

## Article

# Intrasexual competition enhances reproductive isolation between locally adapted populations

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## Abstract

During adaptation to different habitat types, both morphological and behavioral traits can undergo divergent selection. Males often fight for status in dominance hierarchies and rank positions predict reproductive success. Ecotypes with reduced fighting abilities should have low reproductive success when migrating into habitats that harbor ecotypes with superior fighting abilities. Livebearing fishes in the *Poecilia mexicana*-species complex inhabit not only regular freshwater environments, but also independently colonized sulfidic (H<sub>2</sub>S-containing) habitats in three river drainages. In the current study, we found fighting intensities in staged contests to be considerably lower in some but not all sulfidic surface ecotypes and the sulfidic cave ecotype compared with populations from non-sulfidic surface sites. This is perhaps due to selection imposed by H<sub>2</sub>S, which hampers oxygen uptake and transport, as well as cellular respiration. Furthermore, migrants from sulfidic habitats may lose fights even if they do not show overall reduced aggressiveness, as physiological performance is likely to be challenged in the non-sulfidic environment to which they are not adapted. To test this hypothesis, we simulated migration of H<sub>2</sub>S-adapted males into H<sub>2</sub>S-free waters, as well as H<sub>2</sub>S-adapted cave-dwelling males into sulfidic surface waters. We found that intruders established dominance less often than resident males, independent of whether or not they showed reduced aggressiveness overall. Our study shows that divergent evolution of male aggressive behavior may also contribute to the maintenance of genetic differentiation in this system and we call for more careful evaluation of male fighting abilities in studies on ecological speciation.

**Key words:** ecological speciation, extremophile teleost, local adaptation, *Poecilia*, premating isolation, selection against migrants.

Ecological speciation describes the process during which reproductive isolation arises as a consequence of adaptation to different ecological selection pressures (Schluter 2001; Hendry 2004; Nosil et al. 2005; Rundle and Nosil 2005). It is hypothesized that evolution of reproductive isolation is more likely if ecological traits under divergent selection also play a role in sexual selection (i.e., “magic traits”), as is the case for traits that function as signals during mate attraction (Feulner et al. 2009; Servedio et al.

2011). Consequently, previous studies on ecological speciation have often considered components of intersexual selection, that is, mate choice. Mate choice can contribute to reproductive isolation through assortative mating (Endler and Houde 1995; Rundle et al. 2005; Svensson et al. 2006; Langerhans et al. 2007; Grant and Grant 2008; Seehausen et al. 2008; Tobler et al. 2009; Ronald et al. 2012; Plath et al. 2013; Harris and Siefferman 2014).

Although divergent abiotic environments have the potential to directly impact the expression of energetically expensive behaviors, such as aggression, the role of divergent expression (and evolution) of aggressive behavior in fostering reproductive isolation remains much less well-studied (Parzefall 1969, 1974, 1979; Peters et al. 1973; Burchards et al. 1985; Langecker et al. 1995; Espinasa et al. 2005; Bierbach et al. 2012; Qvarnström et al. 2012; Kowalko et al. 2013). In systems in which dominance hierarchies play a central role in determining male reproductive success, reduced aggressiveness should translate into male inferiority in mate competition upon encounter of different behavioral phenotypes (Plath and Strecker 2008; Bierbach et al. 2012). This could partly restrict gene-flow between different locally adapted populations; particularly, if males from different ecotypes fair worse in aggressive encounters in the habitat type they are not locally adapted to (see Winkelmann et al. 2014). In this scenario, reduced gene-flow would also depend on selection for either divergent mate choice or strong female habitat fidelity to prevent hybridization among migratory females and locally adapted males (van Doorn et al. 2009).

Livebearing fishes in the *Poecilia mexicana* species-complex have independently adapted to toxic hydrogen sulfide ( $H_2S$ ) in at least three river systems in Chiapas and Tabasco in southern Mexico (Palacios et al. 2013; Riesch et al. 2015). Hydrogen sulfide-rich aquatic habitats provide extreme conditions for extremophile populations, as  $H_2S$  depletes the water of freely available oxygen, hampers oxygen uptake and transport in the blood, and blocks components of cellular oxidative phosphorylation (Pfenninger et al. 2014; Tobler et al. 2014, 2017). Early studies in this system have identified a number of adaptive traits that allowed successful colonizers to exploit sulfidic habitats (Tobler et al. 2008a, 2017; Riesch et al. 2010b, 2011, 2015; Tobler and Plath 2011; Pfenninger et al. 2014; Kelley et al. 2016). Besides other traits (see the “Discussion” section),  $H_2S$ -adapted populations convergently evolved enlarged heads. It is proposed that the larger buccal cavity allows passage of larger volumes of water over larger gill surfaces, thereby increasing potential for oxygen uptake in oxygen deplete environments (Tobler et al. 2011). Ecotypes that lack specialized adaptations to cope with  $H_2S$ -related toxicity experience high mortality when migrating into sulfidic waters (Tobler et al. 2009; Plath et al. 2013). On the other hand, sulfide-adapted fish have the ability to venture into surrounding non-sulfidic habitats at least for short periods of time, and both sulfide-adapted and not sulfide-adapted ecotypes can be found interacting in non-sulfidic water at the confluence of sulfidic creeks and (larger) non-sulfidic streams (Sommer-Trembo et al. 2016).

Population genetic studies have identified low levels of gene-flow among *P. mexicana* populations from different habitat types but clearly detected evidence for unidirectional migration from sulfidic to non-sulfidic habitats (Plath et al. 2013). Even though female choice is thought to determine the outcome of male mate competition to a large extent as female poeciliids have a preferences for large, dominant males (Sommer-Trembo et al. 2016), and females (at least from non-sulfidic habitats) preferentially mate with males from their own population (Tobler et al. 2009; Plath et al. 2010, 2013; Greenway et al. 2016), dominance hierarchies among males and use of coercive mating tactics (Evans et al. 2003; Plath et al. 2007b; Magurran 2011; Bierbach et al. 2013a) could, in theory, impede the evolution of reproductive isolation. At the interface between habitats where different ecotypes frequently meet,

dominant males may monopolize females from different ecotypes and thus foster hybridization events (Qvarnström et al. 2012).

It has been hypothesized that sulfide-adapted males may lose fights with resident males when migrating into non-sulfidic habitats (Bierbach et al. 2012). Fish from  $H_2S$ -containing waters have lower body condition and fat stores (Tobler et al. 2006; Plath et al. 2007c; Riesch et al. 2010b, 2011), which may be a consequence of low food availability in such extreme habitats: Fishes in these low-oxygen environments spend more time (and energy) at the surface, where water oxygen concentration is higher, reducing the time spent foraging (Plath et al. 2007c). In addition, physiological costs associated with  $H_2S$ -detoxification likely play very important roles (Grieshaber and Völkel 1998; Tobler et al. 2016, 2017).

Previous studies reported a reduction of male sexual activity in extremophile *P. mexicana* populations (Plath et al. 2003; Plath 2008). Assuming that this is due to physiological limitations on energy expenditure, it is likely that these fish will show a reduction in male aggressiveness. This should reduce the reproductive success of immigrant (sulfide-adapted) males in non-sulfidic stream sections through both mate choice and male competition. In theory, a pattern of sulfide-adapted males being inferior in mate competition with males from non-sulfidic habitats could emerge even when sulfide-adapted males are not less aggressive overall in their sulfidic habitats but show impaired physiological performance in non-sulfidic water (see Plath et al. 2010, 2013).

An additional ecological variable in our study system is the presence or absence of light: In the Tacotalpa drainage, *P. mexicana* colonized two limestone caves, the sulfidic Cueva del Azufre and the non-sulfidic Cueva Luna Azufre (Tobler et al. 2008b; Plath and Tobler 2010). Cave populations have reduced but still functional eyes, which are, however, slightly smaller than those of surface-dwelling fish (Tobler et al. 2008a; Plath and Tobler 2010). A previous study demonstrated that males from the sulfidic cave show reduced aggression compared with males from sulfidic surface and non-sulfidic surface populations in that drainage, and that at least a part of those behavioral population differences are heritable (Bierbach et al. 2012).

In our present study, we tested whether sulfidic ecotypes from three different river drainages show reduced aggression compared with males from adjacent non-sulfidic populations. In the Tacotalpa drainage, we also confirmed that males from the sulfidic cave population are less aggressive compared with sulfidic as well as non-sulfidic surface populations. Given the unidirectional migration from sulfidic habitats to non-sulfidic ones as well as from the cave to sulfidic surface and from there to non-sulfidic surface habitats, we furthermore asked whether migrating males would lose fights in aggressive encounters with resident males, thus fostering reproductive isolation.

## Materials and Methods

### Study system and test animals

Wild-caught mollies (*Poecilia* spp.) were collected near the city of Teapa in southern Mexico (federal state of Tabasco, United States of Mexico). In this region, the mountains of the Sierra Madre de Chiapas meet the wide floodplains of northern Tabasco. Several sulfide spring complexes of volcanic origin (Rosales-Lagarde et al. 2008) are distributed across three major tributaries of the Río Grijalva (from east to west: Ríos Tacotalpa, Puyacatengo, and Pichucalco; Figure 1). At least four spring complexes were

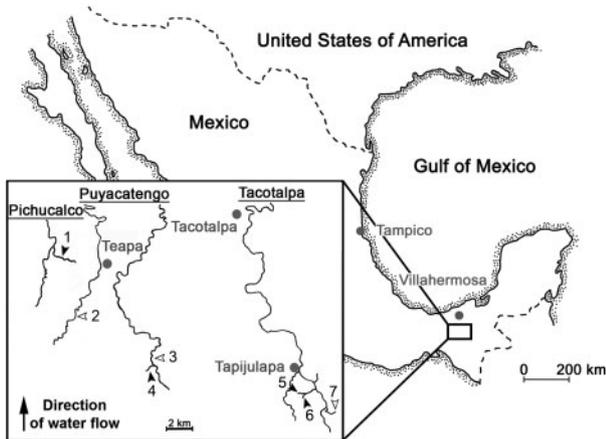
independently colonized by poeciliid fishes (*P. mexicana*, *P. sulphuraria*, *Gambusia eurystoma*, *Pseudoxiphophorus bimaculatus*, and *Xiphophorus hellerii*; Palacios et al. 2013; Plath et al. 2013; Greenway et al. 2014; Riesch et al. 2016). Non-sulfidic and sulfidic (H<sub>2</sub>S-containing) habitats within each drainage are interconnected and not separated by physical barriers that would prevent migration. In the Tacotalpa drainage also two limestone caves were colonized by *P. mexicana*, the sulfidic Cueva del Azufre and the non-sulfidic Cueva Luna Azufre (Tobler et al. 2008b; Plath and Tobler 2010). Thus, the study system provides four distinct ecotypes (surface: sulfidic and non-sulfidic; cave: sulfidic and non-sulfidic). However, in the current study, we were not able to include fish from the non-sulfidic cave Cueva Luna Azufre due to a flooding event that prevented access to this cave. In the Río Pichucalco drainage, the sulfide ecotype has been described as a distinct species, *P. sulphuraria* (Alvarez 1948), which is endemic to sulfide spring complexes at the Baños del Azufre and Rancho La Gloria (Tobler et al. 2008c; Palacios et al. 2013; Greenway et al. 2014). Phylogenetic analyses suggest that colonization of H<sub>2</sub>S-springs occurred first in the Pichucalco drainage by *P. sulphuraria* (between

200 and 500 ka ago; Pfenninger et al. 2014), which show strong phylogenetic affinity to present Northern Mexican *P. mexicana limantouri* (Tobler et al. 2011; Palacios et al. 2013; Plath et al. 2013; Pfenninger et al. 2014). Invasion of H<sub>2</sub>S-springs in the Tacotalpa and Puyacatengo drainages was more recent (approximately 100 ka ago) and those sulfide-adapted ecotypes each are closely related to *P. mexicana mexicana* from adjacent non-sulfidic habitats (Tobler et al. 2011; Plath et al. 2013; Pfenninger et al. 2014; Kelley et al. 2016). In the case of the sulfide cave-dwellers, fish were collected from a front chamber of the Cueva del Azufre (chamber II; after Gordon and Rosen 1962), which receives some light through cracks in the cave roof.

Focal fish (only males; see Tables 1 and 2 for sample sizes) were caught with a seine (4 m long, 4 mm mesh-width) and immediately transported in aerated coolers to a field station in Teapa (*Centro de Investigación e Innovación para la Enseñanza y el Aprendizaje*). Details on collection sites can be found in Figure 1. To avoid ambiguity of species assignment, we did not collect fish in the mixing zones of sulfidic and non-sulfidic waters. Upon capture, all specimens were individually inspected and assigned to ecotypes using distinctive external morphological features (Tobler et al. 2011). Prior to the experiments, fish were acclimated to laboratory conditions in aerated 42-L tanks and in case of sulfide-adapted fish to normoxic (non-sulfidic) conditions for 3 days, which is within the range of previously used acclimation periods (see Plath et al. 2013; Sommer-Trembo et al. 2016). This was done to allow sulfide-adapted ecotypes to gradually habituate to normoxic and sulfide-free conditions, as previous attempts to conduct the experiments under sulfidic conditions failed. We initially held fish in water from the collection site but gradually exchanged the water on a daily basis and replaced it by sulfide-free stream water. We provided *ad libitum* amounts of TetraMin® flake food during the course of the experiment.

### General testing procedure

To test whether sulfide-adapted surface as well as cave-dwelling populations show an overall reduction in male aggressiveness compared with their counterparts from non-sulfidic habitats, we staged contests of size-matched males from the same ecotypes (sulfidic surface, non-sulfidic surface, as well as sulfidic cave). Fighting intensity was scored as the number of aggressive interactions per pair. We decided to sum aggressions per pair in intra-ecotype pairs



**Figure 1.** Map of the study area. (1) Baños del Azufre (sulfidic, black arrow); (2) Río Ixtapangajoya (non-sulfidic, clear arrow); (3) Puyacatengo bridge (non-sulfidic); (4) Puyacatengo springs (sulfidic); (5) Cueva del Azufre (sulfidic cave); (6) El Azufre creek (sulfidic); and (7) Arroyo Bonita (non-sulfidic). Cities are indicated with dots in light gray while river systems are underlined black.

**Table 1.** Overview of populations used to establish mean aggressiveness of different ecotypes in the *Poecilia mexicana*-species complex; numbers of sampling sites follow Figure 1 (see main text)

Population	N	Light	H <sub>2</sub> S	Mean pair size	Mean SL difference	t	df	P
Pichucalco drainage								
<i>P. sulphuraria</i> (1)	9	+	+	27.3 ± 0.7	1.7 ± 0.3	-0.86	8	0.42
<i>P. mexicana</i> (2)	7	+	-	34.6 ± 1.9	1.3 ± 0.4	1.47	6	0.19
Puyacatengo drainage								
<i>P. mexicana</i> (4)	10	+	+	25.0 ± 0.4	0.8 ± 0.6	2.25	9	0.05
<i>P. mexicana</i> (3)	4	+	-	36.2 ± 2.6	1.5 ± 0.3	2.1	3	0.12
Tacotalpa drainage								
<i>P. mexicana</i> (7)	11	+	-	32.3 ± 0.9	1.0 ± 0.3	2.01	10	0.06
<i>P. mexicana</i> (6)	13	+	+	27.3 ± 0.5	0.9 ± 0.2	0.00	10	1.00
<i>P. mexicana</i> (5)	14	-	+	28.2 ± 0.6	1.9 ± 0.3	1.58	4	0.19

**Notes:** Listed are the numbers of fights analyzed, relevant ecological habitat parameters [light absent (-) or present (+); H<sub>2</sub>S absent (-) or present (+)], as well as mean dyad body size (standard length, SL) and mean body size difference [both in (mm)]. Paired *t*-tests were used to compare the body size of winners and losers in fights where one male established dominance.

**Table 2.** Outcome of staged fights between males from different ecotypes (see Figure 1 in the main text for sampling site codes)

Ecotype pairs	<i>N</i>	Resident SL	Intruder SL	<i>t</i>	<i>df</i>	<i>P</i>	Resident won	Intruder won	$\chi^2$	<i>P</i>
Pichucalco drainage										
<i>P. mexicana</i> (2) versus <i>P. sulphuraria</i> (1)	17	25.1 ± 0.4	24.6 ± 0.5	0.89	16	0.39	14	1	11.27	0.001
Puyacatengo drainage										
<i>P. mexicana</i> (2) versus <i>P. mexicana</i> (4)	10	26.2 ± 0.5	25.8 ± 0.5	1.18	9	0.27	7	1	4.50	0.034
Tacotalpa drainage										
<i>P. mexicana</i> (7) versus <i>P. mexicana</i> (6)	15	31.5 ± 0.8	30.8 ± 0.8	1.09	14	0.30	11	2	6.21	0.013
<i>P. mexicana</i> (6) versus <i>P. mexicana</i> (5)	12	26.4 ± 0.9	25.7 ± 0.8	1.87	11	0.09	7	1	4.50	0.034

Notes: We defined the not sulfide-adapted ecotype as “resident” male, while the sulfide- or cave-adapted male was defined as “intruder.” Listed are the numbers of fights, mean body sizes of resident and intruder males (SL in mm), and results from paired *t*-tests comparing male body size of winners and losers. Numbers of fights won by both resident and intruder males are given, along with the results of  $\chi^2$ -tests.

as we showed in a previous study that fight dynamics in dyads of size-matched males are very even until dominance is established (Bierbach et al. 2012). This seems to be an inherent feature of fights in poeciliids (Bierbach et al. 2013a).

To address the question of whether males migrating from sulfidic surface to non-sulfidic surface habitats as well as from the cave to the surface could compete with resident males, or whether they have a disadvantage in intrasexual competition in their ancestral habitats (Bierbach et al. 2012), we simulated possible migration events by staging fights between size-matched males of the resident ecotype and migrating males in all three drainages and assessed the likelihood of migrating males establishing dominance over resident ones. Here, we scored aggressive behaviors for each individual in a pair instead of summing up aggressive behaviors per pair. This was done to see whether fight dynamics may have differed between males of different ecotypes. Due to a lack of males from non-sulfidic sites in the Río Puyacatengo, we staged contests between sulfide-adapted males from the Río Puyacatengo sulfidic springs and males from the nearby, non-sulfidic Río Ixtapangajoya (Table 1 and Figure 1). The Río Ixtapangajoya/Río Teapa is a confluent to the Río Puyacatengo, and overall aggressiveness of males from these two non-sulfidic sites did not differ (Figure 2A; independent-samples *t*-test:  $t_9 = 0.28$ ,  $P = 0.79$ ).

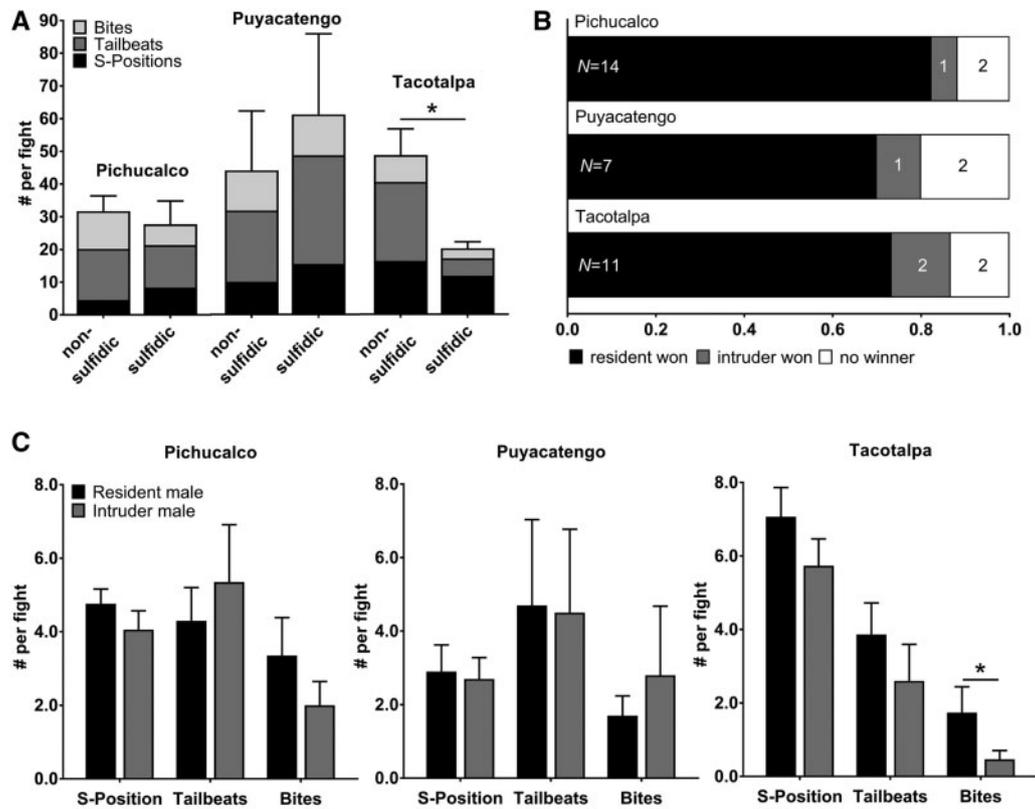
We could not stage fights in H<sub>2</sub>S-containing water, which would provide sulfide-adapted ecotypes which their natural conditions and would have simulated migration from the clear-water habitats into sulfidic ones for three reasons: (i) fish from non-sulfidic habitats had exceedingly low survival rates when translocated into sulfidic habitats (Tobler et al. 2011), (ii) genetic analyses found little migration in this direction (Plath et al. 2013), and (iii) maintaining constant H<sub>2</sub>S concentrations during experimentation is difficult since H<sub>2</sub>S degasses very quickly.

### Behavioral assessment of aggressiveness and dominance

Plexiglas mouse cages (23 × 15 × 16.5 cm), filled with river water, were used to stage dyadic encounters between size-matched males. Males were separated overnight by an opaque plastic divider placed in the center of the test tank. The outer sides were covered with gray paper to minimize external disturbance. In both experiments, male contestants were closely size-matched (see *t*-tests on body size differences in Tables 1 and 2). An air pump provided well-oxygenated water during this period, but the air stone was removed from the tanks directly prior to the testing phase. Males within each dyad had

been kept in different tanks prior to the experiment and were thus not familiar to each other.

Observations took place on the next day between 09:00 and 13:00. At the beginning of the experiment, we removed the partition separating the two males and recorded behavioral interactions for a maximum of 10 min, starting with the first interaction. We separated both contestants immediately after dominance was established to avoid serious injuries. Apart from the loss of single scales, no severe injuries and no mortality occurred during these experiments. If dominance was not established after 10 min of observation we terminated the contest and scored fight outcome as “no winner discernible.” Even though dyads were matched for body size, males differed in aspects of their fin and body coloration and were thus easily distinguishable. We focused on three aggressive behaviors that occur frequently in *Poecilia* spp. (Parzefall 1969; Bierbach et al. 2012, 2013a). (1) S-position: This threat display usually initiates a fight. Males swim in a parallel or anti-parallel position and bend their bodies in an S-shaped manner while all unpaired fins are erected. (2) Tail-beats: The aforementioned behavior is often followed or superimposed by tail-beats, which involve fast movements of head and tail in opposing directions that either touch the opponent’s body or sent shock waves toward the opponent. (3) Bites: We defined all incidences of ramming and attack with the mouth into the direction of the opponent as bites, because these behaviors occur too fast and are too similar to be distinguishable by the human eye. *Poecilia mexicana* males are known to exhibit homosexual behavior; cave-dwelling populations in particular tend to respond to aggressive behavior by showing sexually motivated behaviors (Parzefall 1969; Tobler et al. 2005). Although we observed several homosexual interactions in fights involving cave-adapted males, frequencies were low (mean ± SEM: 0.9 ± 1.6 homosexual behaviors per fight) and so these behaviors were excluded from our analyses. Contest outcome was evaluated based on behavioral differences between the competitors (Morris et al. 1992, 1995). Folded fins, head-down posture, and a position at the periphery of the tank typically characterized the loser of the contest. Winners, on the other hand, constantly chased and further attacked losers with spread fins while occasionally displaying S-positions. We are aware of other methods to establish aggression in fishes (e.g., mirror tests; Balzarini et al. 2014) but decided to use staged-contests throughout our study as these can also be applied to study contests between different ecotypes. After a contest, we measured body size (standard length, SL) of both contestants to the nearest millimeter by briefly laying the fish flat on plastic foil-covered millimeter paper (see Tables 1 and 2).



**Figure 2.** General aggressiveness and intruder inferiority in staged fights involving males from sulfide-adapted and not sulfide-adapted ecotypes. (A) Contests of males from the same ecotype; shown are mean ( $\pm$ SEM) numbers of aggressive behaviors per fight. (B) Intruder inferiority in all three drainages. Shown are fractions of fights won by the “resident” male (not sulfide-adapted ecotype) or by the intruder, and fights with no clear winner. In all three drainages, resident males were significantly more likely to win contests ( $\chi^2$ -tests, see Table 2). (C) Details of fights between resident and intruder males in all three drainages. Shown are mean ( $\pm$ SEM) numbers of aggressive behaviors per fight shown by both types of males. Asterisks indicate significant differences in *post hoc* *t*-tests (see main text).

### Statistical analyses

All analyses were performed using SPSS 23 (IBM). To achieve approximate Gaussian distribution, all dependent data were log-transformed prior to statistical analyses. We compared frequencies of aggressive behaviors (using log-transformed numbers of S-positions, tail beats, and bites/ramming per fight as dependent variables) during fights of males from the same ecotypes by means of multivariate general linear models (GLM; *F*-ratios calculated from Wilk’s lambda). We ran two separate analyses: First, we analyzed only the subset of fights of sulfide-adapted and not sulfide-adapted surface ecotypes, while including the main factor “ecotype” (nested within drainage). Second, we analyzed the subset of ecotypes in the Río Tacotalpa drainage including the cave ecotype, again using “ecotype” as a main factor: (1) the non-sulfidic creek Arroyo Bonita, (2) the sulfidic creek El Azufre, and (3) the sulfidic cave Cueva del Azufre. This approach was chosen as cave ecotypes from the Tacotalpa drainage have no cave-dwelling counterparts in the two other drainages, and a comparison with fish from other drainages is thus biologically irrelevant. *Poecilia mexicana* can differ dramatically in adult male body size (Bierbach et al. 2012, 2013a) which might affect aggressive behavior. Thus, although we staged fights between size-matched males, the mean body size of male dyads differed between trials and so we initially included “mean dyad body size” as a covariate in our models. Furthermore, we accounted for minute differences in body size within pairs and thus initially also included “body size difference” as another covariate.

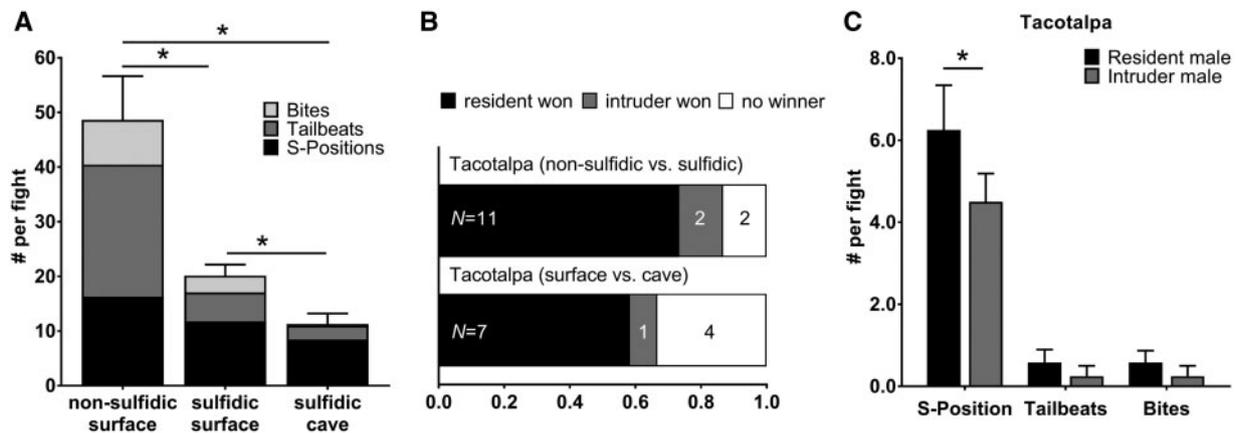
However, both covariates and their interactions with the main factor “ecotype” were excluded from the final models as they had no statistically significant effects ( $F < 0.8$ ,  $P > 0.5$ ). We used independent-samples *t*-tests for *post hoc* comparisons of overall aggressiveness (log-transformed sum of all aggressive behaviors) between ecotypes in all three drainages (sulfidic versus non-sulfidic) and Fisher’s least significant differences (LSD) for comparing the three ecotypes in the Tacotalpa drainage.

For our cross-ecotype fights, we employed  $\chi^2$ -tests to compare numbers of fights won by males from either ecotype. Moreover, we compared numbers of aggressive behaviors shown by individual males from both ecotypes using paired-samples *t*-tests.

### Results

#### Aggressiveness of sulfide-adapted and not sulfide-adapted surface ecotypes

When comparing numbers of aggressive behaviors in dyadic fights between males from sulfidic and non-sulfidic surface habitats from all three drainages investigated herein, we found a significant effect of the main factor “ecotype (nested within drainage)” (multivariate GLM:  $F_{15,127.4} = 4.00$ ,  $P < 0.001$ ), suggesting that ecotypes differed in at least one drainage under investigation. *Post hoc* independent-samples *t*-tests comparing the aggressiveness of ecotypes from sulfidic and non-sulfidic habitats within drainages found the sulfide-adapted ecotype in the Río Tacotalpa drainage to show significantly



**Figure 3.** General aggressiveness and intruder inferiority in the Tacotalpa drainage. (A) Comparison of aggressiveness among ecotypes in contests of males from the same ecotype. Shown are mean ( $\pm$ SEM) numbers of aggressive behaviors per fight. Asterisks indicate significant differences in *post hoc* LSD tests. (B) Intruder inferiority in staged encounters of “migrating” males of the cave-dwelling ecotype into adjacent sulfidic surface waters, or of males from the surface-dwelling sulfide-adapted ecotype into adjacent non-sulfidic surface waters. Shown are fractions of fights won by resident or intruder males, as well as fights in which no winner was discernible. In both cases, resident males were significantly more likely to establish dominance ( $\chi^2$ -tests, see Table 2). (C) Details of staged fights between resident (surface-dwelling sulfide-adapted ecotype) and intruder males (sulfidic cave-ecotype). Shown are mean ( $\pm$ SEM) numbers of aggressive behaviors shown by both types of males per fight. Details on contests between males from the surface-dwelling not sulfide-adapted ecotype and the surface-dwelling sulfide-adapted ecotype (El Azufre) are shown in Figure 2C. Asterisks indicate significant differences in *post hoc* paired *t*-tests.

reduced aggression ( $t_{22} = 4.66$ ,  $P < 0.001$ ; Figure 2A), while ecotypes did not differ significantly in mean aggressiveness in the other two drainages (Río Pichucalco:  $t_{14} = 0.98$ ,  $P = 0.34$ ; Río Puyacatengo:  $t_{14} = 0.98$ ,  $P = 0.34$ ; Figure 2A).

#### Intruder inferiority in fights between sulfide-adapted and not sulfide-adapted surface males

In all staged contests that simulated migration of males from sulfidic into non-sulfidic surface habitats, resident (i.e., not sulfide-adapted) males were significantly more likely to establish dominance (Figure 2B and Table 1). In congruence with the results from the staged contests of males from the same ecotype (see above), we found pronounced differences in numbers of aggressive behaviors between resident and migrant males in the Tacotalpa drainage, and males from the non-sulfidic Arroyo Bonita directed significantly more bites toward sulfide-adapted El Azufre males than vice versa (paired-samples *t*-test:  $t_{14} = 2.73$ ,  $P = 0.016$ ; Figure 2C).

#### Aggressiveness of surface- and cave-dwelling ecotypes

In the Tacotalpa drainage, male aggressiveness differed significantly among ecotypes (multivariate GLM:  $F_{6,66.0} = 9.25$ ,  $P < 0.001$ ). Males of the non-sulfidic surface ecotype (Arroyo Bonita) were significantly more aggressive than males of the surface-dwelling sulfide-adapted ecotype (El Azufre; Fisher’s LSD:  $P = 0.001$ ) and males of the sulfidic cave-dwelling ecotype ( $P < 0.001$ ). Moreover, males from the sulfidic surface habitat (El Azufre) were more aggressive than males of the cave-dwelling ecotype ( $P = 0.002$ ; Figure 3A).

#### Intruder inferiority in fights between cave- and surface-dwelling ecotypes

Similar to the results of staged contests between surface-dwelling males from sulfide-adapted and not sulfidic-adapted ecotypes (see above), our analyses of ecotypes from the Tacotalpa drainage that also included the cave ecotype found resident males to establish dominance over intruders at significantly higher frequencies (Figure 3B). Males from the surface-dwelling sulfide-adapted

ecotype (El Azufre) displayed significantly more S-positions when paired with males of the sulfidic cave ecotype than vice versa ( $t_{11} = 2.25$ ,  $P = 0.045$ ; Figure 3C).

## Discussion

To test the hypothesis that harsh conditions in hydrogen sulfide ( $H_2S$ )-containing and/or perpetually dark (i.e., cave) environments select for the reduction of energetically costly behaviors, we compared male aggressiveness among different locally adapted ecotypes in the *P. mexicana* species-complex (Peters et al. 1973; Parzefall 1974, 2001; Bierbach et al. 2012). Reduced aggressiveness of the surface-dwelling sulfide-adapted ecotype compared with the closely related surface-dwelling ecotype from non-toxic waters was observed in only one of three drainages investigated here (Tacotalpa drainage). The sulfidic cave-dwelling ecotype from the same drainage also showed reduced aggressiveness. Independent of whether or not males showed reduced aggressive behavior overall, males from sulfidic surface and cave habitats were significantly less likely to establish dominance when competing with males from habitats without those physico-chemical stressors. We suggest that this pattern could result in inferiority in mate competition for extremophile males (i.e., sulfidic surface and sulfidic cave ecotypes) that migrate into more benign habitats, which represents the only possible routes of migration in those systems (Tobler et al. 2011; Plath et al. 2013).

We found no general reduction of aggressive behavior in surface-dwelling sulfide-adapted ecotypes from the Pichucalco and Puyacatengo drainages, but males from these river systems were still inferior in aggressive competition when paired with males of the not sulfide-adapted ecotype from their respective drainage. It seems, therefore, that adapting to life in  $H_2S$ -rich environments does not necessarily result in reduced aggressiveness even when tested under non-sulfidic conditions but inevitably lowers males’ resource holding potential (RHP, *sensu* Parker 1974) when competing with fish from non-sulfidic waters. This could have several reasons: First, changes in body morphology of sulfide-adapted ecotypes include larger heads (Tobler et al. 2008a; Fontanier and Tobler 2009;

Tobler et al. 2011), which could negatively affect swimming ability (Van Wassenbergh et al. 2015), and ultimately result in poor fighting performance. Second, H<sub>2</sub>S-adapted ecotypes show altered physiological pathways along with differential gene expression patterns compared with not sulfide-adapted ecotypes (Tobler et al. 2014, 2017). Antioxidant enzymes, in particular, are down-regulated in sulfidic, hypoxic water (Kelley et al. 2016; Passow et al. 2017; Tobler et al. 2017), which could hamper physiological performance upon transfer into sulfide-free waters. More generally speaking, various physiological pathways may not have had enough time to adjust to the new conditions (normoxia, no H<sub>2</sub>S) in our experimental design. Additionally, access to the water surface affects short-term survivability in the toxic environments (Plath et al. 2007c; Tobler 2009b), and *Poecilia* spp. in sulfidic habitats spend a considerable amount of their time budget conducting aquatic surface respiration (Tobler 2009b). This behavior, however, comes at a cost of constraining time available for benthic foraging, as evidenced by behavioral observations in the wild and from the examination of gut fullness (Tobler 2009b; Roach et al. 2011). Our tests, however, did not allow us to uncover the mechanism behind contest inferiority of H<sub>2</sub>S-adapted ecotypes and future experiments, especially using common-garden raised fish from H<sub>2</sub>S habitats (i.e., individuals reared under normoxic and non-sulfidic conditions), are urgently needed. Staging fights between different ecotypes that are all common-garden reared would, for example, allow us to tell apart the effects of current acclimation (or a lack thereof) to non-sulfidic conditions that fish in our tests (and migrants in the wild) will experience.

A different pattern was observed in the Tacotalpa drainage, in which both sulfide-adapted surface and cave ecotypes showed reduced aggressiveness compared with the ecotype from non-sulfidic surface waters. Bierbach et al. (2012) examined male aggressiveness in laboratory-reared fish from all four distinct ecotypes in the Tacotalpa system (non-sulfidic surface, sulfidic surface, sulfidic cave, and non-sulfidic cave) and reported that not only selection from H<sub>2</sub>S but also from darkness led to reduced aggressiveness. This confirmed earlier studies in which cave fish from the sulfidic cave (Cueva del Azufre) showed reduced aggressive behavior, and cross-breeding experiments revealed polygenic Mendelian inheritance of behavioral population differences (Peters et al. 1973; Parzefall 1974, 1979). Similar patterns of reduced aggressive behavior are found in other cave fishes (see Parzefall and Trajano 2010), even though some species, like *Pimelodella kronei*, can be highly aggressive (Trajano 1991; Hoenen and Trajano 1995). Nevertheless, fights between males from the same population reported in Bierbach et al. (2012) were staged with laboratory-reared individuals that had been reared under light conditions for at least two generations. Our current study, using wild-caught fish, confirmed a reduction in aggressive behaviors in both sulfidic surface and sulfidic cave ecotypes.

The exact selective mechanisms (or triggers of plastic changes) leading to reduced aggressiveness in the sulfide-adapted cave and surface ecotypes in the Tacotalpa drainage but not in both others remain elusive, but we argue in favor of independent evolutionary trajectories (Pfenninger et al. 2014, 2015). Gene flow from cave-dwelling *P. mexicana* out of the Cueva del Azufre into the population inhabiting the adjacent sulfidic surface habitat in front of the cave (El Azufre) is detectable, while gene flow in the opposite direction is virtually absent (Plath et al. 2007a; Tobler et al. 2008a, 2009). Thus, it seems possible that reduced aggressiveness—as seen in both sulfide-adapted surface-dwelling males and cave-dwelling males in this particular drainage—is merely a consequence of

introgression of allelic variants stemming from the cave ecotype that may be only weakly (or not at all) counter-selected in the sulfidic surface habitat. As an alternative explanation, one might also argue that extreme habitats in the Tacotalpa drainage were colonized by less aggressive (and competitively inferior) fish from the ancestral population (Winkelman et al. 2014). Thus, future studies should address the question of whether male aggression was involved in initial species divergence, or just in maintaining or shaping of species boundaries and co-existence of divergent phenotypes.

In our study system, natural selection acts mostly through H<sub>2</sub>S-toxicity with high mortality rates of not sulfide-adapted fish in sulfidic waters (and *vice versa* also in the Tacotalpa drainage, see Plath et al. 2013). Furthermore, predation by the aquatic heteropteran *Belostoma* cf. *bakeri* at the interface of cave and surface habitats was found to act against migrants, as surface-dwelling ecotypes are more likely to fall victim to predation under dark conditions, while cave ecotypes suffer greater predation under light conditions [Tobler (2009a), see also Riesch et al. (2010a) for avian predation]. Regarding the contribution of sexual selection, several studies have shown strong preferences in females from non-sulfidic habitats for mating with males from their own population, whereas females from sulfidic environments show no such ecotype-based mating biases (Tobler et al. 2009; Plath et al. 2010, 2013; Sommer-Trembo et al. 2016), which can be ascribed to a large extent to adaptive, divergent evolution of male body shape (Greenway et al. 2016). Divergent evolution of body coloration in cave ecotypes allows surface-dwelling females to choose males from their own ecotype, while cave-dwelling females do not show a preference for males from their own ecotype (Bierbach et al. 2013b). However, in a mating system where dominant males monopolize small groups of females (Bierbach et al. 2014) and typically only those males father the majority of offspring then (but see Schartl et al. 1993), male-male competition could actually play an even stronger role than mate choice.

Similar patterns as reported here are likely to also occur in other systems in which reproductive isolation emerges as a consequence of local adaptation. Our study highlights that inferences about the putative outcome of male contests between different ecotypes cannot be made based on comparisons of mean aggressiveness assessed during contests of opponents from the same ecotype. Even when certain ecotypes do not show reduced aggressiveness overall, cross-ecotype contests need to be conducted to evaluate the role played by male competition for the emergence of reproductive isolation.

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## Conflict of Interest

The authors declare no competing interests. All authors conceived and designed the experiment; D.B. acquired data; D.B. and M.P. analyzed data; D.B. drafted the article; all authors approved the submitted version of the article.

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