poeciliid fishes and arthropods (Evans et al. 2011; Wojcieszek and Simmons 2012; Soto et al. 2013; Heinen‐Kay et al. 2014) provides a solid foundation for studying how genital divergence among lineages impacts speciation processes.

From patterns to mechanisms

Understanding the diversity of poeciliid genitalia and their significance for diversification processes in the future will require the integration of comparative, pattern-based analyses (to understand ecological context) with experimental studies (to understand mechanisms of divergence and functional consequences). Past experiments studying poeciliid reproductive biology have established methods to manipulate genital traits (Rosen 1953; Kwan et al. 2013), quantify insemination success (Pilastro and Bisazza 1999; Riesch et al. 2008), and quantify

fertilization success (Gasparini et al. 2010; Boschetto et al. 2011). Applying these methods to natural systems with among-population variation in genitalia will facilitate a functional perspective of how female and male traits impact sperm-transfer and fertilization.

Similarly, we are missing direct tests of how population divergence in genitalia might actually cause mechanical incompatibilities. Such tests are particularly important because different drivers of genital evolution (lock-and-key, sexual selection, or sexual conflict) potentially lead to different predictions for reproduction isolation among populations, though they might produce similar patterns of genital variation among populations. For example, coevolution of genitalia among the sexes driven by lock-and-key mechanisms should yield a symmetrical increase in reproductive isolation between diverging lineages (Masly 2012; Brennan and Prum 2015). Alternatively, coevolution resulting from sexual conflict predicts that populations should vary in female genital traits that confer resistance to unwanted mating attempts, and persistence traits that help males overcome female resistance (Arnqvist and Rowe 2005). Under this scenario, reproductive barriers may emerge in an asymmetrical fashion, where males from populations with high levels of conflict are effective at inseminating females from populations with low levels of conflict, but not vice versa. Without knowing how female and male traits interact during copulation [e.g. if the female papilla (Figure 1) is a defensive trait against male armaments, or if it is a sensory structure stimulated by male ornaments], our current approaches might lead to inaccurate conclusions about mechanisms that drive genital divergence and the role of genitalia in reproductive isolation.

Others have recently called for mechanistic studies of genitalia in order to understand their functions in pre- and post-copulatory sexual selection (Brennan and Prum 2015; Brennan 2016; Langerhans et al. 2016). We echo these calls, emphasizing that this knowledge is required

for deciphering mechanisms of genital coevolution that produce different outcomes for reproductive isolation. To advance our understanding of the role of genitalia in the speciation process, we highlight that two questions must first be addressed: (1) How do specific female genital traits influence insemination success by enabling cooperation with preferred mates and/or deflecting coercive mating attempts? (2) How do specific male genital traits influence a male's ability to efficiently transfer sperm to the female genital tract? Understanding these questions is essential for subsequently testing how trait variation among lineages influences reproductive isolation.

Conclusions

Our study demonstrated that the genitalia of females and males can rapidly evolve in a correlated fashion during the early stages of ecological speciation, highlighting the potential role of genital evolution as a barrier to gene flow between incipient species. Despite these strong and replicated patterns of divergence and intersexual coevolution among populations, understanding how genital traits actually influence the evolution of reproductive isolation will require identification of the mechanisms driving genital evolution. Though previous studies have made strong claims based solely on pattern-based data, determining which of the several possible mechanisms of genital coevolution are at work is only possible through the use of manipulative experimental approaches that have not yet been attempted.

Figures

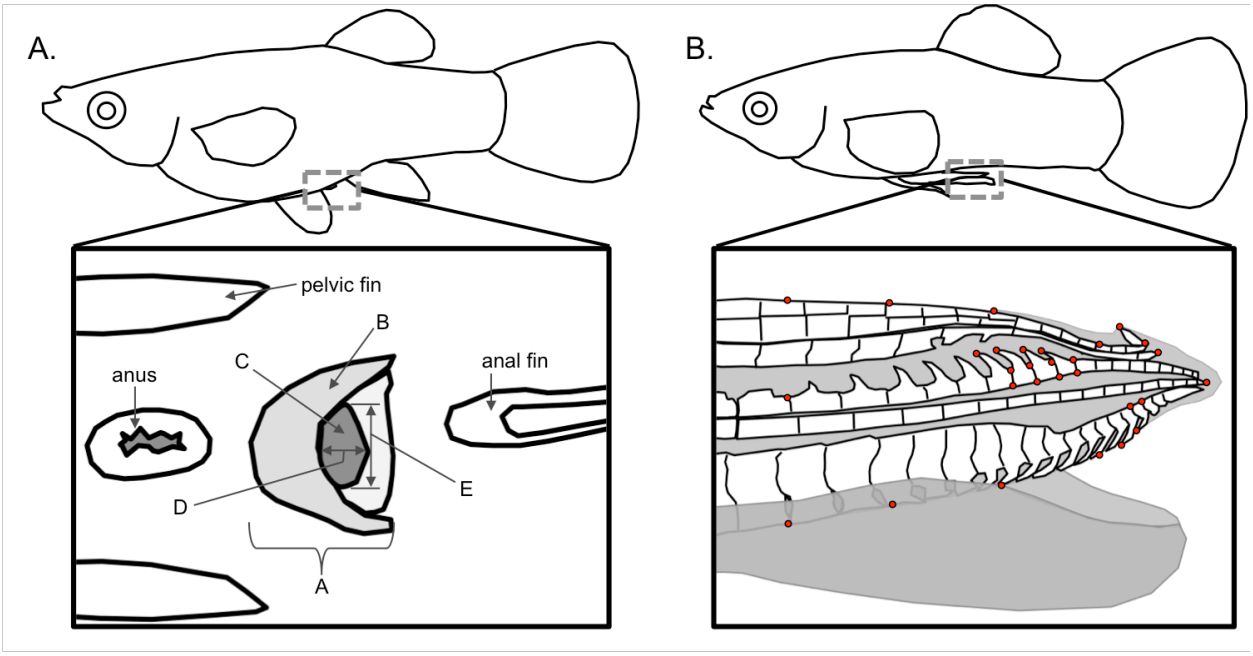

Figure 2.1 Diagrams of *Poecilia mexicana* genital traits

Diagrammatic representations of adult *Poecilia mexicana* and their genitalia. (A) Adult female and ventral view of the urogenital aperture with the five measurements used to calculate the female genital traits analyzed in this study (A – urogenital apertural area; B – apertural papilla area; C – apertural opening area; D – apertural opening length; E – apertural opening width). (B) Adult male and lateral view of the gonopodial distal tip (shaded areas represent soft tissues, light areas represent bony structures), including the 29 homologous landmarks used to measure gonopodial shape (see Table B3 and Figure B1 for detailed descriptions).

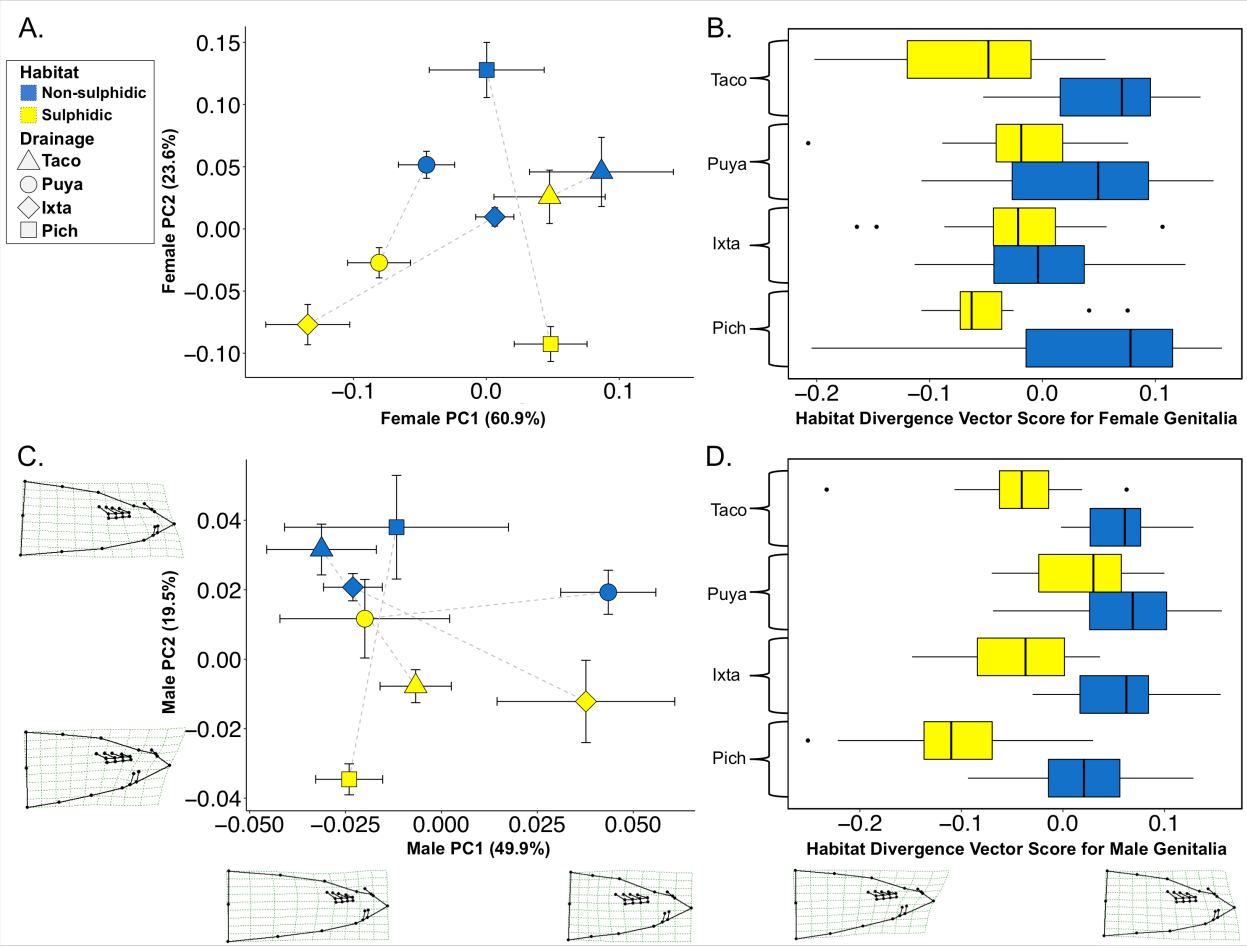

Figure 2.2 Variation in genital morphology of *Poecilia mexicana* populations

Visualization of genital morphological variation in *Poecilia mexicana*. (A) Estimated marginal means (± SE; derived from the Habitat × Drainage term in MANCOVA) of female genital shape variation along the first two principal components. (B) Female genital trait divergence between sulfidic (yellow) and non-sulfidic (blue) populations from different river drainages, relative to the overall average female genital morphology (divergence vector score = 0). (C) Estimated marginal means (± SE; derived from the Habitat × Drainage term in MANCOVA) of male gonopodial distal tip shape variation along the first two principal components. Thin-plate spline transformation grids represent shape variation along each principal component axis. (D) Male gonopodial shape divergence between sulfidic (yellow) and non-sulfidic (blue) populations from different river drainages. Transformation grids depict shape variation along the habitat divergence vector relative to the overall average gonopodium shape (divergence vector score = 0). For PC plots (A, C), dashed lines connect population pairs in the same river drainage. For divergence vector plots (B, D), boxes cover the 25th through 75th percentile of the data; vertical black lines indicate the median and dots indicate outliers. Population abbreviations: Taco = Tacotalpa; Puya = Puyacatengo; Ixta = Ixtapangajoya; Pich = Pichucalco.

Tables

Table 2.1 MANCOVA for female and male genital traits

Results of multivariate analyses of covariance examining variation of genital morphology in female and male *Poecilia mexicana* from sulfidic and non-sulfidic habitats. Effect sizes indicated by η_p^2 (partial eta squared)

Effect	**Hypothesis *df***	**Error *df***	***F***	***P***	η_p^2
Female Genitalia					
Body Size	3	169	6.731	<0.001	0.315
Habitat	3	169	18.115	<0.001	0.482
Drainage	9	411.452	5.403	<0.001	0.151
Body Size × Habitat	3	169	0.983	0.402	0.029
Body Size × Drainage	9	411.452	2.338	0.014	0.027
Habitat × Drainage	9	411.452	4.874	<0.001	0.117
Body Size × Habitat × Drainage	9	411.452	3.554	<0.001	0.059
Male Genitalia					
Body Size	11	181	4.574	<0.001	0.376
Habitat	11	181	5.453	<0.001	0.618
Drainage	33	533.963	2.172	<0.001	0.340
Body Size × Habitat	11	181	1.618	0.097	0.090
Body Size × Drainage	33	533.963	1.193	0.215	0.068
Habitat × Drainage	33	533.963	3.489	<0.001	0.174

Table 2.2 GLMs for relationships among female and male genital traits

Summary of results from model selection based on GLMs predicting the relationship between female and male genital morphology, including the best-supported models ($\Delta AIC_C < 2$) for all dependent variables. Results from post-hoc tests are indicated by the following symbols: $^{\dagger}P \leq 0.1$, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$.

Model	AIC_C	ΔAIC_C	Weight
Female PC1			
Intercept + Male PC1† + Male PC7	-20.553	0	0.877
Female PC2			
Intercept + Male PC2**	-18.722	0	0.611
Female PC3			
Intercept + Male PC9*	-30.847	0	0.370
Intercept	-30.148	0.699	0.261

Chapter 3 - Convergent adaptation and ecological speciation result from unique genomic mechanisms in sympatric extremophile fishes

Ryan Greenway, Anthony P. Brown, Henry Camarillo, Cassie Delich, Kerry L. McGowan, Lenin Arias-Rodriguez, Joanna L. Kelley, and Michael Tobler

Abstract

Whether adaptive divergence and ecological speciation occur in a repeatable manner among lineages experiencing similar selective regimes remains unclear. We analyzed adaptive divergence across levels of biological organization in three species of poeciliid fishes occurring in sympatry in an extremely toxic hydrogen sulfide (H_2S) rich spring and an adjacent benign freshwater stream to identify shared patterns of adaptation among distantly related population pairs. Despite differences in the demographic histories of each population pair, we found evidence for similar patterns of morphological evolution and reproductive isolation mediated by natural selection against migrants between habitats. However, shared mechanisms of adaptive divergence were less common at the molecular level. Although we found evidence for the convergent modulation of gene expression patterns in biochemical pathways associated with H_2S toxicity and detoxification, we uncovered no evidence for selection acting on the same regions of the genome when comparing any two species. Our results suggest that, in the absence of the shared genomic architecture and pools of standing genetic variation shared among closely related lineages, deterministic nature of strong, shared selective regimes may not be sufficient to drive convergence at the genomic level.

Introduction

Replicated cases of ongoing ecological speciation have provided a chance to link convergent aspects of adaptation to the emergence of reproductive isolation (Rundle et al. 2000; Rosenblum and Harmon 2011; Nosil 2012). These replicated speciation events have also allowed characterization of the genomic landscape underlying divergence before the loci mediating adaptation and reproductive isolation are obscured by post-speciation divergence (Jones et al. 2012; Seehausen et al. 2014; Soria-Carrasco et al. 2014). However, studies of repeated ecological speciation often rely on instances of parallel speciation, where geographically disjunct but closely related population pairs undergo ecological speciation as the result of adaptation to similar selective regimes (Schluter and Nagel 1995). Focusing on parallel speciation occurring along separate – albeit qualitatively similar – environmental gradients treated as categorical replicates can introduce unquantified environmental variation, resulting in idiosyncratic and lineage-specific patterns of divergence (Westram et al. 2014; Stuart et al. 2017; Morales et al. 2018). Additionally, the focus on replicated speciation events among closely related lineages might bias our understanding of general patterns of adaptive divergence, potentially leading to an overestimation of the repeatability and predictability of evolution. In particular, the probability of the same loci underlying divergence is relatively high in closely related taxa (Conte et al. 2012), resulting from shared pools of standing genetic variation and the constraints associated with shared genomic architecture (Colosimo et al. 2005; Soria-Carrasco et al. 2014). Consequently, whether aspects of adaptation and speciation driven by divergent selection are predictable and repeatable among distantly related lineages remains an outstanding question. In this study, we used a system where three highly divergent lineages occur in sympatry and are undergoing

speciation along the exact same ecological gradient to ask whether adaptive divergence occurs in a repeatable fashion across levels of biological organization.

Throughout the Neotropics, multiple lineages of livebearing fishes (Poeciliidae) have independently colonized extremely toxic, hydrogen sulfide (H_2S)-rich freshwater springs. Conditions in sulfide springs are lethal to most organisms because H_2S interrupts the oxidative phosphorylation pathway (OXPHOS) in mitochondria, inhibiting aerobic ATP production even in micromolar concentrations (Tobler et al. 2016b). Despite the extreme toxicity, locally adapted populations of sulfide spring poeciliids have repeatedly evolved from ancestral populations in adjacent freshwater habitats (Tobler et al. 2018). Local adaptation has been documented to drive ecological speciation between sulfide spring and ancestral freshwater populations despite the absence of physical barriers between habitats (Plath et al. 2013; Riesch et al. 2016; Tobler et al. 2018). Comparisons among parallel speciation events between replicated populations pairs of *Poecilia mexicana* have revealed convergence in morphology, life history, physiology, and gene expression (reviewed in Tobler et al. 2018). At the genomic level, divergence appears to occur through both lineage-specific and convergent evolution. While some convergence appears to result from *de novo* modification of the same genes or biochemical pathways (Pfenninger et al. 2014; Pfenninger et al. 2015; Brown et al. 2018), there is also evidence for convergent adaptation driven by selection on shared genetic variation, as the result of either standing genetic variation in ancestral populations or introgression among sulfidic populations in separate springs (Brown et al. 2019). As in other systems, focusing on these parallel speciation events may have biased interpretation of the predictability of ecological speciation driven by adaptation to sulfidic conditions. However, populations of three distantly related poeciliid species occur sympatrically within a single sulfide spring and an adjacent non-sulfidic stream in Chiapas, Mexico (Fig.

3.1A). Sulfide spring populations of *Poecilia mexicana*, *Pseudoxiphophorus bimaculatus*, and *Xiphophorus hellerii* differ phenotypically from adjacent populations of the same species and exhibit morphological convergence (*i.e.* increased head size; Fig. 3.1B, Table C12). We took advantage of this unique, spatially restricted system to study whether there are shared patterns of adaptive divergence among these three distantly-related population pairs during replicated ecological speciation along the same environmental gradient.

Results and Discussion

Similar patterns of population structure despite unique demographic histories

We first sought to characterize evidence for and historic dynamics of ecological speciation between sulfidic and non-sulfidic populations of each species. To detect population structure and recent gene flow between sulfidic and non-sulfidic populations of each species, we used a clustering approach based on genotype likelihoods from 19-20 re-sequenced genomes per population (Skotte et al. 2013). This approach unambiguously clustered individuals by their population of origin and detected low levels of gene flow between sulfidic and non-sulfidic populations within each species (Fig. 3.2A). Principal components analysis corroborated these results, consistently separating populations by habitat type along the primary axis of genetic variance in each species (Fig. C1). With the exception of one apparent F1 hybrid in *Poecilia*, recent gene flow appears to be minimal and to have occurred asymmetrically from sulfidic into non-sulfidic populations.

Despite striking similarities in the degree of current population structure between sulfidic and non-sulfidic populations of each species, reconstruction of demographic histories using genome-wide single nucleotide polymorphisms revealed considerable differences in the

historical dynamics of ecological speciation among population pairs (Fig. C2-C4). For each pair, the best-fit demographic model supported divergence with gene flow between sulfidic and non-sulfidic populations over time, but the timing of divergence and levels of migration differed substantially among the three species. Divergence between sulfidic and non-sulfidic *Poecilia* populations started ~20,000 years ago, followed by a period of isolation before gene flow resumed ~5,000 years ago (Fig. C2). For both *Pseudoxiphophorus* and *Xiphophorus*, divergence between sulfidic and non-sulfidic populations started with ongoing gene flow ~16,000 and ~9,000 years ago, respectively (Fig. C3,C4). As in the analyses of population structure, each of these models supported higher levels of gene flow from sulfidic into non-sulfidic populations than vice versa. Together with the evidence for strong population structure, these results indicate that high levels of reproductive isolation quickly evolved following the colonization of sulfide springs, despite differences in the dynamics of historical gene flow and amount of time available for adaptation and speciation to occur.

Reproductive isolation is mediated by natural selection against migrants

We tested whether reproductive isolation between populations in different habitats is mediated by natural selection against migrants through reciprocal translocation experiments. We found strong, habitat-specific differences in the survival of sulfidic and non-sulfidic populations of each species (interaction between "habitat of origin" and "testing habitat": $P < 0.001$; Fig. 3.2B, Table C13). Fish tested in their own habitat had significantly higher survival rates (88% to 100%) than those tested in the opposite habitat type, with the lowest survival observed for fish moved from the non-sulfidic into the sulfidic habitat (0% to ~21%; Fig. 3.2B). Supporting the observed asymmetry of gene flow between habitat types, natural selection is much stronger

against migrants into sulfidic habitats than in the opposite direction. Fish moved from the sulfidic into the non-sulfidic habitat had comparatively high survival (68% to 84%), though lower than resident non-sulfidic fish. We also tested whether the documented selection against migrants into sulfidic habitats is specifically mediated by H_2S toxicity by assessing the tolerance of wild-caught adult fish to acute H_2S exposure. *Poecilia* and *Xiphophorus* from the sulfide spring exhibited significantly higher tolerance to H_2S than the non-sulfidic populations of the same species ("habitat" term, $P < 0.001$; Table C14; Fig. 3.2C, Fig. C5). For *Pseudoxiphophorus*, there was no significant difference in tolerance to acute H_2S exposure between sulfidic and non-sulfidic populations ("habitat" term, $P = 0.101$; Table C14; Fig. 3.2C, Fig. C5). While natural selection against migrants is a strong mechanism of reproductive isolation reducing gene flow between sulfidic and non-sulfidic populations, the specific mechanism of divergent selection may have a different physiological basis in each species as revealed by H_2S tolerance assays. Despite a lack of evidence for increased H_2S tolerance in sulfide spring *Pseudoxiphophorus*, reproductive isolation may be maintained by natural selection against migrants mediated by other physiological stressors characteristic of sulfide springs. Notably, sulfide springs are also hypoxic, have increased specific conductivity, and low pH (Greenway et al. 2014). Natural selection against migrants may stem from the combined effects of these stressors, rather than H_2S alone, for this population pair.

Genes that mediate H_2S toxicity and detoxification exhibit convergent expression patterns across all species

We analyzed variation in gene expression to investigate the role of shared physiological responses to the extreme conditions of the sulfide spring. When looking at overall gene

expression variation in gill tissues from wild-caught individuals, individuals predominantly clustered by habitat type within each species (Fig. 3.3A). Comparisons of gene expression between sulfidic and non-sulfidic individuals of each species revealed significant differential gene expression between habitat types within species. The proportion of differentially expressed genes between sulfidic and non-sulfidic populations was relatively high but variable across species (13.4% *Pseudoxiphophorus*, 20.4% *Poecilia*, 20.9% *Xiphophorus*; false discovery rate < 0.05). Both lineage-specific and convergent physiological responses were evident in the differentially expressed genes of each species. While the vast majority of gene expression differences were species-specific (Tables C4-C6), interpretation of the adaptive significance of genes only differentially expressed in one species in the wild is limited by the fact that a large proportion of gene expression variation can be driven by neutral drift among populations (Whitehead and Crawford 2006). We instead focused on genes that exhibited consistent patterns of differential expression between the sulfidic and non-sulfidic populations of each species (*e.g.* a gene which is upregulated in each sulfidic population), as these are highly unlikely to be the result of drift and represent candidate genes involved in adaptation to shared environmental conditions (Ghalambor et al. 2015; Kelley et al. 2016). In total, 245 genes (1.3% of all analyzed genes; 6.3% to 9.8% of differentially expressed genes) were differentially expressed in the same direction across all three species (153 upregulated; 92 downregulated; Fig. 3.3B, Table C7). The set of shared upregulated genes was significantly enriched for genes associated with mitochondria as well as biological processes associated with H_2S toxicity and detoxification, including aerobic respiration and the electron transport chain (OXPHOS), enzymatic H_2S detoxification, and the processing and transport of sulfur compounds (Table C11). Of particular interest, we found consistent upregulation of genes in the sulfide:quinone oxidoreductase (SQR)

pathway – the primary mechanism of H_2S detoxification – and COX1 – a core subunit of the target of H_2S toxicity (Fig. 3.3C). The biological significance of the shared downregulated genes is not immediately clear. While overall gene expression variation is largely lineage-specific, shared differential expression patterns suggest that convergent modulation of the expression of specific genes with known roles in H_2S toxicity and detoxification plays a role in adaptation to sulfidic conditions. In fact, independent sulfidic populations of *P. mexicana*, notably less divergent from their non-sulfidic ancestors than populations examined in this study, exhibit heritable expression differences of H_2S processing and OXPHOS-related genes that mediate adaptive physiological responses to sulfide springs (Kelley et al. 2016; Passow et al. 2017). Consequently, modification of the expression of these predictable genes with relevance for H_2S toxicity appears to be highly repeatable among divergent lineages and across multiple sulfide springs.

Unique genomic landscapes underlie adaptation and speciation in sulfide springs

Previous studies of two independent population pairs of *P. mexicana* have shown genomic convergence driven both by selection on the exact same genes and by selection on different genes in the same physiological pathway (Pfenninger et al. 2014; Pfenninger et al. 2015; Brown et al. 2018; Brown et al. 2019). At least some aspects of convergence at the genomic level have been driven by selection on standing genetic variation (Brown et al. 2019). We contrasted the landscape of genomic divergence during adaptation to sulfide springs across the three lineages that do not share a recent genomic background to determine if there is evidence for selection on the same genomic regions or pathways. To quantify divergence across the genome and identify regions of the genome subject to natural selection, we calculated estimates of relative and

absolute genomic divergence (F_{ST} and d_{xy}, respectively) in non-overlapping 25-kb windows using 19-20 resequenced genomes per population. Rather than finding a few regions of elevated divergence (indicative of recent speciation with gene flow), we observed substantial divergence between sulfidic and non-sulfidic populations of each species across much of the genome (Fig. 3.4). While genomic divergence was heterogeneous in all cases, global estimates of divergence roughly scaled to estimates of divergence times between the sulfidic and non-sulfidic populations of each species (mean F_{ST} = 0.18 *Poecilia*, 0.17 *Pseuodxiphophorus*, 0.12 *Xiphophorus*). We identified regions of the genome potentially subject to divergent selection between habitats as 25-kb windows in the top 5% percentile for both F_{ST} and d_{xy}, identifying 26 outlier windows in *Poecilia*, 28 in *Pseudoxiphophorus*, and 30 in *Xiphophorus*. Despite some degree of convergence quantified at all other levels of biological organization examined here, none of these outlier regions were shared between any two species. Furthermore, the functional relevance of regions of the genome under selection was not predictable in any species. We found no evidence for divergence of regions with putative functional relevance for mediating adaptation to H_2S toxicity in any of the three species. In fact, gene ontology (GO) enrichment analyses of genes contained in outlier regions (*i.e.*) of each species found no evidence for significant enrichment of any biological processes, functions, or components. Manual inspection of genes contained in outlier windows confirmed the absence of genes with known relevance to the extreme conditions of sulfide springs in these genomic regions.

There are several non-mutually exclusive potential explanations for the observed lack of a link between outlier regions and known mechanisms of adaptation to life in sulfide springs. First, it is possible that genes in these outlier regions play an as of yet uncharacterized role in adaptation to environmental stressors in sulfide springs, requiring further functional examination.

Second, although divergence has occurred relatively recently on a temporal scale (within the last 20,000 years; Fig. C2-C4), each of the population pairs may actually be at later stages of the speciation continuum (Nosil 2012). The low amount of contemporary gene flow and extremely strong selection against migrants are indicative of high levels of reproductive isolation between sulfidic and non-sulfidic populations, suggesting that speciation may have proceeded rapidly. At the genomic level, high levels of background divergence between species is known to obscure the signature of selection on the regions responsible for adaptive divergence during earlier stages of speciation (Renaut et al. 2013; Han et al. 2017). As a result, population genomic methodologies may not be appropriate for identification of specific loci under divergent selection between these population pairs that exhibit such high divergence across the genome. Lastly, detected outlier regions may actually play a role in adaptation to sulfide springs if they include regulatory elements of the genome that mediate adaptive shifts in gene expression, rather than just protein coding regions (Mack and Nachman 2017). Divergent selection acting on regulatory elements in the genome has been implicated to play a prominent role in adaptive divergence for several well-known models of ecological speciation (Baxter et al. 2010; Jones et al. 2012; Hebert et al. 2013; Seehausen et al. 2014; Ishikawa et al. 2017), though most evidence comes from divergence of non-coding genomic regions adjacent to those which encode proteins thought to be involved in adaptation (*i.e. cis*-regulatory regions). Potential regulatory elements captured in the outlier regions we identified would instead be *trans*-regulatory, encoded in separate regions of the genome from the genes which they interact with, as we do not see the presence of genes identified in our differential expression dataset in these outlier regions. Despite ambiguity in the mechanisms of adaptive divergence at the genomic sequence level, our results nonetheless have

documented the rapid and repeated evolution of exceptional genome-wide divergence between sulfidic and non-sulfidic populations.

Conclusions

Overall, our results indicate similar patterns of divergence among three distantly related species evolving in response to an identical environmental gradient with regard to morphology, reproductive isolation, and global levels of genome-wide differentiation. We documented consistent reductions in contemporary gene flow between sulfidic and non-sulfidic populations and similar histories of asymmetric gene flow from sulfidic into non-sulfidic habitats, despite differences in divergence times in each of the population pairs. These results were corroborated for each species by the observed strength of natural selection against migrants into sulfidic habitats, but substantially weaker selection against migration in the opposite direction. However, evidence for shared mechanisms of adaptive divergence begins to erode at lower levels of biological organization. While genes with known relevance for adaptation to sulfide springs are consistently differentially expressed between sulfidic and non-sulfidic populations of each species, patterns of gene expression variation across the transcriptome are largely lineage-specific. Despite clear evidence for some degree of convergence across higher levels of biological organization, we found no evidence for shared mechanisms of divergence acting at the genome sequence level and signals of selection on specific genomic regions were fairly ambiguous. This is in stark contrast to previous studies in sulfide spring poeciliids, which have uncovered evidence for both shared and unique mechanisms of adaptive divergence in the genome. However, these studies have focused on cases of parallel speciation between independent populations of *P. mexicana*. Our results suggest that previously observed genomic convergence may be strongly influenced by the shared genomic background of the *P. mexicana*

clade, biasing evolutionary outcomes in independent sulfide springs. Currently the majority of studies reporting shared patterns of adaptive divergence and ecological speciation at the molecular level have focused on similar instances of parallel speciation in other systems. Our results add a new perspective to this field, and imply that shared genomic architecture and standing genetic variation – more so than the degree of similarity in selective regimes – may have a disproportionately strong influence on shaping genomic convergence. Even in cases of parallel speciation with convergent evolution at the genomic level, genomic convergence rarely involves more than a fraction of the genome (Conte et al. 2012; Soria-Carrasco et al. 2014). The mechanisms by which lineage-specific genomic evolution influences convergence at higher levels of biological organization often remains unresolved in such cases, serving to highlight the disconnect in our understanding of the connection between evolution at genomic and phenotypic levels. Additional studies investigating evolutionary responses across levels of organization during replicated speciation events in distantly related lineages are needed to clarify whether convergence at the molecular level is an exception or a rule of evolutionary diversification.

Methods

Study sites

In the foothills of the Sierra Madre de Chiapas, several freshwater springs rich in naturally occurring H_2S discharge to form the La Gloria spring complex (Tobler et al. 2011a; Greenway et al. 2014). Populations of the poeciliid fishes *Poecilia sulphuraria, Pseudoxiphophorus bimaculatus*, and *Xiphophorus hellerii* have colonized the La Gloria spring complex from ancestral populations in adjacent freshwater habitats (Plath et al. 2013; Greenway et al. 2014). *P. sulphuraria* is a lineage endemic to sulfide springs in the Río Pichucalco drainage, described as a

distinct species divergent from *Poecilia mexicana* populations in adjacent non-sulfidic streams (Palacios et al. 2013). All samples, with the exception of non-sulfidic *Pseudoxiphophorus* gill tissues (originally collected from a nearby stream for a separate study), were collected by seine from the La Gloria sulfide spring complex and non-sulfidic streams of the nearby Arroyo Caracol in the Río Pichucalco drainage of Chiapas, Mexico located near the town of Teapa in Tabasco, Mexico (Fig. 1A; Table C1).

Geometric morphometrics

To test for divergence in body shape among populations in sulfidic and non-sulfidic habitats, adult *Poecilia*, *Xiphophorus*, and *Pseudoxiphophorus* were collected from the two sites using seines and immediately euthanized with buffered MS222 following capture. Tissue samples were collected and stored in 100% EtOH for genomic analyses (see below) before euthanized fish were fixed in 10% formaldehyde solution (n = 424, Table C1). Lateral photographs were taken for all preserved specimens using a Canon EOS 400D digital camera (Canon USA Inc., Lake Success, NY, USA) mounted on a copy stand. We digitized 14 morphological landmarks (Fig. A2) on each photograph using the software program tpsDig (Rohlf 2004). We extracted coordinates of these digitized landmarks to conduct geometric morphometric analyses (Zelditch et al. 2012) using the geomorph package in R (Adams et al. 2019). We first aligned landmark coordinates via least-squares superimposition implemented in the gpagen function to remove effects of translation, rotation, and scale, yielding aligned Procrustes coordinates that describe body shape variation and centroid size as a measure of size. Procrustes coordinates were then analyzed using a Procrustes ANOVA (procD.lm) using residual randomization over 999 iterations and type III sums of square. Predictor variables include “species” (*Poecilia*,

Xiphophorus, *Pseudoxiphophorus*), "habitat" (sulfidic/non-sulfidic), and sex as factors, as well as centroid size as a covariate. *P*-values were based on a Cohen's f-squared sampling distribution. We then calculated divergence vector scores (Langerhans 2009) for each individual based on the first principle component of the among-group covariance matrix for the habitat term from the ANOVA (Klingenberg and Spence 1993). This approach allowed us to visualize aspects of body shape variation associated with the sulfidic or non-sulfidic habitat among species (*i.e.* convergent aspects of body shape), while accounting for all other terms in the model.

Genomic DNA library preparation, whole genome sequencing, and data alignment

We resequenced the genomes of 120 individuals (40 per species, 20 per population; Table C1) for population genomic analyses, including estimating population structure, reconstructing demographic history, and characterizing the genomic landscape of divergence. DNA was extracted from fin clip tissue samples using the NucleoSpin Tissue kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) following the protocol in the user manual (version January 2017 / Rev. 17). For the final elution step, extracted DNA was eluted in 100 µL of elution buffer. All subsequent library preparation and sequencing was performed at the Washington State University Spokane Genomics Core.

Genomic DNA (gDNA) was quantified using the Qubit 2.0 fluorometer with the dsDNA HS assay kit (Thermo Fisher Scientific, Waltham, MA). One hundred ng of gDNA was used as input for library preparation using the TruSeq Nano DNA Library Prep Kit (Illumina, San Diego, CA). Briefly, gDNA was sheared using the M220 Focused-Ultrasonicater (Covaris, Woburn, MA), followed by end repair, size selection (350-bp insert size), dA-tailing, adaptor ligation, and library amplification by eight rounds of PCR. The quality of the DNA libraries was assessed by

Fragment Analyzer with the High Sensitivity NGS Fragment Analysis Kit (Advanced Analytical Technologies, Ankeny, IA). Library concentrations were measured by the StepOnePlus Real-Time PCR System (ThermoFisher Scientific, Waltham, MA) with the KAPA Library Quantification Kit (Kapabiosystems, Wilmington, MA). Each library was labeled with an index sequence during the adaptor-ligation step using Illumina's TruSeq Dual Indexing system (Illumina, San Diego, CA) that contains 96 unique indexes. The 120 libraries were split and pooled into the following two groups at equal molar concentrations: Library pool #1 consists samples P001-P040 and P073-P092; library pool #2 consists samples P041-P072 and P093-P120. Each library had a unique index within its own group. The library pools were diluted to 2 nM with RSB (10 mM Tris-HCl, pH 8.5) and denatured with 0.1 M NaOH. Eighteen pM libraries were clustered in a high-output flow cell using the HiSeq Cluster Kit v4 on a cBot (Illumina, San Diego, CA). After cluster generation, the flow cell was loaded onto HiSeq 2500 for sequencing using the HiSeq SBS kit v4 (Illumina, San Diego, CA). DNA was sequenced with paired-end, 100-bp chemistry. Raw bcl files were converted to fastq files and demultiplexed using the program bcl2fastq2.17.1.14 (https://github.com/thepler/bcl2fastq2).

Raw reads were trimmed to quality 0 to remove adapters with Trimgalore! (Krueger 2014). Trimmed reads for all 120 individuals were mapped to the *Xiphophorus maculatus* reference genome (version 5.0, RefSeq accession number: GCF_002775205.1; Schartl et al. 2013) using BWA-MEM (Li and Durbin 2009). We used this reference genome as *X. maculatus* is a species in the same family (Poeciliidae) with a chromosome scale genome assembly. On average, 93.89% ± 5.08% of trimmed reads mapped across all individuals (Table C2). After mapping, it became clear that one sample labeled as a sulfidic *Pseudoxiphophorus* was likely mislabeled; this sample was excluded from all further analyses. We used the Genome Analysis

Toolkit (GATK, v.3.5; McKenna et al. 2010) and Picard Tools (http://broadinstitute.github.io/picard) to mark duplicate reads (using the MarkDuplicates tool) and realign mapped reads around indels (using the RealignerTargetCreator and IndelRealigner tools) following GATK best practices (DePristo et al. 2011; Auwera et al. 2013). This resulted in a realigned .bam file for each individual to be used in subsequent analyses. Because the genomes generated for this study had a low average depth of sequence coverage (3.801x ± 0.418x; Table C2), genotypes could only be called with substantial uncertainty. Consequently, all population genomic inferences (with the exception of demographic modeling) were based on genotype likelihoods estimated with ANGSD (Korneliussen et al. 2014), which accounts for the inherent uncertainty of genotypes called from low-depth sequencing data.

Analyses of population structure

We assessed structure between populations in each habitat using two methods optimized for low-coverage sequencing data based on genome-wide genotype likelihood data. Genotype likelihoods were estimated for combined sulfidic and non-sulfidic populations of each species using the GATK method (McKenna et al. 2010), and data was filtered to exclude sites with sequence data in fewer than 35 individuals per species (*-minInd* 35), a minor allele frequency below 5% (*-minMaf* 0.05), mapping quality below 30 (*-minMapQ* 30), and a base q-score below 20 (*-minQ* 20). This resulted in the retention of 4,061,776 sites for *Poecilia*, 7,849,617 for *Pseudoxiphophorus*, and 6,049,047 for *Xiphophorus*. We investigated the extent of gene flow between populations using NGSadmix (Skotte et al. 2013), a maximum likelihood based assignment method that estimates admixture proportions for each individual. NGSadmix was run with the number of populations (*K*) set as 2 to test if individuals cluster by habitat of origin.

Population structure was further examined by principal component analysis based on a covariance matrix computed using *ngsCovar* in the ngsTools program suite (Fumagalli et al. 2014). Principal components were calculated with the function *eigen* in R (R Core Team 2017). Inspection of PC axes revealed clear separation of individuals along the primary axis in each species, forming distinct clusters of sulfidic and non-sulfidic individuals (Fig. C1).

Demographic modeling

We used diffusion approximations for demographic inference (*∂a∂i*) (Gutenkunst et al. 2009) to reconstruct demographic histories for each of the three population pairs using the resequenced genomes described above. Because demographic analyses using *∂a∂i* rely on discrete single nucleotide polymorphisms (SNPs) as input data, we initially identified SNPs in each of our realigned sample .bam files using the GVCF output mode in the HaplotypeCaller from GATK (v.3.5; McKenna et al. 2010), following recommended best practices (DePristo et al. 2011; Auwera et al. 2013). Samples were then genotyped jointly on a per-species basis using GATK's GenotypeGVCFs tool. To obtain a set of SNPs suitable for demographic analysis, we filtered our species vcf files using VCFtools (Danecek et al. 2011). Specifically, we only kept sites if they were bialleleic (--*max-alleles* 2 and --*min-alleles* 2), present at a depth of at least 8x (--*minDP* 8) in at least 70% of the individuals (--*max-missing* 0.7), and had a quality score of at least 30 (--*minQ* 30). SNPs passing these filters were retained for subsequent demographic history reconstruction using *∂a∂i*. Three general models were compared for each pair: 1) A population split followed by migration (full migration model); 2) A population split followed by a period where migration was allowed, but with migration stopping at some point in time (ancient migration model); 3) A population split followed by a period without migration, but with

migration starting at some point later in time (secondary contact model). To more fully explore the parameter space, we started each model from 20 different, randomly selected points in the parameter space, and we performed additional runs of each model where we restricted migration in one or both directions. Singletons were masked to reduce any potential effects of sequencing errors on the models. We also down-sampled to 24 chromosomes per population to account for missing genotypes. AICc was used to compare the fit of the models. We assumed a mutation rate of 6.6×10^{-8} mutations per site per generation (Recknagel et al. 2013) and a generation time of six months (Jourdan et al. 2014; Riesch et al. 2014) to convert *∂a∂i* parameters to effective population sizes, migration rates per generation, and times of events (*i.e.* divergence times or times when migratory patterns changed). We performed 100 bootstrap replicates of the best model for each population pair to obtain 95% confidence intervals for each parameter.

To confirm that these results were not biased by SNPs in regions of the genome under selection or by recent gene flow, we ran additional models excluding admixed individuals and with only putatively neutral SNPs as input. In order to obtain a set of putatively neutral SNPs, we filtered out all SNPs found within gene regions using the *intersect* tool within BEDTools v. 2.25.0 (Quinlan and Hall 2010). We removed an apparent F1 admixed individual from the sulfidic *Poecilia* population to limit its effect on our demographic models using the *–remove-indv* option within VCFtools v. 0.1.15 (Danecek et al. 2011). Models run with this set of SNPs recovered quantitatively similar results with overlapping 95% confidence intervals to model parameters estimated with SNPs from all regions of the genome.

Reciprocal translocation experiments

To test for natural selection against migrants between sulfidic and non-sulfidic habitats, we conducted reciprocal translocation experiments using previously established approaches (Tobler et al. 2009a; Plath et al. 2013). 20-L plastic buckets were placed into the two habitats as experimental mesocosms. Two holes (18 × 32 cm) were cut into opposite sides of each bucket and subsequently sealed with 1.5 mm plastic mesh to allow the free exchange of water with the environment. Approximately 50 small holes (<1 mm) were drilled into the bucket lids to facilitate air exchange. Mesocosms were placed into shallow areas of the sulfidic and non-sulfidic streams, seeded with a ~ 3-4 cm layer of natural substrate, and allowed to settle overnight before the first experimental introduction. We established ten mesocosms in each habitat. Water conditions in these mesocosms have been shown to closely match conditions in the surrounding stream (Tobler et al. 2009a).

We collected adult *Poecilia*, *Xiphophorus*, and *Pseudoxiphophorus* to be used in these mesocosms by seine and placed them in insulated coolers with aerated water for transport to the mesocosm locations (n = 299; Table C1). Five haphazardly chosen individuals of the same species and habitat type were introduced into a mesocosm. Half of the mesocosms in each habitat were used as controls to test the survival of resident fish, the other half to test survival of fish from the opposite habitat type. Transportation and handling times were minimal (~1 hour) and were balanced for resident and translocated individuals. Fish were measured for standard length (SL) prior to introduction. Experiments ran for ~20 hours before termination. Following the experimental period, we quantified survival and returned surviving individuals to their original collection site.

To analyze differences in the survival of potential migrants between habitat types for each species, we used generalized linear mixed models (GLMMs) with a binomial error

distribution and a logit link function as implemented in the R package lme4 (Bates et al. 2014); survival (binary data: 0 = died; 1 = survived) was included as the response variable. We included "habitat of origin" (sulfidic/non-sulfidic), and "testing habitat" (sulfidic/non-sulfidic) as independent variables, the interaction between these terms, and "SL" (log_{10}-transformed) as a covariate. Additionally, "bucket ID" was included as a random factor.

Sulfide tolerance

To test for differences in sulfide tolerance between individuals collected from the sulfidic and non-sulfidic habitat, we conducted acute H_2S exposure trials, subjecting wild-caught fish to increasing concentrations of H_2S (Tobler et al. 2011a). We first collected adult *Poecilia*, *Xiphophorus*, and *Pseudoxiphophorus* from the two sites using seines, transferred them to insulated coolers filled with water from the collection site, and transported to a nearby field station (n = 78; Table C1). Fish were kept in population and species-specific holding tanks with aeration for at least 18 hours prior to testing. To standardize experimental conditions, water from the collection sites was replaced with H_2S-free well water over the first 8 hours in the holding tanks. Water was continuously aerated and filtered during this time, and the fish received no food.

For the experiment, we prepared stock solutions of 10 mM aqueous H_2S solution by dissolving 2.40 g sodium sulphide hydrate (Na_2S*6H_2O) into 1 L water deoxygenated with nitrogen (Butler et al. 1994). Individual fish were placed into clear plastic containers with 150 mL water from the holding tanks and allowed to acclimate for 5 minutes. Following acclimation, 10 mL of H_2S solution was added to the experimental container at 2-minute intervals using a syringe placed under the water surface. Each fish was observed as H_2S concentration increased

in the experimental container. We measured the time until the fish lost equilibrium (*i.e.* stopped swimming and was unable to right itself), at which point the fish was removed from the container, sexed, weighed, and placed into a heavily aerated recovery tank. Experiments were ended after 32 minutes (15 sulfide additions) if a fish did not lose equilibrium. We analyzed variation in sulfide tolerance separately for each species using a survival analysis (Cox regression) with the coxph function implemented in the R package survival (Therneau 2015). We used time to loss of equilibrium as the response variable (Individuals that did not lose equilibrium during the course of the experiment were censored) and included "mass" (log_{10}-transformed), "sex" (female/male), and "habitat" (sulfidic/non-sulfidic) as independent variables. Adjusted survival curves were generated for the habitat term of the Cox regression to visualize expected survival curves, using the ggadjustedcurves function from the survminer package (Kassambara and Kosinski 2018).

Transcriptome sequencing and data alignment

We used an RNA-seq approach to identify differentially expressed genes between the sulfidic and non-sulfidic populations of each species. We collected adult female individuals (n = 6 per population; Table C1) of *Poecilia*, *Pseudoxiphophorus*, and *Xiphophorus* in La Gloria sulfide spring, as well as individuals from the same species in proximate non-sulfidic habitats (Table S1). Following capture with a seine, fish were instantly euthanized, gill tissues were extracted from both sides of the head and then immediately preserved in 2mL of RNAlater (Ambion, Inc.). We selected gill tissue because the gills mediate physiological processes necessary for the maintenance of homeostasis (Evans et al. 2005), are in direct contact with the H_2S-rich water (Tobler et al. 2016b), and show strong, heritable transcriptional responses upon H_2S exposure

(Kelley et al. 2016; Passow et al. 2017). RNA was isolated from gill tissues and cDNA libraries were constructed for each individual as described for samples in previous studies (see Barts et al. 2018 and Kelley et al. 2016 for details). Briefly, we extracted total RNA from pulverized gill tissues using the NucleoSpin RNA kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) following the manufacturer's protocol. We conducted mRNA isolation and cDNA library preparation with the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs, Inc., Ipswich, Mass., USA) and NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs, Inc., Ipswich, Mass., USA) following the manufacturers' protocol with minor modifications. Each cDNA library was assigned a unique barcode, quantified, and pooled in sets of 11–12 samples, split so that samples from different species and habitat types were sequenced together. cDNA libraries were sequenced on an Illumina HiSeq 2500 with paired-end, 100-bp reads at the Washington State University Spokane Genomics Core.

Raw RNA-seq reads were sorted by barcode and trimmed twice (quality 0 to remove adapters, followed by quality 24) with Trimgalore! (Krueger 2014). Trimmed reads for all 36 individuals were mapped to the same version of the *X. maculatus* reference genome used for genomic analyses (version 5.0, RefSeq accession number: GCF_002775205.1; Schartl et al. 2013) using BWA-MEM (Li and Durbin 2009). On average, 97.27% ± 1.32% of reads mapped across all individuals (Table C3). We functionally annotated genes from the *X. maculatus* reference genome by extracting the longest transcript per gene (using the perl script gff2fasta.pl, available from https://github.com/ISUgenomics/common_scripts/blob/master/gff2fasta.pl), and then searching against the human SWISSPROT database (critical E-value 0.001; access date 11/15/2018) using BLASTx (Camacho et al. 2009). Each *X. maculatus* gene was annotated with the top BLAST hit against the human database based on the top high-scoring segment pair.

These annotations were used for analysis of differentially expressed genes as well as for analyses of outlier regions from genomic data (*Population genomic statistics and genome-wide divergence* below).

We used STRINGTIE (v.1.3.3b; Pertea et al. 2015, Pertea et al. 2016) to quantify the number of RNA-seq reads mapped to each gene in the *X. maculatus* reference genome for each individual (measured as counts per million mapped reads) and used a Python script provided with STRINGTIE (prepDE.py) to generate a read counts matrix (Pertea et al. 2016). We then removed genes that did not have at least two counts per million in 3 or more individuals across all species, resulting in a set of 18,598 genes to be analyzed for patterns of gene expression. To examine overall patterns of gene expression variation, we conducted hierarchical cluster analysis on the full set of retained genes. The hierarchical cluster analysis was performed on log-transformed counts per million and visualized with the heatmap.2 function in the 'gplots' package in R (Warnes et al. 2016).

To identify differentially expressed genes, we used generalized linear models (GLMs) as implemented in the Bioconductor package edgeR (Robinson et al. 2010) in R. We used glmFit to fit a negative binomial GLM to the normalized read counts of each gene based on tagwise dispersion estimates and a design matrix describing the comparison between the sulfidic and non-sulfidic population of each species. We assessed statistical significance with the GLM likelihood ratio test with a false discovery rate of < 0.05 calculated with the Benjamini-Hochberg correction (Benjamini and Hochberg 1995). We then intersected the significantly up-regulated and down-regulated genes from all three species-specific comparisons separately in order to identify genes with consistent evidence for differential expression among all three species. After identifying the set of genes with differential expression between sulfidic and non-sulfidic

populations of each species, and genes with evidence for shared differential expression across all three species-specific analyses (Table C4-C7), we used a Gene Ontology (GO) enrichment analysis to explore the putative biological functions of these candidate sets of genes (Table C8-C11). We first annotated all genes that had a match in the human SWISSPROT database (described above) with GO IDs (Gene Ontology Consortium 2004) and then tested for the enrichment of specific GO IDs in these sets of genes with differential expression relative to the full dataset of 18,598 analyzed genes using GORILLA (*p*-value threshold: 0.0001, accessed 04/11/2019; Eden et al. 2009). In total, 10,817 unique SWISSPROT annotations were associated with a term in the GO database.

Population genomic statistics and genome-wide divergence

To characterize the landscape of genomic divergence, we calculated genomic differentiation ($F_{ST,}$ relative divergence) and divergence (d_{xy}, absolute divergence) between the sulfidic and non-sulfidic population of each species across non-overlapping 25-kb windows spanning the genome. Divergent selection between habitats should lead to elevated levels of both F_{ST} and d_{xy} at and around loci under selection (those contributing to the maintenance of adaptive divergence), while regions of the genome with reduced diversity in one or both populations (likely a consequence of background selection or low recombination rates) are expected to exhibit elevated $F_{ST,}$ but not d_{xy} (Cruickshank and Hahn 2014; Ravinet et al. 2017; Delmore et al. 2018).

To quantify F_{ST}, we estimated the folded site frequency spectrum for each population separately using ANGSD (Korneliussen et al. 2014), filtering sites based on the same criteria as used for analyses of population structure (*-minInd* 35, *-minMaf* 0.05, *-minMapQ* 30, *-minQ* 20). We used the population-specific site frequency spectra to obtain a joint frequency spectrum for

the combined population pairs of each species with the *realSFS* tool. The joint frequency spectrum was then used as a prior for allele frequencies at each site to estimate the average F_{ST} across each 25-kb window.

We used a similar approach to quantify d_{xy}, modifying the method employed by Marques et al. (2018). In brief, we first estimated the folded site frequency spectrum for each population in 25-kb windows (using identical filtering) and then used these windowed site frequency spectra to obtain joint frequency spectra for each 25-kb window for the population pair of each species using *realSFS*. We subsequently estimated average d_{xy} across each of these windows using a custom python script (modified from the script used by Marques et al. 2018; provided by D. Marques from https://github.com/marqueda/PopGenCode).

We identified probable targets of divergent selection between sulfidic and non-sulfidic populations with an empirical outlier approach. We first identified 25-kb windows above the top 5% percentile for both F_{ST} and d_{xy} separately. We then classified genomic regions likely experiencing divergent selection in each species as those windows with outlier signatures for both statistics. Lastly, we looked for outlier regions that were shared across all three species, but found none.

We investigated the link between divergent selection and the putative biological functions of genes found in these outlier windows for each species using a GO enrichment analysis (as described above). We first identified genes in these outlier regions from the *X. maculatus* genome annotation file (GFF) using the *intersect* tool in bedtools (v.2.25.0; Quinlan and Hall 2010). We then annotated all genes that had a match in the human SWISSPROT database (21,891 genes) with GO IDs and tested for enrichment of specific GO IDs in these outlier regions relative to all human annotated genes in the *X. maculatus* genome using

GORILLA (*p*-value threshold: 0.0001, accessed 04/20/2019; Eden et al. 2009). In total, 12,247 unique SWISSPROT annotations were associated with a GO term.

Figures

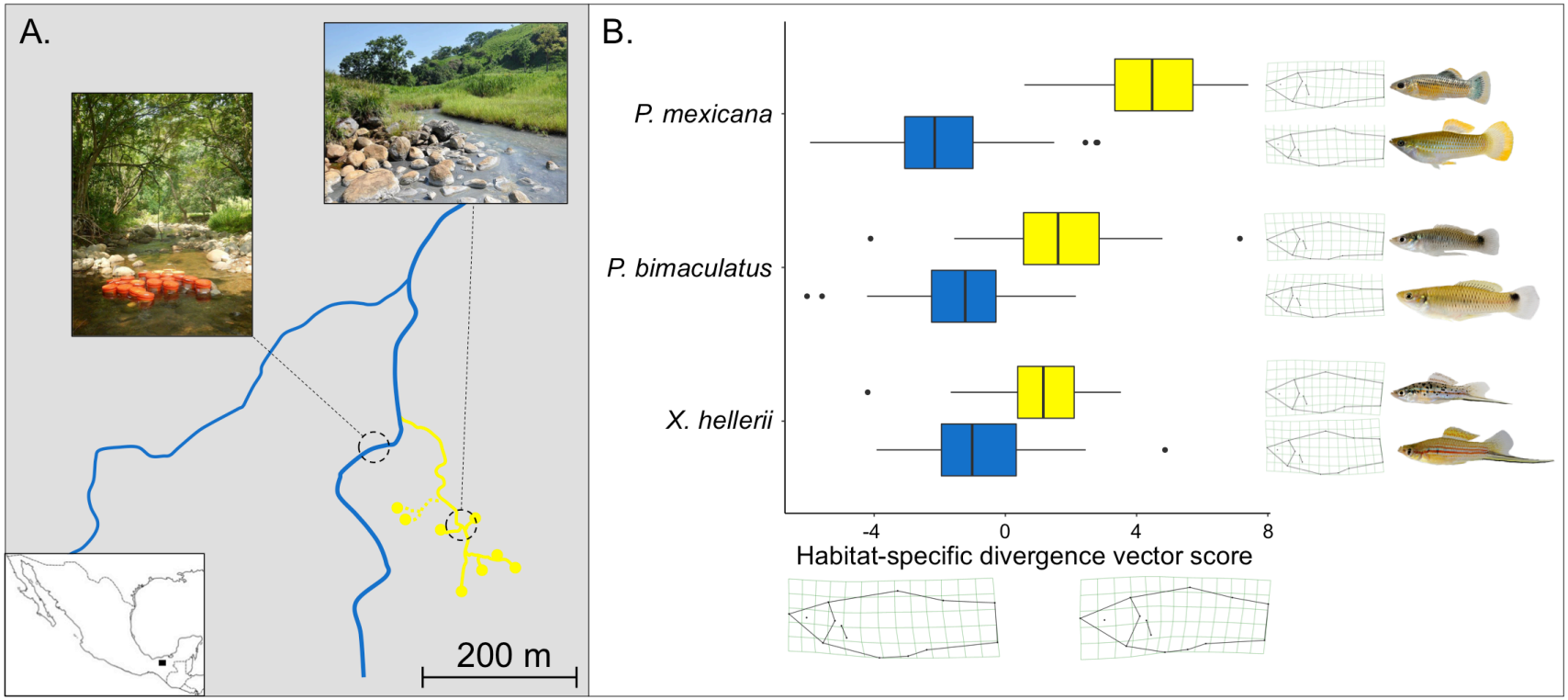

Figure 3.1 Sampling location and habitat-specific morphological variation

(A) Overview of the study area and sampling locations of *Poecilia*, *Pseudoxiphophorus*, and *Xiphophorus* in the La Gloria sulfidic spring system (yellow; circles represent springheads) and the non-sulfidic Arroyo Caracol (blue). Inset photos depict photographs of each habitat. (B) Morphological divergence between sulfidic (yellow) and non-sulfidic (blue) populations of each species. Transformation grids depict shape variation along the habitat divergence vector relative to the overall average body shape (divergence vector score = 0). Shared morphological responses to the sulfidic and non-sulfidic habitat are depicted along the X axis. Species-specific morphological responses to these habitats are visualized along the left side, with corresponding images of male individuals from each population (top sulfidic, bottom non-sulfidic).

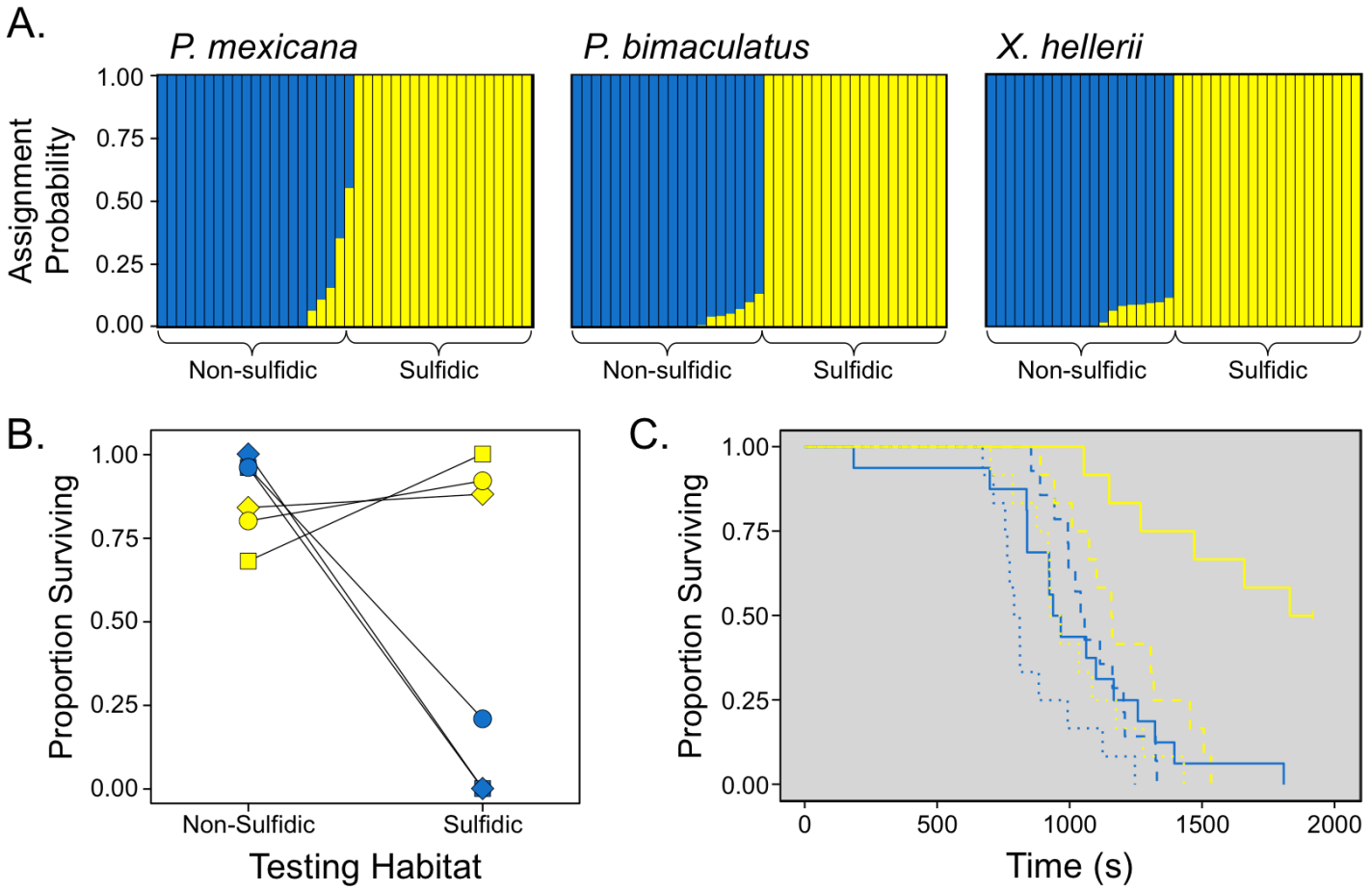

Figure 3.2 Population structure, natural selection against migrants, and H_2S tolerance

(A) Estimates of individual admixture proportions using genome-wide genotype likelihood data for $K = 2$ genetic clusters per species. Brackets indicate the true habitat of origin (sampling location) for each individual. (B) Proportion of individuals surviving translocation experiments between sulfidic and non-sulfidic habitats, as well as control experiments within the habitat of origin. Yellow symbols indicate sulfidic populations, blue symbols non sulfidic. ■= *Poecilia*, ◆ = *Pseudoxiphophorus*, ● = *Xiphophorus*. (C) Survival curves of fish from sulfidic (yellow) and non-sulfidic (blue) populations upon acute exposure to H_2S. Solid line = *Poecilia*, dotted line = *Pseudoxiphophorus*, dashed line = *Xiphophorus*.

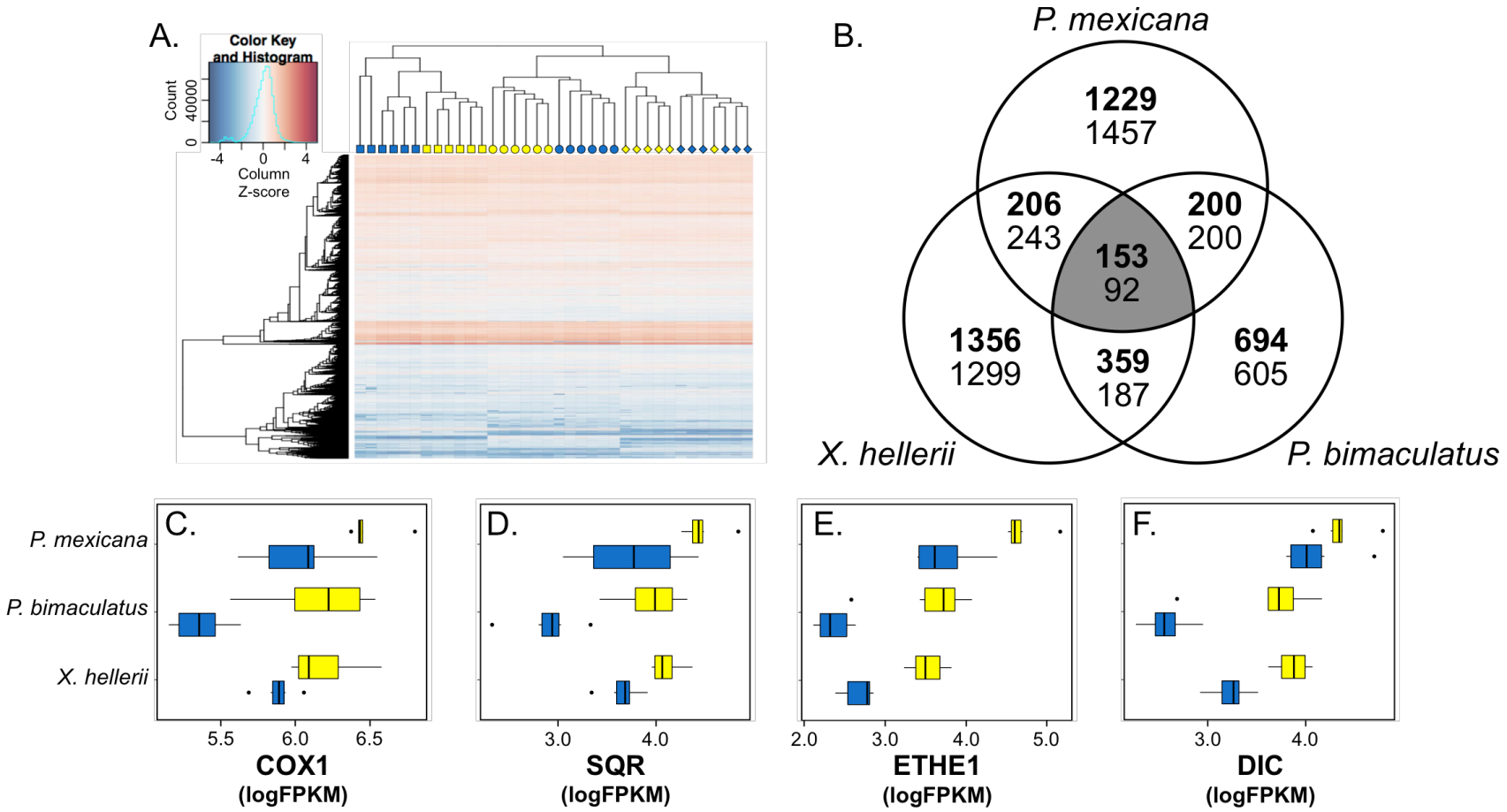

Figure 3.3 Patterns of gene expression variation

(A) Results of a hierarchical cluster analysis and heat map depicting expression variation across 18,598 genes. Yellow symbols indicate sulfidic populations, blue symbols non sulfidic. ■= *Poecilia*, ◆ = *Pseudoxiphophorus*, ● = *Xiphophorus*. (B) Venn diagram showing shared and unique gene expression variation between sulfidic and non-sulfidic populations of the three species examined. (C-F) Gene expression variation (log-transformed Fragments Per Kilobase of transcript per Million mapped reads [logFPKM]) of genes with known functional relevance for H_2S toxicity (C; COX1 – target of toxicity) and detoxification (D-F; genes in the sulfide oxidation pathway) across sulfidic (yellow) and non-sulfidic (blue) populations of each species.

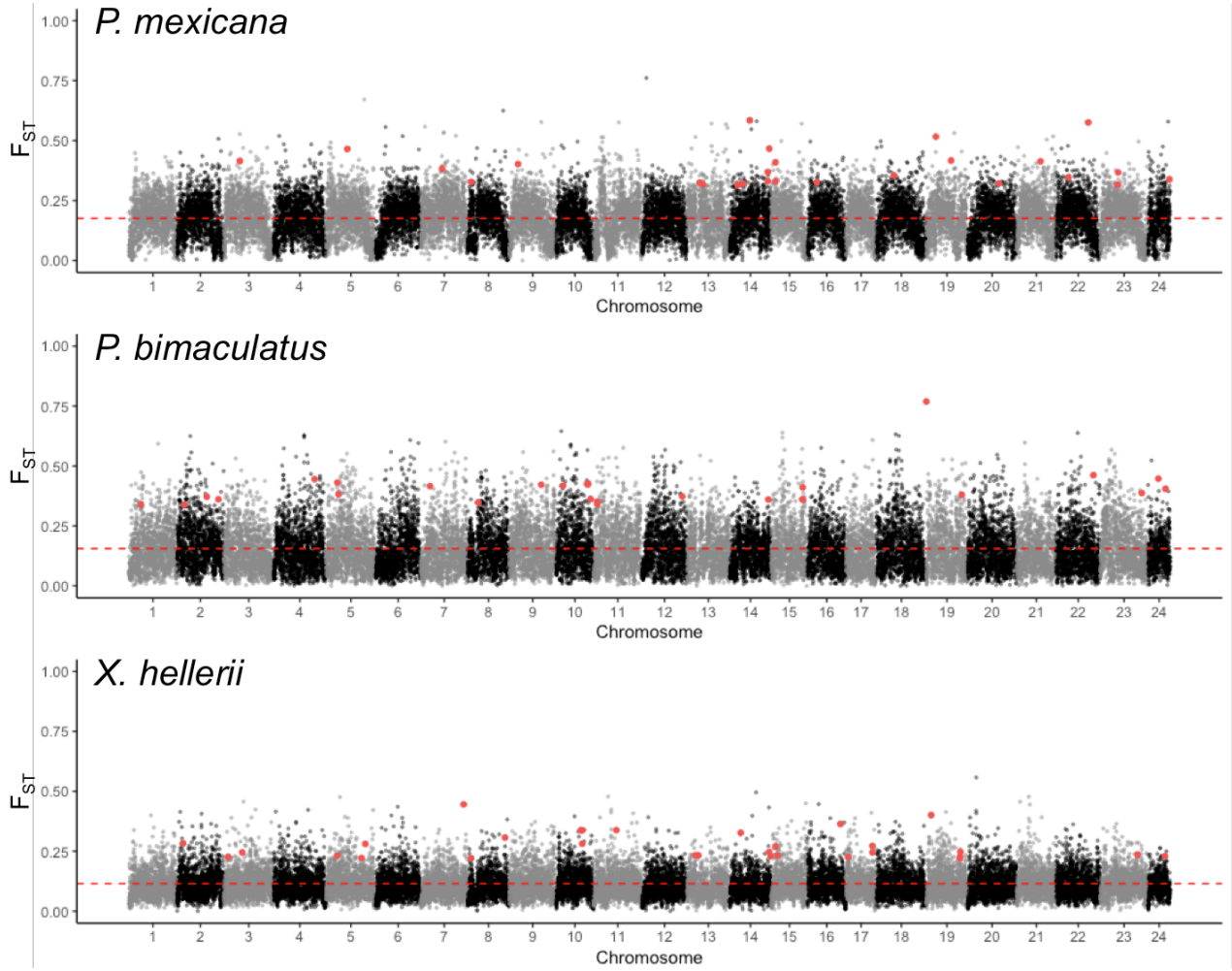

Figure 3.4 Genome-wide divergence and outlier regions

Genetic divergence (F_{ST}) between sulfidic and non-sulfidic populations in 25-kb non-overlapping windows across the genome for three sympatric species. Alternating shading represents the different chromosomes and dashed red lines denote the mean F_{ST}. Red points indicate windows identified as being above the top 5% percentile for both F_{ST} and d_{xy}.

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Appendix A - Adaptive, but not condition-dependent, body shape differences contribute to assortative mating preferences during ecological speciation

Supplementary Figures

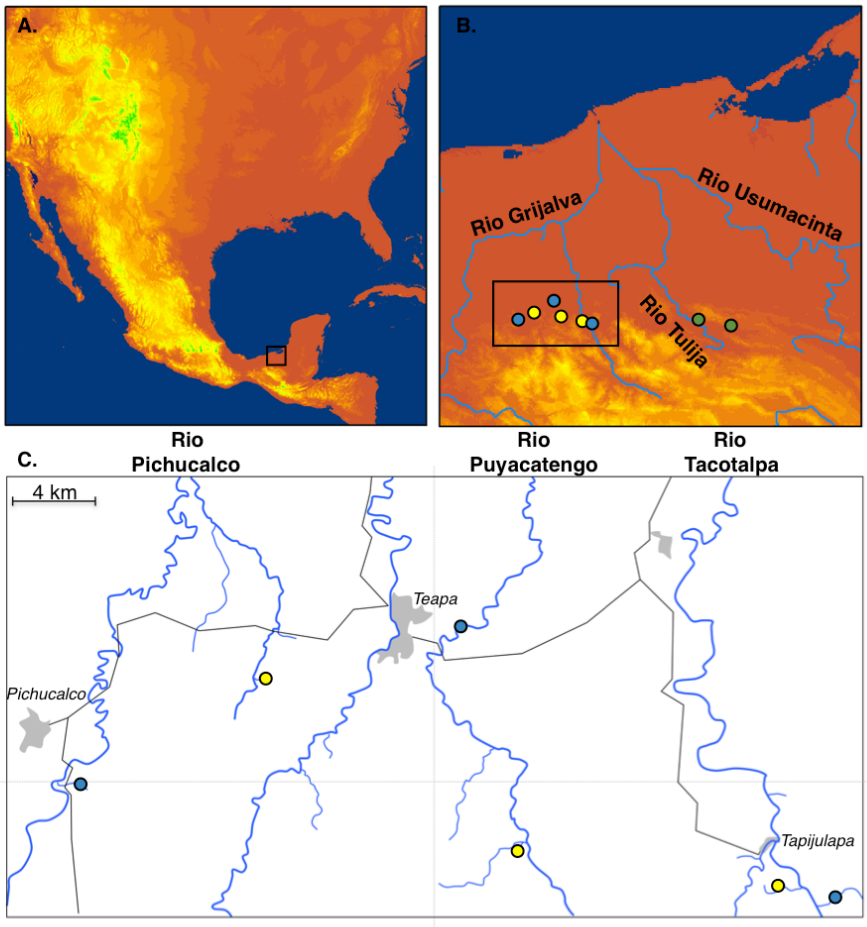

Figure A1. (A) Location of the general study area in southern Mexico. (B) Overview of the study area and sampling localities of *Poecilia* sp. The colors represent sulfidic (yellow), non-sulfidic sympatric (blue), and non-sulfidic allopatric (green) populations from river drainages included in this study as described in Table S1. There are no known records of sulfide springs in the Río Tulija and Río Usumacinta drainages. (C) Detailed view of sympatric sulfidic and non-sulfidic sampling sites in the Ríos Pichucalco, Puyacatengo, and Tacotalpa.

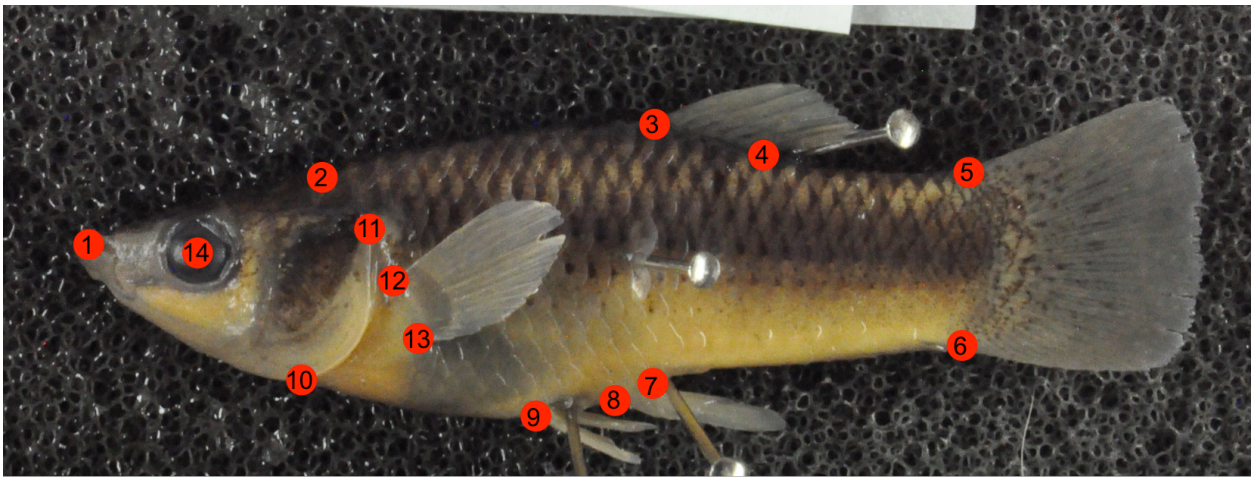

Figure A2. Landmark locations used for geometric morphometric analyses. Landmarks included the tip of the upper jaw (1); the posterior head region (2); the anterior (3) and posterior (4) insertions of the dorsal fin; the dorsal (5) and ventral (6) insertions of the caudal fin; the posterior (7) and anterior (8) insertions of the anal fin; the anterior insertion of the pelvic fin (9); the bottom of the head where the operculum breaks away from the body outline (10); the posterodorsal corner of the operculum (11); the ventral (12) and dorsal (13) insertion of the pectoral fin; and the center of the eye (14).

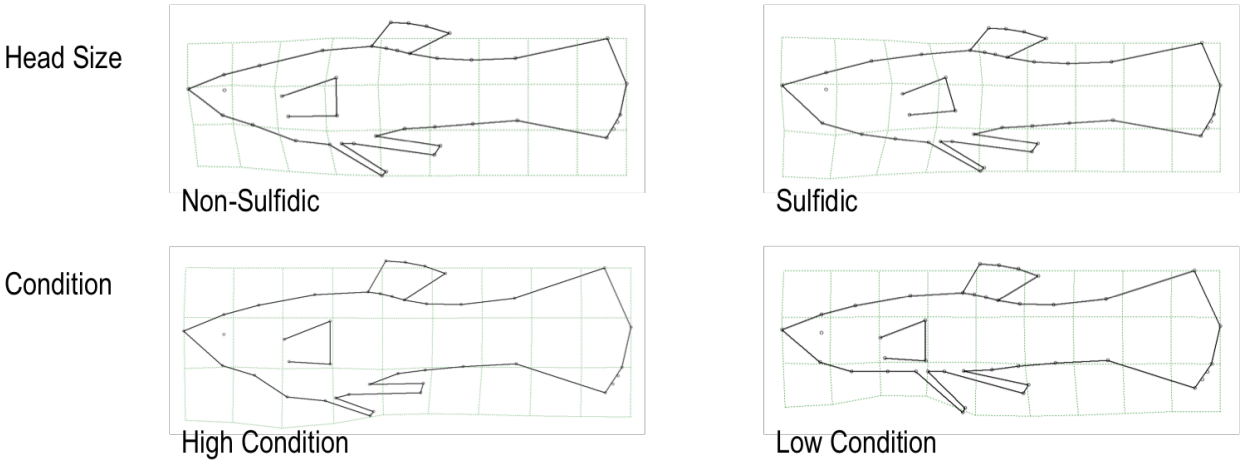

Figure A3. Visualization of *Poecilia mexicana* shape variation in the head and abdomen based on the 42 geometric morphometric landmarks and semilandmarks used by *anyFish*. These outputs from the tps program suite were used as rigs, or "skeletons," in *anyFish* to generate male fish animations.

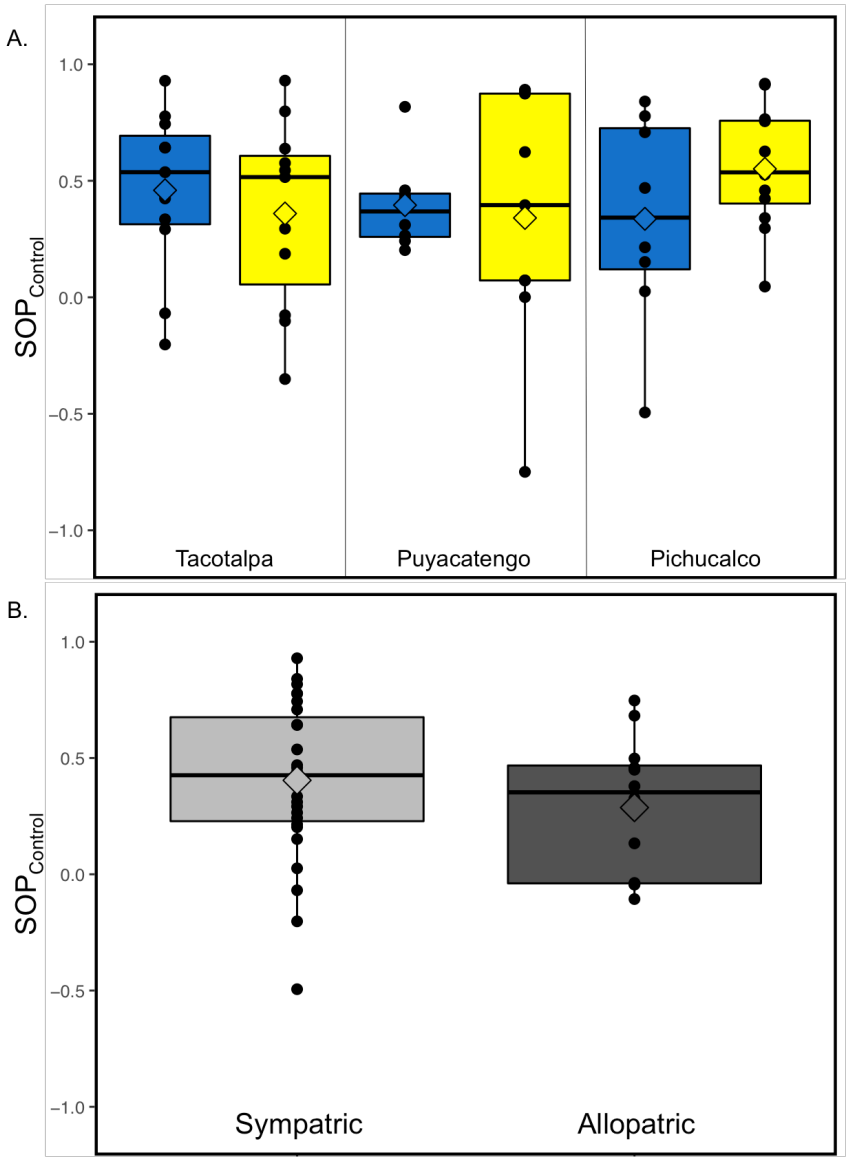

Figure A4. Box plots showing female *Poecilia mexicana* strength of preference (SOP) for animated stimulus male versus a blank background animation, where positive SOP values indicate a preference for the male fish animation over the blank background. (A) Depicts SOP values for sympatric non-sulfidic and sulfidic populations, (B) depicts SOP for sympatric and allopatric non-sulfidic populations.

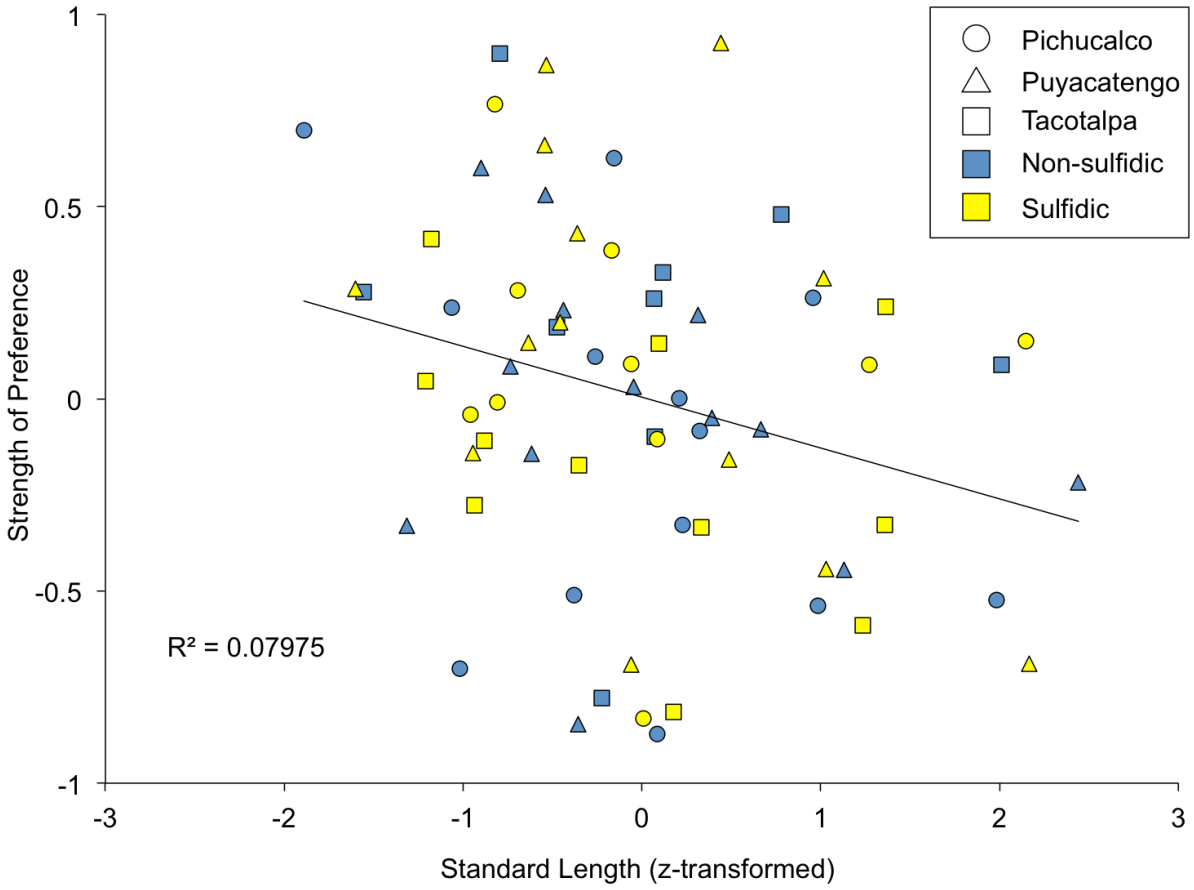

Figure A5. Female strength of preference (SOP) as a function of female size (standard length) for male animations in high (positive SOP) or low condition (negative SOP).

Supplementary Tables

Table A1. Overview of collection sites used in this study. We also provide sample sizes used for the assessment of male body shape variation among populations (wild-caught specimens) and food treatments (laboratory experiment), as well as the number of females tested per site before and after unresponsive females were removed from analyses.

Site	H_2S	Latitude, Longitude	*N* Wild Males	*N* Males fed high/normal/low diet	*N* Females Tested/Responsive
Sympatric populations					
Río Tacotalpa Drainage					
Arroyo Bonita	-	17.427, -92.752	10	8 / 10 / 8	45 / 28
El Azufre I	+	17.442, -92.775	10	10 / 10 / 9	45 / 32
Río Puyacatengo Drainage					
Puyacatengo, at Teapa	-	17.580, -92.898	10		45 / 28
La Lluvia, big spring	+	17.464, -92.896	10		45 / 31
Río Pichucalco Drainage					
Arroyo Rosita	-	17.485, -93.104	10		45 / 30
La Gloria springs	+	17.532, -93.015	10		45 / 34
Allopatric populations					
Río Tulija Drainage					
Tiemopa	-	17.427, -92.193			33 / 18
Río Misol-Há	-	17.387, -91.988			24 / 19

Table A2. Comparison of the strength of preference (SOP) for animated stimulus male versus a blank background animation in females across drainages and habitats, as well as a comparison of the preferences between non-sulfidic females in different geographic contexts.

Effect	***df***	***Mean Square***	***F***	***P***	$\eta_p 2$
Sympatric Populations ($SOP_{Control}$)					
SL (*z*-transformed)	1	0.000	0.001	0.977	<0.001
Drainage	2	0.044	0.297	0.744	0.011
H_2S	1	0.004	0.028	0.868	0.001
Allopatric-Sympatric ($SOP_{Control}$)					
SL (*z*-transformed)	1	0.015	0.126	0.725	0.004
Geographic Context	1	0.077	0.660	0.422	0.020
Population (Geographic Context)	3	0.052	0.452	0.718	0.039

Appendix B - Correlated divergence of female and male genitalia in replicated lineages with ongoing ecological speciation

Supplementary Figures

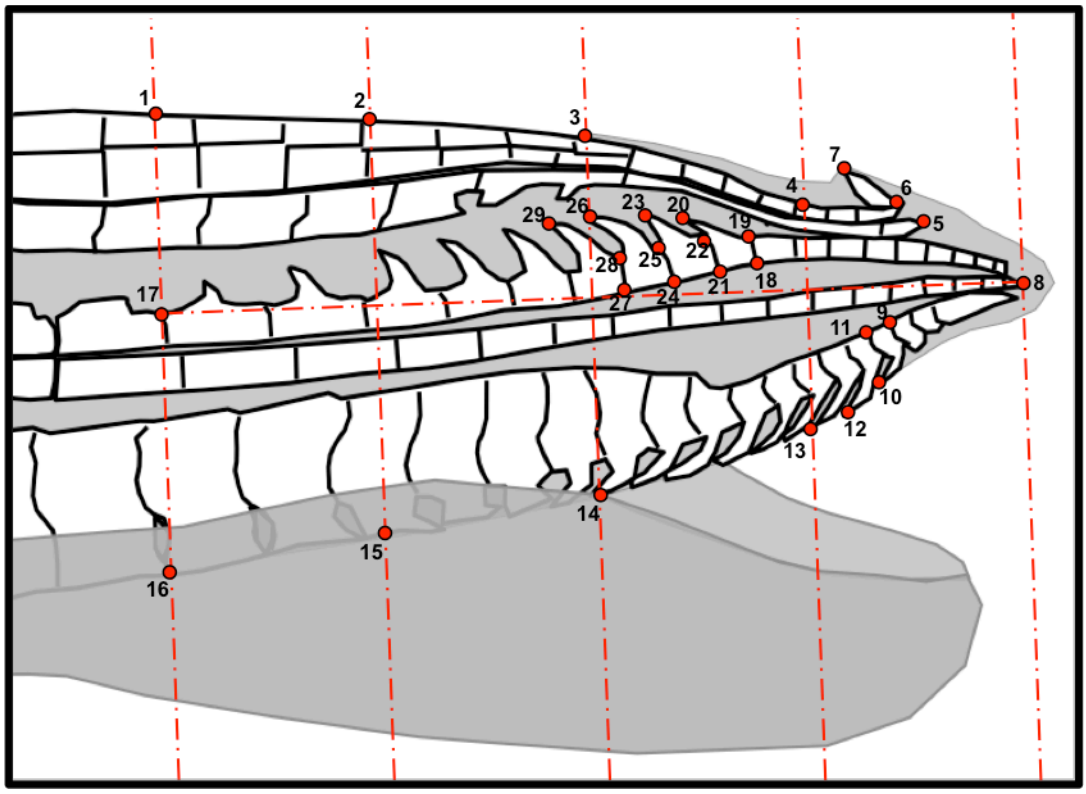

Figure B1. Locations of male landmarks on the gonopodial distal tip, see Table S3 for landmark descriptions. Red dashed lines represent evenly spaced lines added to each gonopodium image used to evenly space landmarks 1-4 along the dorsal edge of the gonopodium and landmarks 13-16 along the ventral edge of the gonopodium.

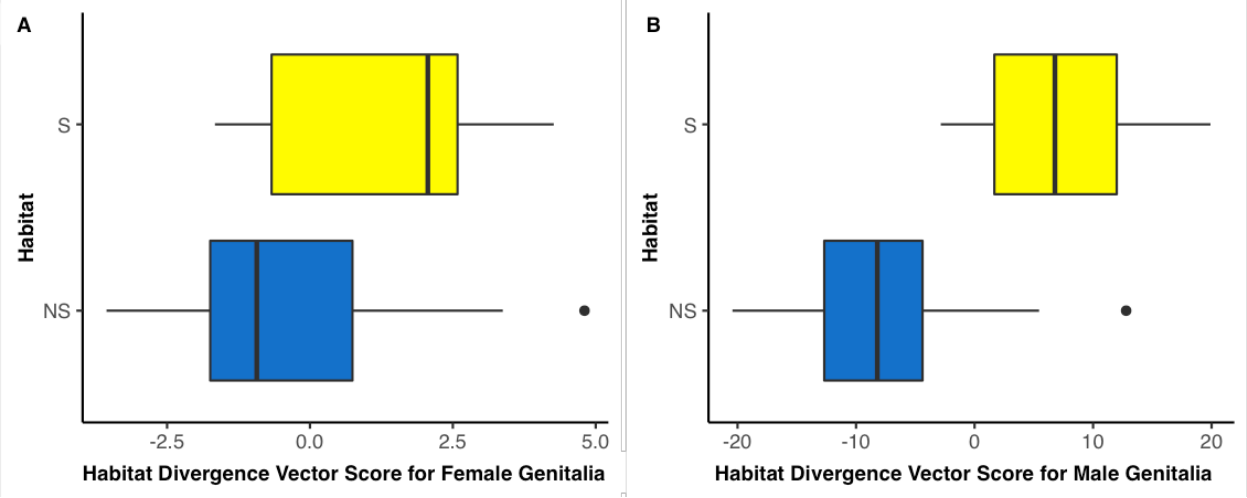

Figure B2. Divergence vector scores for laboratory-reared females (A) and males (B) based on the habitat term in sex-specific MANCOVAs (as described in main text). Note that yellow represents the sulfidic and blue the non-sulfidic population in both panels.

Supplementary Tables

Table B1. Collection locations and sample sizes.

Site	H_2S	Latitude, Longitude	*N* Females	*N* Males
Río Tacotalpa Drainage				
Arroyo Bonita	-	17.427, -92.752	23	17
El Azufre I	+	17.442, -92.775	17	27
Río Puyacatengo Drainage				
Río Puyacatengo at Teapa	-	17.580, -92.898	19	22
La Lluvia, small spring	+	17.464, -92.896	23	25
Rio Ixtapangajoya				
Tributary to Río Ixtapangajoya	-	17.510, -92.980	36	40
La Esperanza, large spring	+	17.511, -92.983	34	21
Río Pichucalco				
Arroyo Rosita	-	17.485, -93.104	23	21
La Gloria springs	+	17.532, -93.015	12	31

Table B2. Female genital trait PC loadings and % variance explained per axis

Trait	PC1 (61.0%)	PC2 (23.6%)	PC3 (10.5%)
Urogenital apertural area	-0.021	-0.390	0.519
Apertural opening length	0.089	-0.396	-0.061
Apertural opening width	-0.152	-0.473	0.000
Apertural opening area	-0.003	-0.437	0.020
Apertural papilla area	-0.014	-0.170	0.610
Proportional opening area	-0.023	-0.481	-0.592
Apertural elongateness (width/length)	-0.692	-0.033	-0.052
Apertural opening aspect ratio	-0.700	0.122	0.036

Table B3. Male gonopodial distal tip landmark descriptions

Landmark #	Landmark description
1-4	Semilandmarks evenly spaced along the dorsal edge of fin ray 5 between landmarks 8 and 17
5	most distal tip of fin ray 5
6	bottom of the bony hook on fin ray 5
7	top of the bony hook on fin ray 5
8	most distal tip of fin ray 4, most distal bony segment of the gonopodium
9	distal edge of the base of the most distal spine on fin ray 3 (spine 1)
10	pointed edge of the most distal spine on fin ray 3 (spine 1)
11	distal edge of the base of the second most distal spine on fin ray 3 (spine 2)
12	pointed edge of the second most distal spine on fin ray 3 (spine 2)
13-16	Semilandmarks evenly spaced along the ventral edge of fin ray 3 between landmarks 8 and 17
17	most proximal-dorsal edge of the most proximal bone segment with a serra along fin ray 4
18	distal-ventral edge of the most distal bone segment with a serra along fin ray 4
19	base of the most distal serra on fin ray 4 (serra 1)
20	tip of the most distal serra on fin ray 4 (serra 1)
21	distal-ventral edge of the second most distal bone segment with a serra along fin ray 4
22	base of the second most distal serra on fin ray 4 (serra 2)
23	tip of the second most distal serra on fin ray 4 (serra 2)
24	distal-ventral edge of the third most distal bone segment with a serra along fin ray 4
25	base of the third most distal serra on fin ray 4 (serra 3)
26	tip of the third most distal serra on fin ray 4 (serra 3)
27	distal-ventral edge of the fourth most distal bone segment with a serra along fin ray 4
28	base of the fourth most distal serra on fin ray 4 (serra 4)
29	tip of the fourth most distal serra on fin ray 4 (serra 4)

Table B4. Eigenvector coefficients of the divergence vector (**DV**) used to determine which principal components (PCs) most contribute to female genital shape variation between habitat types (sulfidic and non-sulfidic).

Variable	**DV**
PC1	-0.02
PC2	1.01
PC3	-0.99

Table B5. Eigenvector coefficients of the divergence vector (**DV**) used to determine which principal components (PCs) most contribute to gonopodial-tip shape differences between habitat types (sulfidic and non-sulfidic).

Variable	**DV**
PC1	0.34
PC2	1.80
PC3	1.37
PC4	-1.82
PC5	0.49
PC6	-0.03
PC7	-0.50
PC8	-0.82
PC9	-0.50
PC10	-0.21
PC11	-0.11

Appendix C - Convergent adaptation and ecological speciation result from unique genomic mechanisms in sympatric extremophile fishes

Supplementary Figures

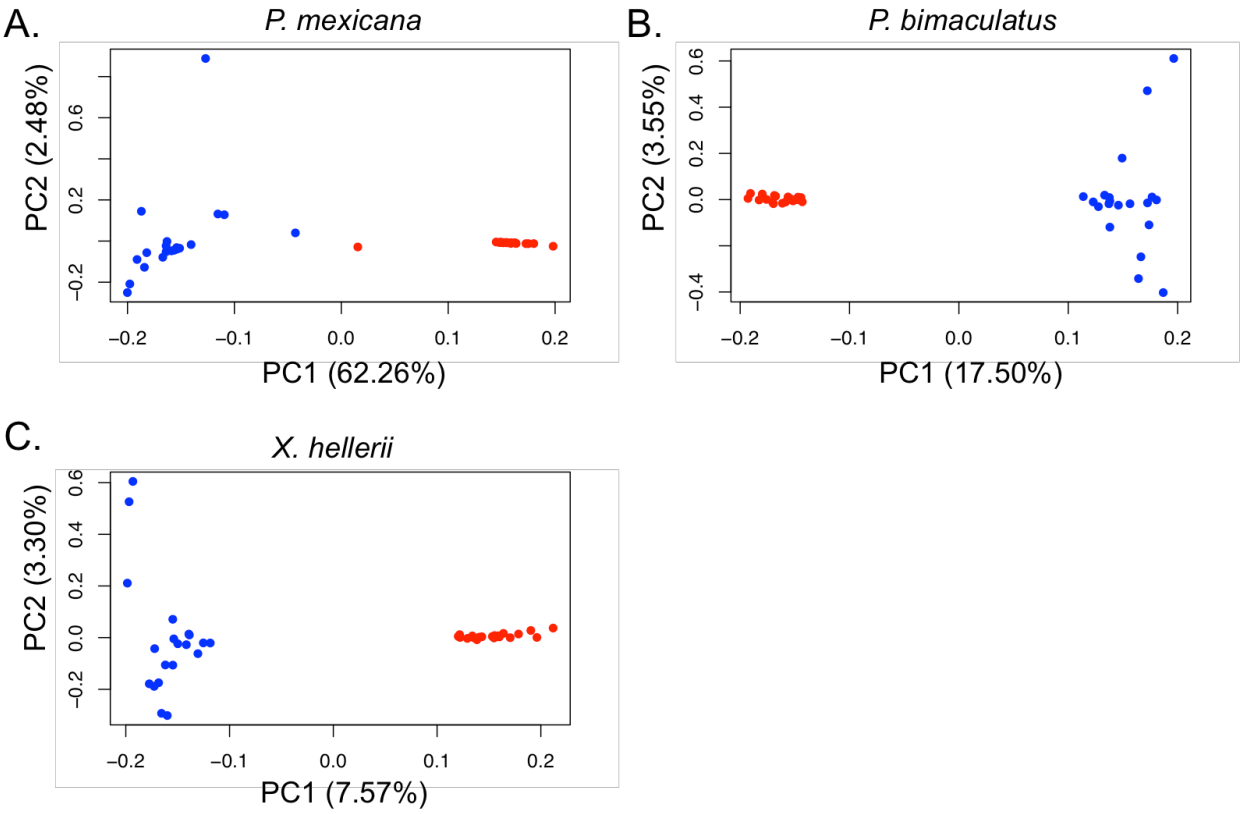

Figure C1. Patterns of genetic variation between sulfidic (red) and non-sulfidic (blue) populations of each species along the first two principal components.

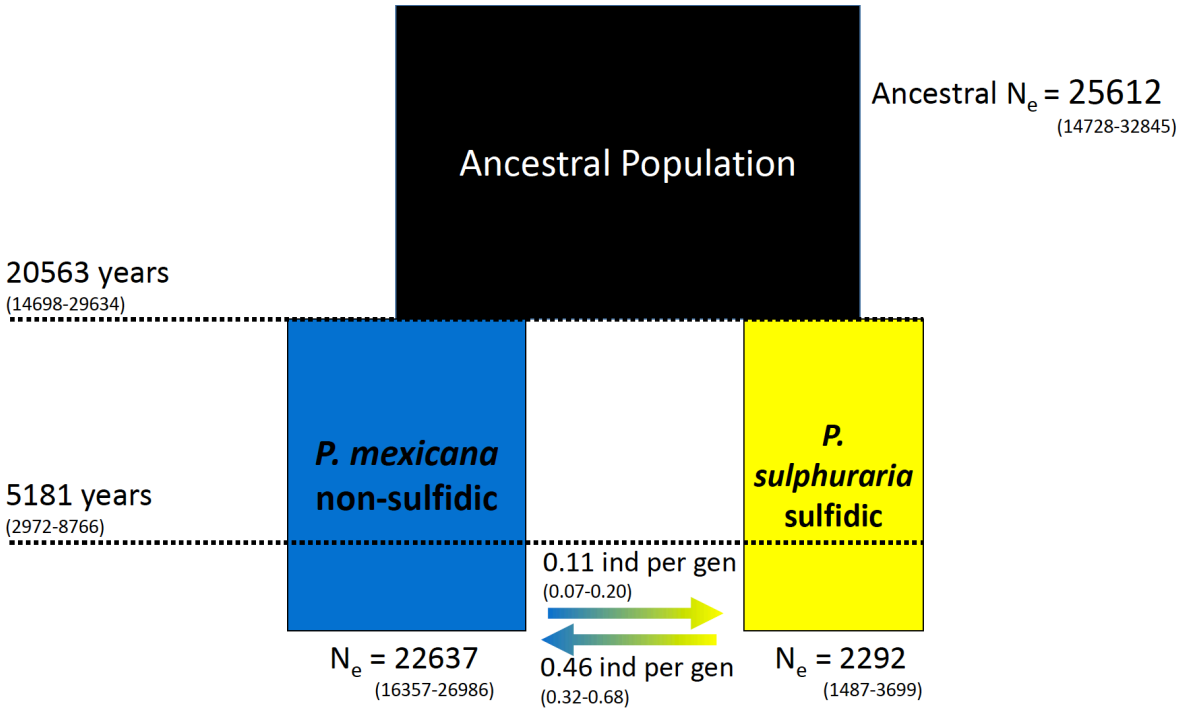

Figure C2. Best supported demographic model for the sulfidic and non-sulfidic *Poecilia* populations as inferred using ∂a∂i. Parentheses denote 95% confidence intervals calculated from 100 bootstrap replicates, N_e values indicate estimates of effective population sizes, dashed lines denote the estimated timing of divergence (top) and secondary contact (bottom), and colored arrows represent estimates of gene flow between populations per generation. Not to scale.

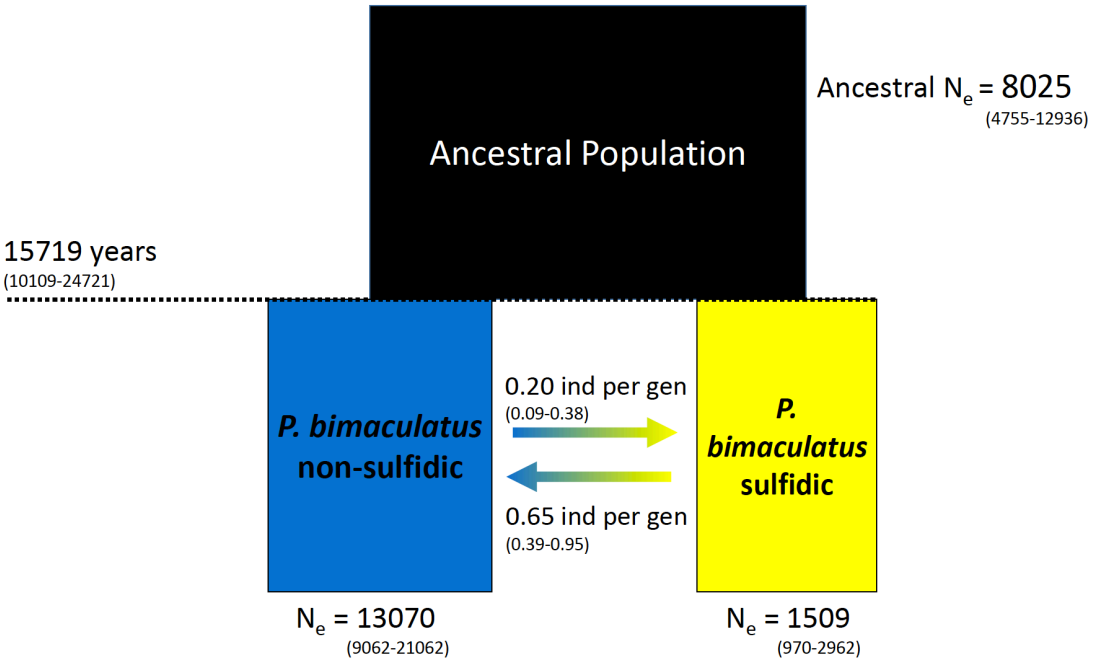

Figure C3. Best supported demographic model for the sulfidic and non-sulfidic *Pseudoxiphophorus* populations as inferred using ∂a∂i. Parentheses denote 95% confidence intervals calculated from 100 bootstrap replicates, N_e values indicate estimates of effective population sizes, the dashed line denotes the estimated timing of divergence, and colored arrows represent estimates of gene flow between populations per generation. Not to scale.

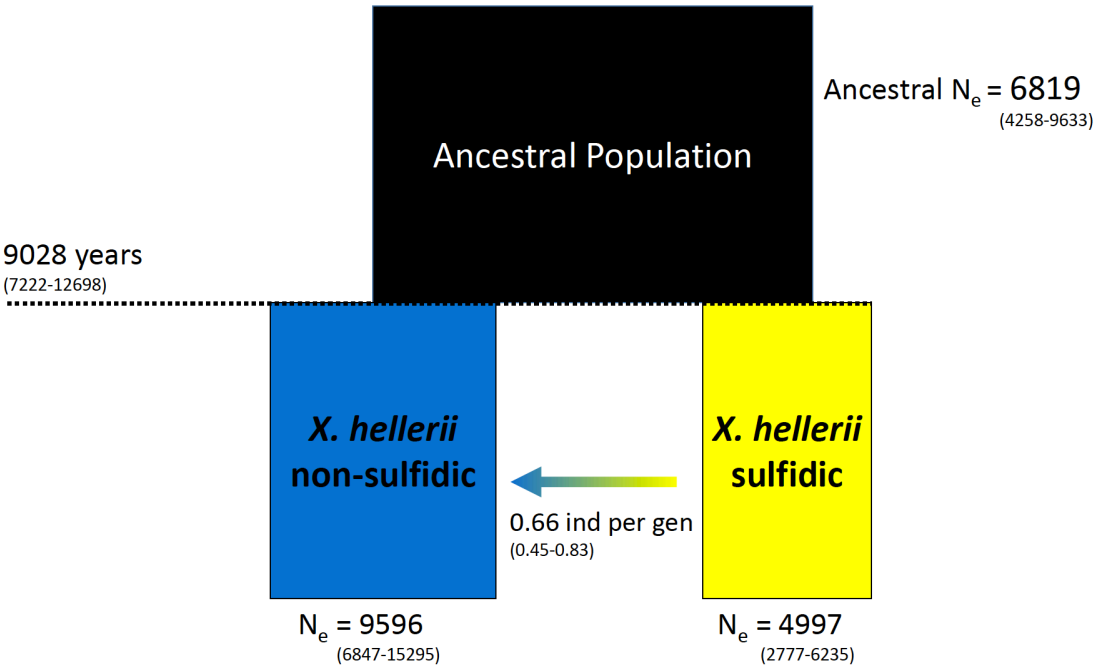

Figure C4. Best supported demographic model for the sulfidic and non-sulfidic *Xiphophorus* populations as inferred using ∂a∂i. Parentheses denote 95% confidence intervals calculated from 100 bootstrap replicates, N_e values indicate estimates of effective population sizes, the dashed line denotes the estimated timing of divergence, and the colored arrow represent estimates of gene flow from the sulfidic population into the non-sulfidic population per generation. Not to scale.

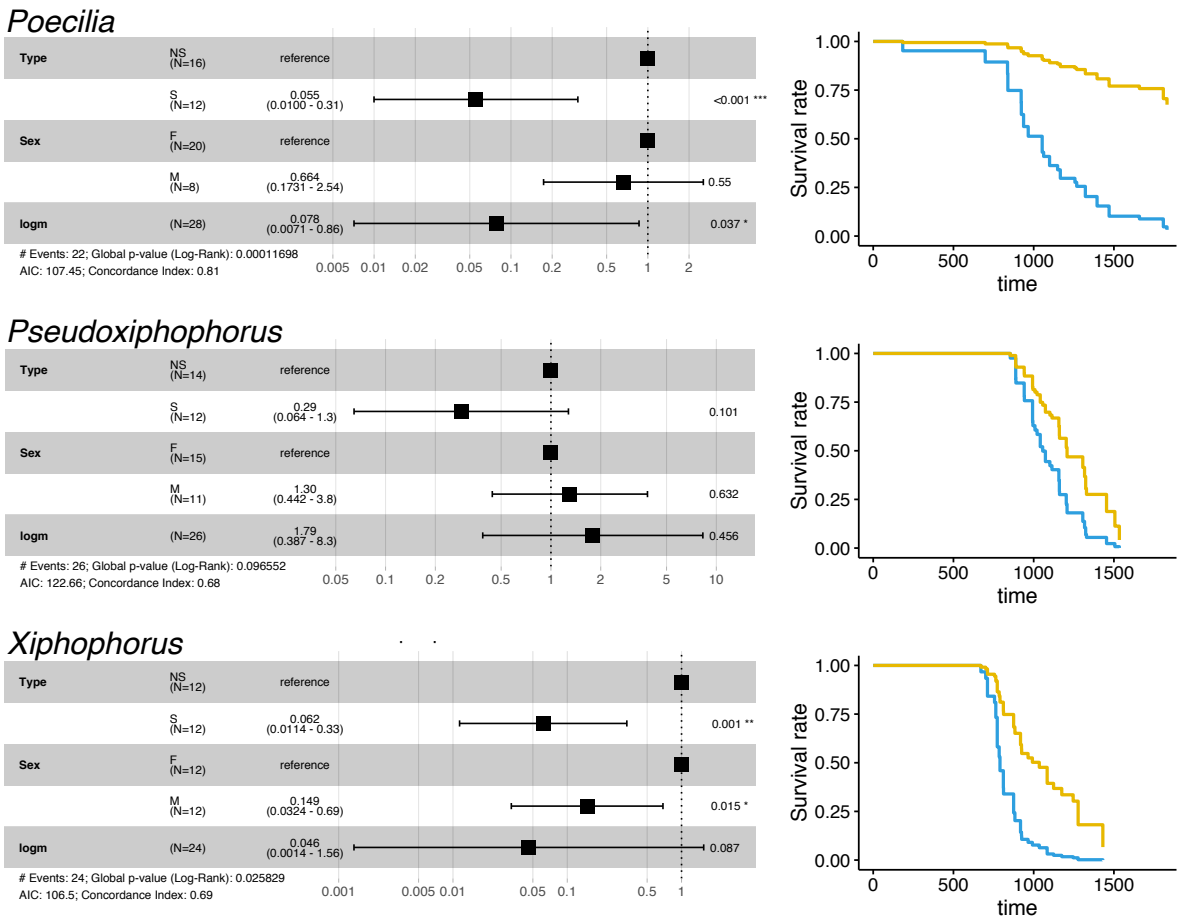

Figure C5. Forest Plot showing hazard ratios and adjusted survival curves from Cox models.

Supplementary Tables

Table C1. Collection locations and sample sizes.

Locality	H_2S	Lat., long.	*N* Morphometrics	*N* Genomes	*N* Translocated (Resident/Migrant)	*N* H_2S Tolerance	*N* RNAseq
La Gloria springs, Rio Pichucalco drainage, Chiapas, Mexico	+	17.532, -93.015					
Poecilia mexicana			52	20	25/25	12	6
Pseudoxiphophorus bimaculatus			63	19	25/25	12	6
Xiphophorus hellerii			99	20	25/25	12	6
Arroyo Caracol, Rio Pichucalco drainage, Chiapas, Mexico	-	17.537, -93.017					
Poecilia mexicana			115	20	25/25	16	6
Pseudoxiphophorus bimaculatus			52	20	25/25	14	
Xiphophorus hellerii			43	20	25/24	12	6
Arroyo Pujil, Rio Ixtapangajoya drainage, Chiapas, Mexico	-	17.476, -92.986					
Pseudoxiphophorus bimaculatus							6

Table C2. Average read counts after quality control, the average percentage of quality-controlled reads that mapped, and the average depth of coverage of genomic DNA reads mapped to the *X. maculatus* genome per population (*n* = 19-20 per population).

Species	Habitat	Paired Reads After Trimming (Mean)	% Paired Reads Aligned (Mean)	Depth of Coverage (Mean)
P. mexicana	NS	32251543.60	87.52	3.330
P. mexicana	S	30998540.60	87.61	3.204
P. bimaculatus	NS	30295782.70	95.53	3.748
P. bimaculatus	S	32404180.11	95.57	4.018
X. hellerii	NS	31606354.70	98.5-	4.321
X. hellerii	S	30549363.70	98.62	4.186

Table C3. Average read counts after quality control and the average percentage of quality-controlled reads that mapped for RNAseq reads mapped to the *X. maculatus* genome per population (*n* = 6 per population).

Species	Habitat	Paired Reads After Trimming (Mean)	% Paired Reads Aligned (Mean)
P. mexicana	NS	83042097.67	97.02
P. mexicana	S	76339057.33	96.30
P. bimaculatus	NS	35247808.00	96.89
P. bimaculatus	S	39545078.33	95.71
X. hellerii	NS	30761274.33	98.75
X. hellerii	S	29296035.00	98.97

Table C4. List of up- and downregulated genes in sulfidic *Poecilia*. Blue rows are upregulated genes, and red are downregulated genes Due to the large size, the table is provided in a separate excel spreadsheet titled "Table C4 – Differential Expression *Poecilia*.xlsx"

Table C5. List of up- and downregulated genes in sulfidic *Pseudoxiphophorus*. Blue rows are upregulated genes, and red are downregulated genes Due to the large size, the table is provided in a separate excel spreadsheet titled "Table C5 – Differential Expression *Pseudoxiphophorus*.xlsx"

Table C6. List of up- and downregulated genes in sulfidic *Xiphophorus*. Blue rows are upregulated genes, and red are downregulated genes Due to the large size, the table is provided in a separate excel spreadsheet titled "Table C6 – Differential Expression *Xiphophorus*.xlsx"

Table C7. List of consistently up- and downregulated genes across all sulfidic populations. Blue rows are upregulated genes, and red are downregulated genes Due to the large size, the table is provided in a separate excel spreadsheet titled "Table C7 – Shared Differential Expression.xlsx"

Table C8. Results of the GO enrichment analysis of differentially expressed genes in *Poecilia* conducted in Gorilla, including a list of GO terms with evidence for significant enrichment (the identification number and description of each GO term), the total number of genes in the reference set (N), the total number of genes associated with a specific GO term (B), the number of genes in the target set (upregulated or downregulated genes; n), and the number of genes in the intersection (b). Enrichment was calculated from these values ([b/n]/[B/N]). Also included is the P-value associated with enrichment and the false discovery rate adjusted significance (q-value). Due to the large size, the table is provided in a separate excel spreadsheet titled "Table C8 – *Poecilia* GO Enrichment.xlsx"

Table C9. Results of the GO enrichment analysis of differentially expressed genes in *Pseudoxiphophorus* conducted in Gorilla, including a list of GO terms with evidence for significant enrichment (the identification number and description of each GO term), the total number ofgenes in the reference set (N), the total number of genes associated with a specific GO term (B), the number of genes in the target set (upregulated or downregulated genes; n), and the number of genes in the intersection (b). Enrichment was calculated from these values ([b/n]/[B/N]). Also included is the P-value associated with enrichment and the false discovery rate adjusted significance (q-value). Due to the large size, the table is provided in a separate excel spreadsheet titled "Table C9 – *Pseudoxiphophorus* GO Enrichment.xlsx"

Table C10. Results of the GO enrichment analysis of differentially expressed genes in *Xiphophorus* conducted in Gorilla, including a list of GO terms with evidence for significant enrichment (the identification number and description of each GO term), the total number of genes in the reference set (N), the total number of genes associated with a specific GO term (B), the number of genes in the target set (upregulated or downregulated genes; n), and the number of genes in the intersection (b). Enrichment was calculated from these values ([b/n]/[B/N]). Also included is the P-value associated with enrichment and the false discovery rate adjusted significance (q-value). Due to the large size, the table is provided in a separate excel spreadsheet titled "Table C10 – *Xiphophorus* GO Enrichment.xlsx"

Table C11. Results of the GO enrichment analysis of differentially expressed genes shared across all species conducted in Gorilla, including a list of GO terms with evidence for significant enrichment (the identification number and description of each GO term), the total number of genes in the reference set (N), the total number of genes associated with a specific GO term (B), the number of genes in the target set (upregulated or downregulated genes; n), and the number of genes in the intersection (b). Enrichment was calculated from these values ([b/n]/[B/N]). Also included is the P-value associated with enrichment and the false discovery rate adjusted significance (q-value). Due to the large size, the table is provided in a separate excel spreadsheet titled "Table C11 – Shared GO Enrichment.xlsx"

Table C12. Results of Procrustes analysis of variance examining variation in body shape in *Poecilia*, *Pseudoxiphophorus*, and *Xiphophorus* from sulfidic and non-sulfidic populations. Effect sizes indicated by *Z* values.

Term	***df***	**SS**	**MS**	**R^2**	***F***	***Z***	***P***
Centroid size	1	0.020	0.020	0.004	7.939	4.901	0.001
Sex	1	0.048	0.048	0.010	19.260	6.220	0.001
Habitat	1	0.006	0.006	0.001	2.286	2.033	0.020
Species	2	0.054	0.027	0.011	10.907	7.505	0.001
Sex × Habitat	1	0.005	0.005	0.001	2.013	1.796	0.034
Sex × Species	2	0.113	0.057	0.023	22.692	8.450	0.001
Habitat × Species	2	0.069	0.034	0.014	13.780	7.488	0.001
Centroid size × Habitat	1	0.002	0.002	0.000	0.929	0.121	0.457
Centroid size × Species	2	0.015	0.007	0.003	2.994	3.698	0.001
Centroid size × Sex	1	0.006	0.006	0.001	2.500	2.279	0.010
Sex × Habitat × Species	2	0.015	0.007	0.003	2.970	3.596	0.001
Residuals	407	1.015	0.002	0.210			
Total	423	4.833					

Table C13. Results of generalized linear mixed models (with binomial error distribution and logit link function) on survival rates during the reciprocal translocation experiments. "Bucket ID" was included as a random factor.

Term	Estimate	SE	*z*	*P*
Poecilia				
Habitat of Origin	3.982	1.503	2.649	0.008
Testing Habitat	7.789	1.893	4.115	< 0.001
$\log_{10}$(SL)	12.904	7.061	1.828	0.068
Habitat of Origin × Testing Habitat	-10.542	2.333	-4.518	< 0.001
Pseudoxiphophorus				
Habitat of Origin	1.937	1.190	1.627	0.104
Testing Habitat	6.629	1.302	0.479	< 0.001
$\log_{10}$(SL)	-5.946	1.474	4.499	0.150
Habitat of Origin × Testing Habitat	-7.026	1.699	-4.135	< 0.001
Xiphophorus				
Habitat of Origin	1.896	1.302	1.456	0.145
Testing Habitat	5.203	1.508	3.451	< 0.001
$\log_{10}$(SL)	-6.813	4.526	-1.505	0.132
Habitat of Origin × Testing Habitat	-6.248	1.879	-3.326	< 0.001

Table C14. Results of survival analysis (Cox regression) of sulfidic and non-sulfidic populations exposed to increasing H_2S concentrations.

Term	β	Hazard Ratio	SE	Wald	*P*
Poecilia					
Habitat	-2.892	0.055	0.875	-3.305	0.001
Sex	-0.410	0.664	0.686	-0.598	0.550
$\log_{10}$(mass)	-2.545	0.078	1.223	-2.081	0.037
Habitat:Sex	1.048	2.852	1.093	0.959	0.337
Pseudoxiphophorus					
Habitat	-1.249	0.287	0.762	-1.640	0.101
Sex	0.264	1.302	0.551	0.479	0.632
$\log_{10}$(mass)	0.583	1.791	0.782	0.745	0.456
Habitat:Sex	1.736	5.673	0.962	1.805	0.071
Xiphophorus					
Habitat	-2.787	0.062	0.860	-3.242	0.001
Sex	-1.902	0.149	0.780	-2.440	0.015
$\log_{10}$(mass)	-3.077	0.046	1.798	-1.711	0.087
Habitat:Sex	2.059	7.837	1.010	2.039	0.041