# Functional consequences of morphological variation between locally adapted populations

by

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# Abstract

Natural selection drives the evolution of traits to optimize organismal performance, but optimization of one aspect of performance can often influence other aspects of performance. Here, we asked how phenotypic variation between locally adapted fish populations affect locomotion and ventilation, testing for functional trade-offs as well as trait-performance correlations. Specifically, we investigated two populations of livebearing fish (*Poecilia mexicana*) that inhabit distinct habitat types (hydrogen-sulfide-rich springs and adjacent non-sulfidic streams). For each individual fish, we quantified different metrics of burst-start swimming during simulated predator attacks, steady swimming, as well as gill ventilation. Coinciding with theoretical predictions, we documented significant population differences in all aspects of performance, with fish from sulfidic habitats exhibiting higher steady swimming performance and higher ventilation capacity but slower burst-starts. There was a significant functional trade-off between steady and burst-speed swimming, but not between different aspects of locomotion and ventilation, indicating modularity of traits associated with either aspect of function. While our findings about population differences in locomotion performance largely parallel the results from previous studies, we provide novel insights about how morphological variation might impact ventilation and ultimately oxygen acquisition. Overall, our analyses provided insights into the functional consequences of previously documented phenotypic variation, which will help to disentangle the effects of different sources of selection that may coincide along complex environmental gradients.

# Table of Contents

List of Figures ..................... v

List of Tables ..................... vi

Acknowledgements ..................... vii

Chapter 1 - Functional consequences of morphological variation between locally adapted populations ..................... 1

- Introduction ..................... 1
- Materials and methods ..................... 5
  - *Study organisms and general experimental design* ..................... 5
  - *Escape performance* ..................... 6
  - *Steady swimming performance* ..................... 8
  - *Gill Ventilation Capacity* ..................... 11
  - *Testing for functional trade-offs and linking performance to morphology.* ..................... 13
- Results ..................... 14
  - *Variation in performance* ..................... 14
  - *Functional trade-offs and morphology-performance relationships* ..................... 16
- Discussion ..................... 16
  - *Swimming in sulfidic and non-sulfidic habitats* ..................... 17
  - *Oxygen acquisition in sulfidic and non-sulfidic habitats* ..................... 19
  - *Trait-performance correlations and functional trade-offs* ..................... 21
- Conclusions ..................... 22
- Figures and Tables ..................... 24

References ..................... 35

# List of Figures

Figure 1: Plot of a Principal Component Analysis between Component 1 and 2 of burst speed divided between populations (sulfidic: yellow; nonsulfidic; blue). ..................................... 24

Figure 2: (A) Scatter plot showing differences in critical swimming speed between fish from sulfidic (yellow) and nonsulfidic environments (blue). (B) Plot showing the best fitting model for PC1 of steady swimming kinematics as speed fish swam increased. Population differences are shown, with sulfidic environments in gold and nonsulfidic environments in blue. (C) Plot showing the best fitting model for PC3 of steady swimming kinematics as speed fish swam at increased. Population differences are observed with different colors showing population differences. ......................................................................... 25

Figure 3: (A) Boxplot comparing differences in gill ventilation frequency square root transformed between populations. (B) Scatter plot showing differences in buccal cavity size between sulfidic (yellow) and nonsulfidic (blue) populations. Buccal cavity size was log transformed. (C) Scatter plot showing differences in volume of water taken in per second (log transformed) between sulfidic (yellow) and nonsulfidic (blue) populations................ 26

Figure 4: Correlation of coupled partial least-squares latent vectors of body shape and different performances for sulfidic and nonsulfidic populations of *Poecilia mexicana* females. Vector diagrams illustrate the body shapes at either extreme body shape. Y axis depicts differences in performance extremes with fish more negative on the performance axis demonstrating greater critical swimming speeds and weaker burst speeds and gill ventilation frequencies. Fish with more positive values exhibited the opposite performance (better burst swimming and gill ventilation with weaker critical swimming speeds)............................................... 27

# List of Tables

Table 1: Statistics of all fish used for experiments separated by population and sex with average mass(g) included ........ 28

Table 2: Results of principal component analyses on burst speed metrics. Provided are component loadings, eigenvalues, and per cent variance explained by each of the axes. .... 29

Table 3: Results of principal component analyses on steady swimming data based on different kinematic data. Provided are component loadings, eigenvalues and per cent variance explained by each of the axes. ........ 30

Table 4: Results of (M)ANCOVA on different response variables quantified in this study: (A) burst speed, (B) $U_{crit,}$ (C) maximum gill ventilation frequency, (D) buccal cavity size, and (E) total respiratory volume. Body mass was $\log_{10}$-transformed prior to analyses. Effect sizes were quantified with partial eta squared. ........ 31

Table 5 Analyses principal component axes (PC 1-3) describing swimming kinematics during steady swimming . For each response variable, models are listed from best to least supported based on ΔAICc values. ........ 32

Table 6: Results of partial least-squares analysis examining covariation between performance variables and body shape for (A) males (B) females. ........ 34

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# Chapter 1 - Functional consequences of morphological variation between locally adapted populations

## Introduction

Divergent selection along environmental gradients is a primary driver of phenotypic evolution and local adaptation (Kawecki & Ebert, 2004). Establishing the origins of phenotypic diversity requires an understanding of how variation in selection favors different traits that mediate organismal performance and how trade-offs potentially prevent the simultaneous optimization of multiple aspects of organismal function and the evolution of general-purpose phenotypes. Trade-offs in the expression of traits frequently arise as a consequence of constraints associated with the genetic architecture underlying phenotypic traits (Scarcelli, Cheverud, Schaal, & Kover, 2007), developmental mechanisms (Jeffery, 2009), or energy availability (Emlen, 2001). In addition, there may be functional constraints where optimizing a particular trait for one function impairs the utility of that trait for other functions (Ghalambor, Walker, & Reznick, 2003; Johnson, Burt, & DeWitt, 2008). In the context of complex environmental gradients with coinciding sources of selection, establishing how trait variation impacts multiple aspect of organismal performance facilitates a better understanding of the causal drivers of phenotypic evolution and functional trade-offs that limit evolutionary outcomes.

Fishes provide an excellent example of staggering morphological variation within and among species, making them conducive to the investigation of adaptive evolution at micro- and macro-evolutionary scales (Jacquemin & Pyron, 2016). Fishes vary broadly in their general body shape and streamlining (Domenici, 2003), fin size and positioning (Webb, 1982; Webb, Kostecki, & Stevens, 1984), head morphology  (Farina, Near, & Bemis, 2015; Hulsey & Garcia De Leon, 2005), as well as traits important for reproduction (Langerhans, Layman, & DeWitt, 2005). The ultimate causes underlying variation in fish morphology are tied to diverse functions that include locomotion (Domenici, 2003), feeding (Hulsey & Garcia De Leon, 2005; Webb et al., 1984) , oxygen acquisition (Farina et al., 2015), as well as mating and reproduction (Basolo, 1990; Bisazza, 1993). The field of eco-morphology seeks to understand how different sources of selection (e.g., flow regimes, presence of predators, or prey availability) are related to morphological variation within and among species, and how morphological variation in turn influences different functions contributing to organismal performance across environmental contexts (Arnold, 1983). Linking selection, phenotypic variation, and organismal performance is perhaps best investigated in the context of body shape variation and associated trade-offs between steady and unsteady swimming performance (Blake, 2004; Domenici, 2003; Langerhans & Reznick, 2010; Videler, 1993; Webb, 1984). Streamlined body shapes are optimal for energy efficient steady swimming, which is favored in high flow environments, open and structure-poor habitats, and habitats with high levels of competition (Domenici, 2003; Langerhans & Reznick, 2010; Plaut, 2001; Rice & Hale, 2010). In contrast, body shapes with an enlarged caudal area are optimal for burst swimming that mediate quick bursts of acceleration(Blake, 2004; Langerhans, Layman, Shokrollahi, & DeWitt, 2004; Walker, 1997; Webb & Weihs, 1983). Such body shapes are favored in structurally complex habitats and high predation environments that require rapid acceleration during predator attacks (Kerfoot & Sih, 1987). Functional trade-offs shape morphological evolution depending on whether local environmental conditions favor optimization of steady or burst swimming (Langerhans, 2009b). Similarly, a trade-off between the generation of suction pressure and the volumetric expansion of the buccal cavity impacts foraging habits in centrarchid fishes (Carroll, Wainwright, Huskey, Collar, & Turingan, 2004). While we have a thorough understanding of trade-offs between different modes of swimming or foraging, there are fewer examples of how phenotypic diversification is potentially impacted by trade-offs among different types of organismal function (locomotion *vs*. foraging, locomotion *vs*.

reproduction, etc.). For example, the evolution of the length of the gonopodium (a modified anal fin) in livebearing fishes is shaped by a functional trade-off between reproduction (long gonopodia are preferred by females during mate choice) and survival during predator attacks (short gonopodia increase escape probability; Langerhans et al. 2005). In this study, we were particularly interested in investigating the functional implications of morphological variation in the context of locomotion and respiration using a well-established study system of local adaptation to divergent environmental conditions.

*Poecilia mexicana* is a widespread livebearing fish occurring in various freshwater habitats of Mexico and Central America (Palacios, Voelker, Arias Rodriguez, Mateos, & Tobler, 2016). In addition, this species has independently colonized multiple toxic, hydrogen sulfide ($H_2S$) rich springs in southern Mexico (Greenway, Arias-Rodriguez, Diaz, & Tobler, 2014; Tobler et al., 2011). Populations in adjacent non-sulfidic and sulfidic habitats face strong and multifarious divergent selection that includes variation in abiotic as well as biotic environmental conditions (Tobler, Kelley, Plath, & Riesch, 2018). Fish inhabiting sulfide springs are exposed to high levels of toxicity associated with the presence of $H_2S$, extreme hypoxia, high densities of intra-specific competitors, and reduced species-richness with relatively few aquatic predators and inter-specific competitors (Plath et al., 2007; Riesch, Schlupp, Tobler, & Plath, 2006; Tobler et al., 2006). In contrast, fish inhabiting normal freshwater streams experience higher oxygen concentrations and more complex communities with higher levels of inter-specific competition and predation. Divergent selection across habitat types has led to locally adapted *P. mexicana* populations that are undergoing ecological speciation (Tobler et al., 2018). Fish occupying the different environments diverged phenotypically (e.g., phyisology: Pfenninger et al., 2014; morphology: Tobler & Hastings, 2011; life history: Riesch, Plath, Schlupp, Tobler, & Langerhans, 2014), and reduced gene flow led to significant population genetic differentiation despite small spatial scales and a lack of physical barriers preventing fish movement (Plath et al., 2013). Reproductive isolation is in part mediated by natural and sexual selection against migrants (Greenway, Drexler, Arias-Rodriguez, & Tobler, 2016; Plath et al., 2013).

Given the complexity of selective regimes and phenotypic variation, it remains largely unclear what selective pressures ultimately drove the evolution of specific traits. For example, previous studies have demonstrated morphological variation between fish from the two habitat types, with sulfide spring fish exhibiting significantly enlarged heads, different caudal peduncle proportions, and lower body depths compared to populations in non-sulfidic environments (Tobler et al., 2011). Although $H_2S$ is a strong source of selection impacting the evolution of biochemical and physiological traits in this system (Bagarinao & Lantin-Olaguer, 1998; Li, Rose, & Moore, 2011; Tobler, Passow, Greenway, Kelley, & Shaw, 2016), it is unlikely that $H_2S$ *per se* directly caused the observed morphological divergence (Tobler et al., 2018). Two alternative scenarios have been proposed: (1) Body shape differences between sulfidic and non-sulfidic populations may have arisen through selection on locomotion. Fish in the sulfidic habitats face constraints in organismal energy budgets (Passow, Arias-Rodriguez, & Tobler, 2017) and consistently exhibit lower body condition (Tobler, 2008) than those from non-sulfidic habitats. Energy limitation – in conjunction with high levels of intraspecific competition (Culumber et al., 2016) – in sulfide springs should select for energy-efficient steady swimming. In contrast, higher levels of predation in non-sulfidic habitats (Riesch et al., 2010; Tobler et al., 2006) should select for a higher escape performance to evade predator attacks. (2) Body shape differences between sulfidic and non-sulfidic populations may have arisen through selection on oxygen acquisition. Respiratory adaptations have previously been shown to facilitate survival in the toxic and hypoxic environment. Sulfide spring fish spend a substantial amount of time engaging in aquatic surface respiration (Plath et al., 2007), exhibit increased gill surface areas

(Tobler et al., 2011), and have physiological adaptations that impact oxygen transport and consumption (Barts et al., 2018; Passow et al., 2017). Therefore, hypoxia in sulfidic habitats might select for a higher ventilation capacity, allowing fish to maximize the amount of water they can pump over their gills. Naturally, these two hypotheses are not mutually exclusive. For example, fish from sulfidic environments may simultaneously have exhibit more efficient steady swimming and higher ventilation capacity. However, functional trade-offs may cause discrepancies between predicted and observed performance differences between fish from different habitats (Ghalambor et al., 2003). Based on previously established trade-offs between steady and burst swimming, we expect that neither sulfidic nor non-sulfidic fish can optimize energy-efficient swimming and escape performance simultaneously. Similarly, selection for higher ventilation capacity may adversely impact aspects of swimming performance, or *vice versa*.

To understand how morphological variation between fish from sulfidic and nonsulfidic environments impacts performance, we quantified multiple aspects of organismal performance related to locomotion (escape performance, critical swimming speed, and steady swimming kinematics) and ventilation (maximum ventilation frequency and volume of water an individual can pump over its gills). Performance metrics were used to test hypotheses about adaptive differences between populations, identify potential functional trade-offs, and relate individual variation in performance to variation in morphology.

## Materials and methods

### *Study organisms and general experimental design*

In Southern Mexico, several sulfide springs inhabited by fish of the *P. mexicana* species complex occur in the states of Tabasco and Chiapas (Palacios et al., 2016). For the present study, we used fish from a sulfidic and a nonsulfidic population located in the Río Tacotalpa drainage of the Río Grijalva basin. Specifically, fish from the non-sulfidic population were collected from a site in the Río Tacotalpa by the village of Tapijulapa (latitude/longitude: N 17.2752/W 92.4622); fish from the sulfidic population were collected from El Azufre I (lat/long; see Tobler et al., 2011). Upon collection, fish from both populations were transported to Kansas State University (Manhattan, KS), where they were housed in 80-liter tanks and given two months of acclimation to laboratory conditions. After the acclimation period, fish were separated into groups of 3-4 individuals and housed in 40-liter tanks, facilitating the tracking individual fish across different portions of the experiment. During the entire experimental period, fish were kept at 25 °C, a 12L:12D photoperiod, and were fed a diet of flake food and frozen brine shrimp twice daily.

For each fish, we quantified three aspects of swimming and ventilation: 1) escape performance upon a simulated predator attack, 2) steady swimming performance, and 3) gill ventilation. The order of performance assays was randomized across individuals, and individual fish were allowed to recover for at least one week in between experiments. Fish were fasted for a 24-hour period prior to each performance trial to ensure they had time to process previously consumed food, were in a post-absorptive state, and exhibited uniform levels of gut-fullness (Kieffer, 2000; Niimi & Beamish, 1974). After the completion of all three performance assays, individuals were euthanized in MS-222 for subsequent morphological analyses. A total of *N*=71 fish were tested (see Table 1 for descriptive statistics). Unless stated otherwise, all statistical analyses described below were conducted in R version 3.2.2 (R Core Team, 2013).

### *Escape performance*

We quantified escape performance of individual fish during their reflexive escape response (C-start) to simulated predatory attacks. During a C-start, a single pair of neurons called the

Mauthner cells (Eaton, Bombardieri, & Meyer, 1977; Eaton & Hacket, 1984) initiates an escape response, where the fish's head rotates away from predatory stimulus, the body bends into a 'C' shape, and then produces a propulsive stroke of the caudal region in the escape direction, resulting in a sudden high energy burst of unsteady swimming activity (Domenici & Blake, 1997; Eaton et al., 1977; Harper & Blake, 1990; Howland, 1974; Weihs, 1973). Fish from high-predation environments have previously been documented to perform faster at C-starts than fish from low-predation environments (Langerhans, Gifford, & Joseph, 2007; Langerhans et al., 2004; Langerhans & Reznick, 2010; Walker, 1997), and several studies have linked escape performance with survival in the presence of predators (e.g., Ingley & Johnson, 2016; Walker, Ghalambor, Griset, Mckenny, & Reznick, 2005).

Methods for the quantification of escape responses were adapted from previous studies in livebearing fishes (Ingley, Camarillo, Willis, & Johnson, 2016; Langerhans et al., 2004). For each trial, we gently transferred the test fish from their holding tank to a clear acclimation cylinder (5.5 cm in diameter) within a larger test arena (circular tank with a 40-cm diameter). To minimize vertical displacement and approximate two-dimensional escape responses, the water level was maintained at a depth of ~3 cm. After a 10-minute acclimation period, the acclimation cylinder was gently removed, and we struck the arena with a wooden probe (6 mm in diameter and 90 cm long) within ~1 body length of the fish's caudal region to evoke an escape response. After the first trial, fish were placed back into the clear cylinder, given the same acclimation period, and tested twice more with the same procedures, yielding a total of three escape performance trials per fish. Each trial was filmed from above with a Sony NXCAM NEX-FS700 high-speed camera (Sony Corporation, Tokyo, Japan) at 120 frames per second (fps).

Videos were analyzed frame by frame using the DLTdv6 tracking software (Hedrick, 2008) in MATLAB 2016a (Mathworks Inc., Natick, MA, USA) to quantify four metrics of escape performance (Walker, Ghalambor, Griset, McKenney, & Reznick, 2005) following methods established by Langerhans (2009): (1) total distance traveled ($d_{net}$ [cm]) is the net distance a fish traveled within 1/12 of a second after bending into the 'C-shape'; (2) rotational velocity ($\omega_S$ [°/s]) is the average rotational velocity of the head from the moment the fish begins bending into the 'C-shape' until it has completely bended (rotational angle of bend divided by duration); (3) maximum velocity ($v_{max}$ [cm/s]) is the greatest change in distance between two consecutive frames (1/120 of a second) during the escape response; (4) maximum acceleration ($a_{max}$ [cm/s$^2$]) is the greatest positive change in velocity between two consecutive frames. For each individual fish, we calculated the average of each metric across the three recorded trials.

To reduce data dimensionality, we conducted a principal component analysis (PCA) on the different metrics of escape performance ($D_{net}$, $\omega_S$, $v_{max}$, $a_{max}$) using a correlation matrix, and we retained two PC axes with eigenvalues >1 for subsequent analyses (see Table 2). PC axis 1 was negatively correlated with maximum velocity, maximum acceleration, and distance traveled, while PC axis 2 was positively correlated with rotational velocity. To compare escape performance between populations, we used PC scores associated with the first two axes as dependent variables in a multivariate generalized linear model (GLM) using the car package (Fox & Weisberg, 2011) in R. Habitat of origin (sulfidic vs. non-sulfidic) and sex were used as factors and body mass ($\log_{10}$-transformed) as a covariate. Note that interactions between predictor variables were not significant ($F < 0.071$, $P > 0.932$) and excluded from the final model.

### *Steady swimming performance*

We also quantified metrics for steady swimming performance, which is defined the ability of a fish to maintain a constant-speed using periodic body movements (Blake, 2004; Domenici, 2003;

Plaut, 2001). Steady swimming is an energy-efficient mode of locomotion fish use during place-holding against water flow, foraging, and mate searching (Blake, 2004; Langerhans, 2008; Plaut, 2001; Roff, 2002; Vogel, 1994). Steady swimming performance is expected to be optimized in environments that require constant movement, exhibit low predation but high competition, or are resource limited (Blake, 2004; Domenici, 2003; Plaut, 2001). To quantify steady swimming performance, we measured the critical swimming speed ($U_{\text{crit}}$) as well as swimming kinematics at different swimming speeds for each individual.

$U_{\text{crit}}$ is defined as the maximum flow speed at which an individual fish can maintain constant swimming (Blake, 2004; Domenici, 2003; Plaut, 2001) and can be quantified by incrementally increasing the swimming speed until an individual fatigues (Brett, 1964). Critical swimming speed can then be calculated as $U_{\text{crit}} = U_f + U_s \times (t_f/t_s)$, where $U_f$ is the highest flow velocity maintained for a full time-interval, $U_s$ is the velocity increment, $t_f$ is the time to fatigue at the last flow speed, and $t_s$ is the time interval at which increases in flow speed occur (Brett, 1964).

To do so, individual fish were placed in a 5-L swim tunnel (Loligo Systems ApS, Viborg, Denmark), in which they were exposed to laminar flow with adjustable speed (Ellerby & Herskin, 2013). Trials started with an initial 20-minute acclimation period, including 10 minutes of acclimation without flow and 10 minutes of acclimation at a flow speed of one body length per second ($\text{BLs}^{-1}$). After acclimation, fish were incrementally exposed to higher flow speeds ($U_s = 1\ \text{BLs}^{-1}$) every 10 min ($t_s = 600$ s). Methods were modified from previous studies that measured $\text{U}_{\text{crit}}$ with the same acclimation times and body length increments (Hammill, Wilson, & Johnston, 2004; Oufiero & Garland, 2009; Sfakianakis, Leris, & Kentouri, 2011). Trials were immediately terminated once a fish reached fatigue, defined as the moment when fish did not have energy to swim against the flow and was pinned against the back end of the tunnel (Ingley & Johnson, 2016).

During each fish's steady swimming trial, fish were also filmed at 120 (fps) using a Sony NXCAM NEX-FS700 high-speed camera twice during each swimming interval (once immediately after swimming had been increased, and a second time halfway into each swimming speed). After each trial, four videos were selected at speeds ~25%, 50%, 75%, and 100% of each individual fish's critical swimming speed (Oufiero & Garland, 2009). At each swimming speed, we quantified five kinematic variables relevant for the hydrodynamics of steady swimming (see Langerhans, 2009; McHenry, Pell, & Long, 1995): (1) tail-beat frequency ($f$ [Hz]) was measured as the inverse average period of ten complete tail-beat cycles (Langerhans, 2009b); (2) rostral amplitude ($R$ [mm]) was measured as half the distance between right and left excursions of the anterior tip of the rostrum (Langerhans, 2009b); (3) tail-beat amplitude ($H$ [mm]) was measured as half the distance between right and left excursions of the fin (Langerhans, 2009b) ; (4) propulsive wavelength ($\lambda$ [mm]) was measured as doubling the posterior half-wavelength (Webb, 1982; Webb, 1984); and (5) propulsive wave speed ($c$ [mm/s]) ) was calculated by multiplying the propulsive wavelength with the tail-beat frequency ($c= \lambda *f$) (Langerhans, 2009b). For $R$, $H$, and $\lambda$ measurements were taken by averaging the values of each across three complete tail beats. Videos were analyzed frame by frame using the DLTdv6 tracking software (Hedrick, 2008) in MATLAB 2016a (Mathworks Inc., Natick, MA, USA).

Variation in $U_{\text{crit}}$ (in $\text{BLs}^{-1}$) was analyzed using a GLM with habitat of origin (sulfidic vs. non-sulfidic) and sex as factors, as well as body mass ($\log_{10}$-transformed) as a covariate. Note that interactions between predictor variables were not significant ($F < 0.049$, $P > 0.826$) and excluded from the final model. To analyze swimming kinematics, we conducted a PCA on the different metrics ($f$, $R$, $H$, $\lambda$, $c$) using a correlation matrix, and we retained three PC axes with eigenvalues >1 for subsequent analyses (see Table 3). PC axis 1 was negatively correlated with all five kinematic variables, PC axis 2 was positively correlated with tail-beat frequency and negatively correlated with the remaining kinematic variables, and PC axis 3 was positively correlated for tail-beat frequency, rostral amplitude, tail-beat amplitude, and negatively correlated for propulsive wavelength and propulsive wave speed. Since we had multiple measurements of swimming kinematics per individual fish at different swimming speeds, we used the three PC scores as dependent variables in mixed models as implemented in R's lme package (Bates et al., 2018). We contrasted alternative models to illuminate the effects of swimming speed, sex, population of origin (sulfidic *vs*. non-sulfidic), as well as their interactions. The null model included body size and the individual identification number as a random factor. Models were evaluated based on Akaike Information Criterion for small sample sizes (AICc) (Johnson & Omland, 2004), and models with ΔAICc < 2 were assumed to be well supported (Burnham & Anderson, 2004).

### *Gill Ventilation Capacity*

Gill ventilation in teleost fishes has been described as a two-pump system, in which water is taken through the mouth into the buccal cavity, pumped into opercular cavity, and out through the opercular openings, resulting in a unidirectional flow across the respiratory surfaces of the gills (Hughes, 1958). The rate at which water is pumped over the gills consequently should be limited by the capacity of the first pump (i.e., number of respiratory cycles an individual can perform as well as the buccal volume). We quantified these variables for each individual to estimate the maximum gill ventilation capacity.

To measure the maximum frequency of respiratory cycles, fish were driven to peak aerobic performance using chase trials similar to those used by Brennan, Hwang, Tse, Fangue, &

Whitehead, (2016). Fish were placed in a circular chase arena (28 cm in diameter), given a 10-minute acclimation period, and then chased along the outer edge of the arena using a plastic handle for 6 minutes. A pilot experiment indicated that this time period was sufficient to induce maximum ventilation frequency. After chasing, individuals were immediately transferred into a photo tank (18 × 10.5 × 10 cm) with aerated water and filmed at 60 fps using a Sony NXCAM NEX-FS700 high-speed camera. Fish were recorded eight times for 3 s each, 15 s apart. Based on the videos, we counted the number of respiratory cycles for each 3-second interval (in pumps per second), retaining the highest value for subsequent analyses.

Since the total volume of water pumped over the gills per unit time is not only dependent on the ventilation frequency, but also the volume taken per respiratory cycle, we estimated the volume of the buccal cavity for each fish. To do so, we first performed a sagittal cut from the center of the body toward the mouth and a second cut in a planar fashion just behind the opercula, yielding in two separate halves of the head. Digital images were than taken of each sagittal plane using a Canon Rebel T5i camera (Canon USA Inc., Lake Successes, NY, USA) mounted on a Nikon ZMZ1000 dissecting microscope (Nikon Instruments Inc., Melville, NY, USA), allowing for the measurement of the length of the buccal cavity. Additional images were taken of the planar planes at the anterior and posterior edge of the buccal cavity, allowing for the measurement of the corresponding radii of the buccal cavity. Measurements on the two halves of the head were averaged. To approximate the buccal volume, we treated the shape of the cavity as a circular truncated cone, for which the volume can be calculated as $V_b=(1/3)*h*\pi*(r_1^2+r_1r_2+r_2^2)$, where $h$ is the length of the buccal cavity, and $r_1$ and $r_2$ are the radii of the anterior and posterior ends, respectively.

Maximum gill ventilation frequency (in $s^{-1}$; square-root-transformed) was analyzed using a GLM with habitat of origin (sulfidic vs. non-sulfidic) and sex as factors, as well as body mass

($\log_{10}$-transformed) as a covariate. Note that interactions between predictor variables were not significant ($F < 0.005$, $P > 0.943$) and excluded from the final model. Buccal cavity volume (in $mm^3$) was $\log_{10}$-transformed and also analyzed with GLM using the same predictor variables. Interactions between predictor variables were not significant ($F < 1.488$, $P > 0.227$) and excluded from the final model. Finally, we approximated the volume of water an individual can pump per unit time ($V_W$ in $mm^3s^{-1}$) by multiplying the maximum gill ventilation frequency with the buccal volume for each individual. $\text{Log}_{10}$-transformed values of $V_W$ were analyzed using GLM with the same models described above; interactions between predictor variables were not significant ($F < 0.9083$, $P > 0.344$) and excluded from the final model.

### *Testing for functional trade-offs and linking performance to morphology.*

To identify potential functional trade-offs, we tested for correlations between performance metrics (first two PC axis describing escape performance, $U_{crit}$, and maximum gill ventilation frequency) using Pearson correlation using the Hmsci package in R (Harrell Jr, 2018). To understand how different performance metrics relate to variation in body shape, we quantified body shape for each individual using a geometric morphometric approach. For all specimens, lateral photographs were taken with a Canon Rebel T5i (Canon USA Inc., Lake Successes, NY, USA) mounted on a copy stand. We digitized 14 landmarks using the software program tpsDig version 2.10 (Rohlf, 2006): (1) tip of the upper jaw; (2) the center of the orbit; (3) postereo-dorsal corner of the head; (4) anterior and (5) posterior insertions of the dorsal fin; the (6) dorsal and (7) ventral insertions of the caudal fin; (8) the posterior and (9) anterior junctions of the anal fin; (10) the anterior junction of the pelvic fin; (11) the bottom of the heed where the operculum breaks away from the body outline; (12) the dorsal endpoint of the opercular bone; (13) dorsal and (14) ventral insertions of the pelvic fin (see Palacios et al 2013).

We conducted a two-block partial least-squares analysis (PLS) to test for covariation among performance metrics and correlation with body shape as implemented in the program tpsPLS (Rohlf, Corti, & Olmstead, 2000). PLS reduces data dimensionality by creating new linear combinations between the two different sets of data (i.e., body shape and performance), and singular axes are generated to maximize the covariation between these two sets of variables (Rohlf et al., 2000). Males and females were analyzed separately due to strong sexual dimorphism in body shape (Culumber & Tobler, 2017). Different performance metrics (two PC axes describing escape performance, $U_{\text{crit}}$, and maximum gill ventilation frequency) were designated as the first variable block, landmark coordinates describing body shape as the second variable block.

## Results

### *Variation in performance*

Analysis of the two PC axis describing variation in escape performance revealed significant differences between *P. mexicana* from sulfidic and non-sulfidic habitats, but no effects of sex or body mass (Table 4A). Population differences were evident along PC axis 1 (but not PC axis 2; Fig. 1). Compared to fish from sulfidic habitats, those from non-sulfidic habitats on average exhibited higher velocities, accelerations, and longer distances traveled (see Table 2).

In contrast, *P. mexicana* from the sulfidic habitat exhibited significantly higher critical swimming speeds than those from non-sulfidic habitats (Fig. 2A). Neither sex nor body mass were significant predictors of $U_{\text{crit}}$ (Table 4B). Population differences in steady swimming were also reflected in swimming kinematics. For PC 1 and PC3, the sole supported model ($\Delta$AICc < 2) indicated that swimming speed and habitat of origin impacted swimming kinematics (Table 3). For PC1 (negatively correlated with all kinematics variables), scores declined with increasing swimming speeds in both populations, and fish from the sulfidic population overall exhibited higher PC scores than those from the non-sulfidic populations (Fig. 2B). In other words, for any given swimming speed, fish from the sulfidic habitat exhibited lower propulsive wavelength, tail-beat frequency, rostral amplitude, tail-beat amplitude, and propulsive wave speed. For PC3 (positively correlated with tail beat frequency, rostral amplitude, and tail beat amplitude; negatively correlated with propulsive wave length and speed), scores increased with swimming speed in both populations, but the slope of increase was significantly higher for individuals from the non-sulfidic population (Fig. 2C). So, non-sulfidic fish disproportionally increased their tail beat frequency, rostral amplitude, and tail beat amplitude and disproportionally decreased their propulsive wave length and speed as they swam faster. For PC2, the null model performed best and a model only including swimming speed exhibited ΔAICc = 2 (Table 5). There was a week positive relationship between PC2 scores and swimming speed, indicating that trail beat frequency and propulsive wave speed increased with speed, and rostral amplitude, tail beat amplitude, and propulsive wave length degreased with speed.

Analysis of maximum gill ventilation rates revealed no effects of size or sex, but fish from non-sulfidic habitats were able to go through significantly more respiratory cycles than those from sulfidic habitats (Table 4C, Fig. 3A). As expected, buccal volumes were significantly correlated with body mass, and sulfidic fish exhibited significantly larger buccal values compared to non-sulfidic ones (Table 4D, Fig. 3B). The larger buccal volumes in sulfidic fish offset lower maximum ventilation rates; the estimated volume of water an individual can pump per unit time correlated with body size and was significantly higher in fish from the sulfidic than the non-sulfidic population (Table 4E).

### *Functional trade-offs and morphology-performance relationships*

The Pearson correlations provided significant evidence for a trade-off (negative correlation) between the first principle component of burst speed and critical swimming speed ($r_p = -0.29$, $P = 0.016$). In addition, there was a positive correlation between the first principle component of burst speed and maximum gill ventilation frequency ($r_p = 0.28$, $P = 0.018$), indicating facilitation. Correlations among all other performance metrics were not significant ($|r_p| < 0.16$, $P > 0.184$).

The PLS analysis produced four dimensions of covariation between body shape and performance variables for both sexes. For males, there were no significant correlations between variation in performance and body shape (Table 6A). In contrast, variation in performance was significantly correlated with body shape in females along the first PLS dimension (Table 6B). Variation in performance along this axis was positively associated with metrics of burst speed and maximum gill ventilation frequency, and negatively with $U_{crit}$ (Table 6B). The body shapes associated with faster burst speeds and higher gill ventilation were predominantly characterized by smaller heads and a more posterior anal fin position, while body shapes associated with higher $U_{crit}$ were predominately characterized with larger heads (Fig. 4).

## Discussion

We investigated populations of *Poecilia mexicana* that are locally adapted to divergent environmental conditions to test how phenotypic variation impacts different aspects of organismal performance and to identify potential functional trade-offs. Consistent with our predictions, fish from sulfidic habitats exhibited a higher steady swimming performance and a higher ventilation capacity, while those from non-sulfidic habitats had faster escape responses to simulated predator attacks. Functional trade-offs were evident between different modes of swimming (steady vs. burst swimming), but not between aspects of swimming and ventilation, potentially indicating modularity of traits associated with either aspect of function. Overall, our analyses provided insights into the functional consequences of previously documented phenotypic variation, which will help to disentangle the effects of different sources of selection that may coincide along complex environmental gradients.

### *Swimming in sulfidic and non-sulfidic habitats*

Quantifying different aspects of swimming performance in fish from sulfidic and non-sulfidic populations revealed significant differences, both in metrics associated with burst and steady swimming. During simulated predator attacks, fish from non-sulfidic habitats exhibited faster escape responses, a performance metric that predicts survival in the presence of predators in other fish species (Langerhans, 2009a; Langerhans, Gifford, et al., 2007; Langerhans et al., 2004; Langerhans & Reznick, 2010; Walker, 1997). In contrast, fish from sulfidic habitats appeared to be more energy-efficient swimmers. They exhibited higher critical swimming speeds and had to exert less effort (*i.e.*, lower trail-beat frequency, rostral amplitude, trail-beat amplitude, propulsive wave length and speed) to maintain any given swimming speed. These population differences in burst and steady swimming were evident in a functional trade-off, where individuals with high escape performance exhibited lower critical swimming speeds and *vice versa*. Functional trade-offs between burst and steady swimming have been widely documented in poeciliids (Ingley et al., 2016; Langerhans, 2009b) and other fish groups (Ellerby & Gerry, 2011; Yan, He, Cao, & Fu, 2012). Functional constraints associated with swimming consequently underlie population differences in morphology when divergent selection favors different aspects of locomotion performance even across small spatial scales (Langerhans, Layman, Langerhans, & Dewitt, 2003). Fundamental constraints in combination with replicated environmental gradients may therefore be important contributors to convergent morphological evolution, both across independent lineages that have colonized $H_2S$-rich environments (Tobler et al., 2011) as well as in other systems (Ingley et al., 2016; Langerhans, 2009b).

Differences in swimming performance between fish from sulfidic and non-sulfidic environments largely match *a priori* predictions based on the prevailing ecological conditions in each habitat type. Sulfidic habitats are characterized by toxic levels of $H_2S$ and extreme hypoxia (Bagarinao & Lantin-Olaguer, 1998; Tobler et al., 2016), but also exhibit species-poor communities with few inter-specific competitors and aquatic predators (Greenway et al., 2014). Despite the lack of predatory fishes, it remains unclear whether predation pressure is actually relaxed in sulfidic habitats, because aerial predation by birds (Riesch et al., 2010) and insects (Tobler, 2009) can be considerable. Thus, we hypothesize that increases in steady swimming performance at the cost of escape performance is primarily driven energetic constraints. Energy limitation in sulfidic environments arises because the presence of $H_2S$ and hypoxia requires fish to trade off time between benthic feeding and aquatic surface respiration (Tobler, Riesch, Tobler, & Plath, 2009), which mediates short-term survival in the extreme environment (Plath et al., 2007). In addition, the maintenance of homeostasis in sulfide springs is energetically costly (Tobler et al., 2018). Constraints in energy acquisition and increased maintenance costs are reflected in fish from sulfidic habitats having comparatively low body condition (Tobler, 2008). Since fish from different habitats that we used in our experiment were acclimated to the same environmental conditions, including *ad libitum* access to food, energy limitation is unlikely to be a proximate cause for differences in steady swimming performances between populations; rather, we suggest that exposure to energetic constraints in the natural habitats has caused evolutionary shifts in traits underlying steady swimming. Energy limitation has also been hypothesized to be the key driver underlying the evolution of other phenotypic traits in *P. mexicana* that have colonized sulfidic habitats, including changes in body size and routine metabolic rates (Passow, Greenway, Arias-Rodriguez, Jeyasingh, & Tobler, 2015), the expression of energetically-costly behaviors (Plath, 2008) and organs (Schulz-Mirbach et al., 2016), as well as reproductive life history traits (Riesch, Plath, Schlupp, Tobler, & Langerhans, 2014). Faster escape performance in fish from non-sulfidic habitats indicates that escaping predators has more substantial fitness impacts than energetic constraints associated with steady swimming. Variation in predation regimes alone has been documented to impact morphological evolution in other poeciliid species, particularly favoring body shapes associated with escape responses (Ingley et al., 2016; Langerhans, 2009a, 2009b). Ultimately, our analyses of swimming performance in sulfidic and non-sulfidic populations of *P. mexicana* largely match the findings in other study systems, even if the relative importance of different sources of selection that push populations along the unsteady-steady swimming continuum may vary.

### *Oxygen acquisition in sulfidic and non-sulfidic habitats*

Habitats with high levels of $H_2S$ are typically characterized by extreme hypoxia (Bagarinao, 1992), which tends to exacerbate the toxic effects of $H_2S$ itself (Bagarinao & Lantin-Olaguer, 1998). Fish in sulfidic habitats should therefore be selected for efficient oxygen acquisition, and we predicted that they would exhibit higher maximum gill ventilation rates and higher buccal volumes compared to relatives in non-sulfidic habitats, ultimately maximizing the volume of water that can be pumped over the gas-exchange surfaces of the gills through time. While we did find that fish from sulfidic habitats exhibited higher rates of water being pumped per unit time, this was primarily driven by differences in buccal volumes between populations. In fact, we documented a trade-off between ventilation frequency and buccal volume, where larger buccal volumes in fish from sulfidic habitats were associated with significantly lower ventilation frequencies, compared to relatives in non-sulfidic habitats. Our finding parallels a similar trade-off between the ability to either generate high fluid speeds or high volumetric flow rates during suction feeding in centrarchid fishes (Higham, Day, & Wainwright, 2006).

The higher capacity for ventilation in fish from sulfidic compared to non-sulfidic habitats adds further evidence for the importance of trait modifications associated with oxygen acquisition, distribution, and use during adaptation to sulfide spring environments. On a molecular level, *P. mexicana* populations in sulfidic habitats exhibit differential expression of and positive selection on oxygen transport genes (Barts et al., 2018) as well as upregulation of genes associated with anaerobic ATP production (Kelley et al., 2016). On a behavioral level, fish from sulfidic habitats heavily engage in aquatic surface respiration (Plath et al., 2007), which is a common response in hypoxia-tolerant fish species (Kramer, 1983; Kramer & McClure, 1982; Kramer & Mehegan, 1981; Lewis, 1970; Timmerman & Chapman, 2004; Winemiller, 1989). And on a morphological level, increased head size is not only correlated with ventilation efficiency (as documented here), but also with significant increases in gill surface areas (Tobler et al., 2011). *I.e.*, fish from sulfidic habitats are able to pump higher volumes of water per unit time, and that larger volume of water simultaneously is in contact with larger respiratory surfaces. Interestingly, evolutionary increases in head size and concomitant changes in gill surface area are common in other lineages of sulfide spring poeciliids (Tobler & Hastings, 2011) and other fishes exposed to chronic hypoxia, most prominently including cyprinids (Chapman & Liem, 1995; Langerhans, Chapman, & Dewitt, 2007; Schaack & Chapman, 2004) and cichlids (Bouton, Visser, & Barel, 2002; Chapman, Galis, & Shinn, 2000). Whether variation in head size (and presumably correlated differences in buccal volumes) also impacts ventilation frequency (negatively) and the volume of water pumped through time (positively) in other fish species exposed to hypoxic environments remains to be investigated. In this context, it may also be worthwhile investigating how selection on ventilation capacity might impact other aspects of organismal function associated with variation in head morphology, which are especially associated with foraging in fishes (López-Fernández et al., 2014; Wainwright, 1988). Nothing is known about how the jaw kinematics of feeding in *Poecilia* (Gibb, Ferry-Graham, Patricia Hernandez, Romansco, & Blanton, 2008) might impact ventilation efficiency, but potential trade-offs between ventilation and foraging could in fact be the reason why we observe higher ventilation frequency and lower buccal volumes in individuals from non-sulfidic habitats. In essence, our study adds additional evidence for how increases in head size might be adaptive in the context of hypoxia (increased ventilation capacity), but we still fail to understand the putative costs associated with these traits in normoxic environments.

### ***Trait-performance correlations and functional trade-offs***

Our experiments have revealed clear trade-offs between different modes of swimming (as various previous studies; Ellerby & Gerry, 2011; Ingley et al., 2016; Langerhans, 2009b; Oufiero, Walsh, Reznick, & Garland, 2011; Yan et al., 2012), but no evidence for trade-offs between swimming and ventilation. In fact, there was positive association between critical swimming speed and gill ventilation frequency. This is not surprising because an increased capacity to acquire oxygen should directly impact aerobic performance (Kiceniuk & Jones, 1977). Selection on ventilation efficiency consequently is not expected to negatively impact fish locomotion, or *vice versa*. Instead, there may be functional facilitation between ventilation and steady swimming performance (Ghalambor et al., 2003).

The final intriguing observation from our study is that we could document significant correlations between phenotypic traits (body shape) and organismal performance, with body shape differences along the performance gradients largely reflecting previously documented differences between sulfidic and non-sulfidic populations. However, trait-performance correlations were only evident for females and not for males. This may in part be caused by the strong patterns of sexual dimorphism in poeciliid fishes, and variation in male morphology has previously been documented to be correlated with sexual selection, while variation in female morphology is primarily associated with ecology (Culumber & Tobler, 2017). Despite strong sexual dimorphism, none of our analyses actually found significant performance differences between the sexes (Culumber & Tobler, 2017), perhaps suggesting that traits other than body shape (e.g., muscle mass, aerobic capacity, etc.) are important to predict variation in performance, compensating for morphological differences.

## Conclusions

Our study revealed that previously documented phenotypic differences between *P. mexicana* from sulfidic and non-sulfidic habitats has significant consequences for organismal function in the context of locomotion and ventilation. While documented trade-offs between steady and burst swimming performance large match the findings of previous studies, we also documented how variation in head size impacts ventilation in fish. Hypoxia-induced morphological variation is common in fishes (Bouton et al., 2002; Chapman et al., 2000; Chapman & Liem, 1995; Langerhans, Chapman, et al., 2007; Nyboer & Chapman, 2013; Reid, Farrell, Luke, & Chapman, 2013; Schaack & Chapman, 2004; Timmerman & Chapman, 2004), but its functional significance for ventilation remains largely unexplored. Future studies should also focus on characterizing trade-offs and facilitation among different aspects of organismal function (*e.g.,* locomotion, ventilation, foraging, and reproduction) (Ghalambor et al., 2003). A better understanding of interdependencies between different aspects of organismal performance will improve our understanding of phenotypic evolution, just like the recognition of pleiotropic effects has in the past (Yamamoto, Byerly, Jackman, & Jeffery, 2009).

## Figures and Tables

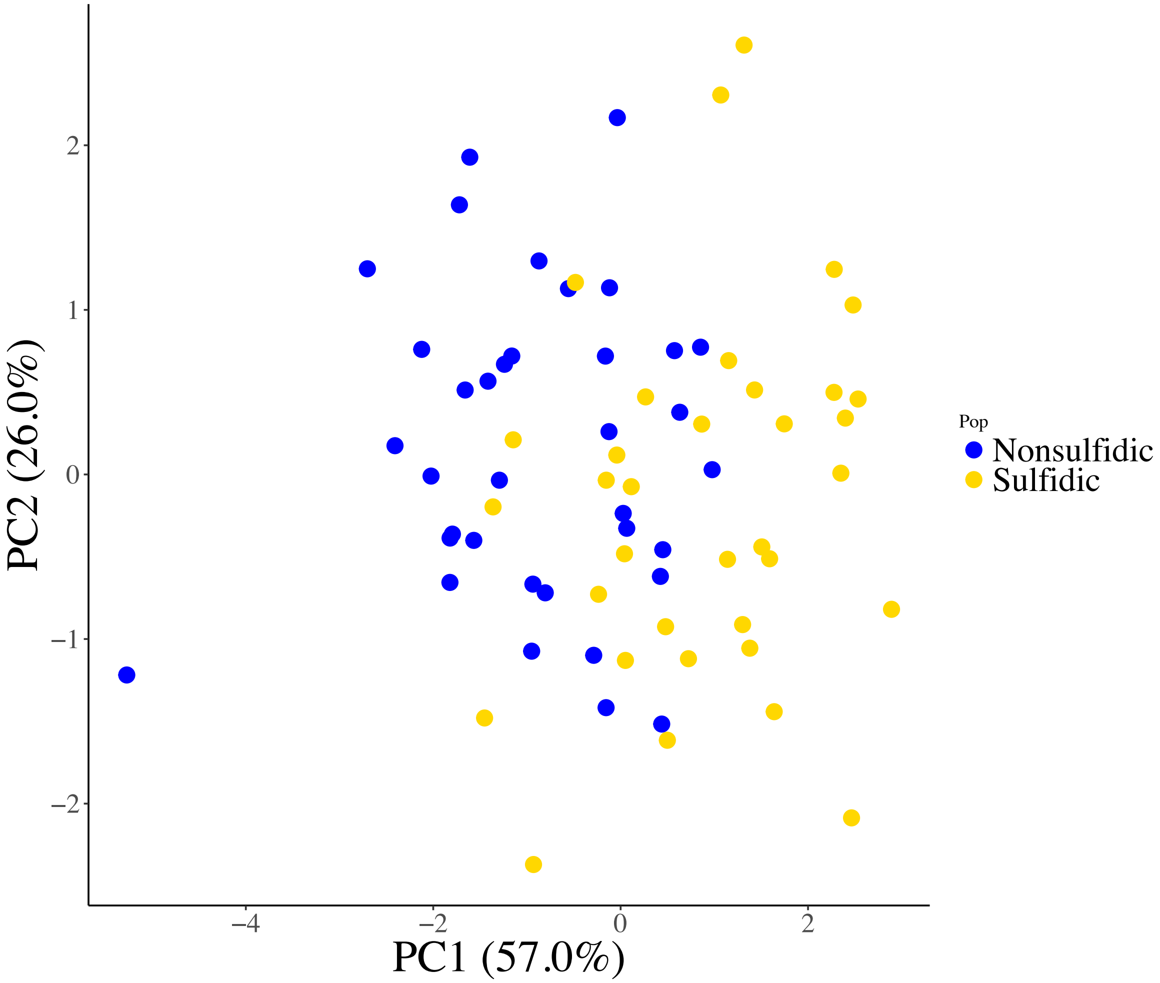

**Figure 1: Plot of a Principal Component Analysis between Component 1 and 2 of burst speed divided between populations (sulfidic: yellow; nonsulfidic; blue).**

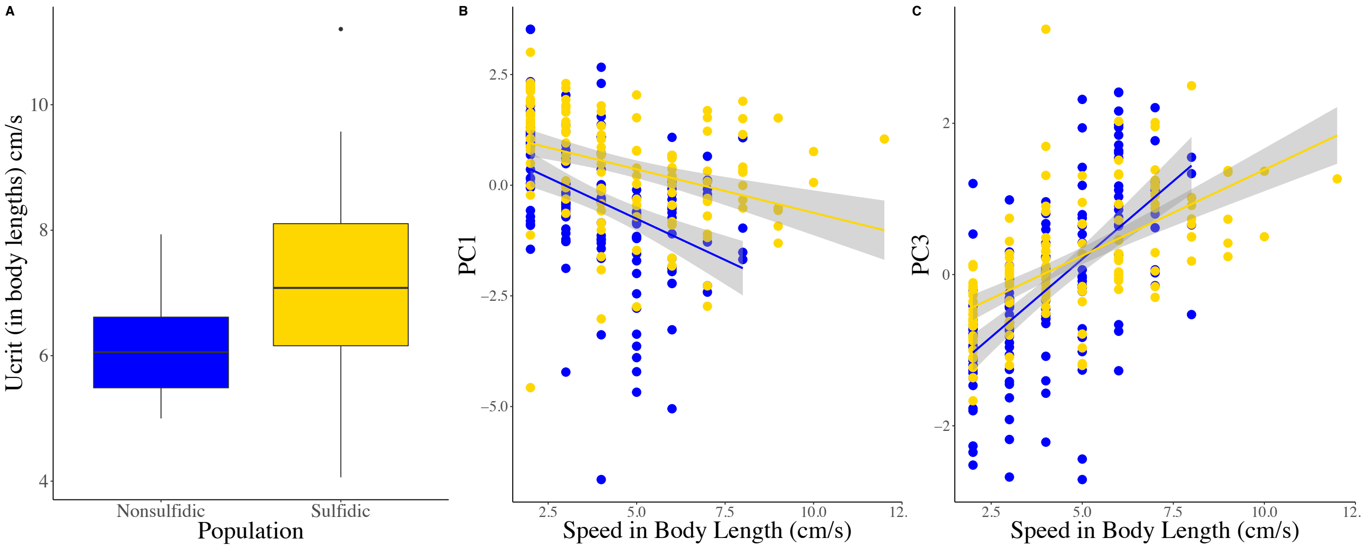

**Figure 2: (A) Scatter plot showing differences in critical swimming speed between fish from sulfidic (yellow) and nonsulfidic environments (blue). (B) Plot showing the best fitting model for PC1 of steady swimming kinematics as speed fish swam increased. Population differences are shown, with sulfidic environments in gold and nonsulfidic environments in blue. (C) Plot showing the best fitting model for PC3 of steady swimming kinematics as speed fish swam at increased. Population differences are observed with different colors showing population differences.**

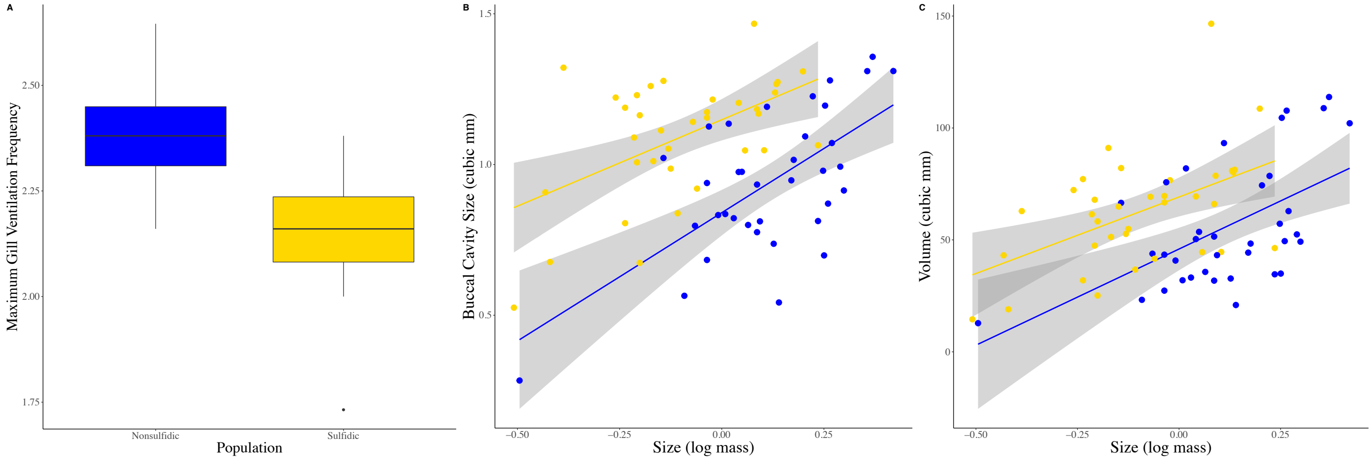

**Figure 3: (A) Boxplot comparing differences in gill ventilation frequency square root transformed between populations. (B) Scatter plot showing differences in buccal cavity size between sulfidic (yellow) and nonsulfidic (blue) populations. Buccal cavity size was log transformed. (C) Scatter plot showing differences in volume of water taken in per second (log transformed) between sulfidic (yellow) and nonsulfidic (blue) populations.**

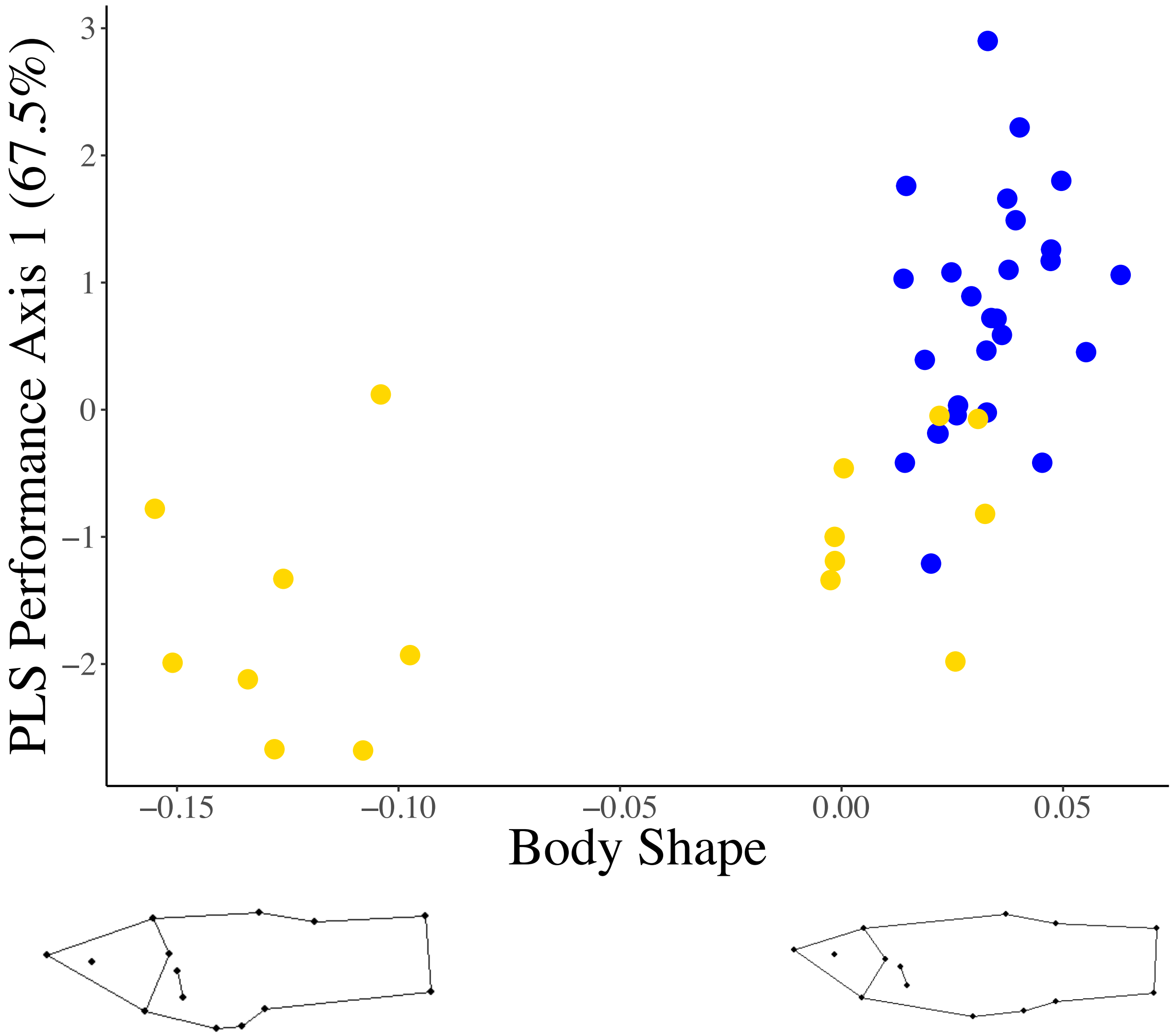

**Figure 4: Correlation of coupled partial least-squares latent vectors of body shape and different performances for sulfidic and nonsulfidic populations of *Poecilia mexicana* females. Vector diagrams illustrate the body shapes at either extreme body shape. Y axis depicts differences in performance extremes with fish more negative on the performance axis demonstrating greater critical swimming speeds and weaker burst speeds and gill ventilation frequencies. Fish with more positive values exhibited the opposite performance (better burst swimming and gill ventilation with weaker critical swimming speeds).**

**Table 1: Statistics of all fish used for experiments separated by population and sex with average mass(g) included**

| Population | Sex | Sample size | Mass ± SD [g] |
|---|---|---|---|
| Sulfidic | Male | 19 | 0.798 ± 0.126 |
| | Female | 17 | 0.942 ± 0.113 |
| Non-sulfidic | Male | 10 | 1.140 ± 0.399 |
| | Female | 26 | 1.510 ± 0.493 |

**Table 2: Results of principal component analyses on burst speed metrics. Provided are component loadings, eigenvalues, and per cent variance explained by each of the axes.**

| | PC1 | PC2 |
|---|---|---|
| Velocity | -0.644 | -0.003 |
| Acceleration | -0.504 | -0.247 |
| Rotational Velocity | -0.023 | 0.952 |
| Distance Traveled | -0.575 | 0.182 |
| Eigenvalue | 2.281 | 1.042 |
| % variance | 57.0 | 26.0 |

**Table 3: Results of principal component analyses on steady swimming data based on different kinematic data. Provided are component loadings, eigenvalues and per cent variance explained by each of the axes.**

| | PC1 | PC2 | PC3 |
|---|---|---|---|
| Tail-beat frequency | -0.077 | 0.830 | 0.418 |
| Rostral amplitude | -0.437 | -0.174 | 0.605 |
| Tail-beat amplitude | -0.531 | -0.226 | 0.262 |
| Propulsive wave length | -0.571 | -0.126 | -0.379 |
| Propulsive wave speed | -0.441 | 0.462 | -0.498 |
| Eigenvalue | 2.261 | 1.132 | 1.036 |
| % variance | 45.2 | 22.6 | 8.0 |

**Table 4: Results of (M)ANCOVA on different response variables quantified in this study: (A) burst speed, (B) $U_{crit}$, (C) maximum gill ventilation frequency, (D) buccal cavity size, and (E) total respiratory volume. Body mass was $log_{10}$-transformed prior to analyses. Effect sizes were quantified with partial eta squared.**

| | *F* | *df* | *P* | Effect Size |
|---|---|---|---|---|
| *(A) Burst Speed* | | | | |
| Sex | 0.089 | 2, 66 | 0.915 | 0.003 |
| Population | 12.102 | 2, 66 | <0.001 | 0.268 |
| Body Mass | 1.109 | 2, 66 | 0.336 | 0.033 |
| *(B)* $U_{crit}$ | | | | |
| Sex | 0.195 | 1 | 0.660 | 0.003 |
| Population | 5.393 | 1 | 0.023 | 0.075 |
| Body Mass | 1.038 | 1 | 0.312 | 0.015 |
| *(C) Maximum Gill Ventilation Frequency* | | | | |
| Sex | 0.029 | 1 | 0.866 | <0.001 |
| Population | 46.645 | 1 | <0.001 | 0.411 |
| Body Mass | 0.940 | 1 | 0.362 | 0.012 |
| *(D) Buccal Cavity Size* | | | | |
| Sex | 1.009 | 1 | 0.319 | 0.015 |
| Population | 37.391 | 1 | <0.001 | 0.358 |
| Body Mass | 28.052 | 1 | <0.001 | 0.295 |
| *(E) Respiratory Volume* | | | | |
| Sex | 1.284 | 1 | 0.261 | 0.019 |
| Population | 19.105 | 1 | <0.001 | 0.222 |
| Body Mass | 28.137 | 1 | <0.001 | 0.296 |

**Table 5 Analyses principal component axes (PC 1-3) describing swimming kinematics during steady swimming . For each response variable, models are listed from best to least supported based on ΔAICc values.**

| | -2 × log-likelihood | N | AICc | ΔAICc | AICc weight |
|---|---|---|---|---|---|
| *(A) PC1* | | | | | |
| Speed × Population +Body Mass + (1\|ID) | -452.56 | 280 | 931..40 | 0.0 | 0.8388 |
| Speed + Body Mass + (1\|ID) | -458.79 | 280 | 931.00 | 3.6 | 0.1415 |
| Speed × Sex × Population + Body Mass + (1\|ID) | -449.33 | 280 | 940.30 | 8.8 | 0.0101 |
| Speed × Sex +Body Mass + (1\|ID) | -457.09 | 280 | 940.40 | 8.9 | 0.0096 |
| Body Mass + (1\|ID) | -486.03 | 280 | 982.50 | 51.1 | <0.001 |
| Population +Body Mass + (1\|ID) | -484.62 | 280 | 982.90 | 51.5 | <0.001 |
| Sex × Population + Body Mass + + (1\|ID) | -482.07 | 280 | 983.20 | 51.8 | <0.001 |
| Sex +Body Mass + (1\|ID) | -485.69 | 280 | 985.00 | 53.5 | <0.001 |
| *(B)* PC2 | | | | | |
| Body Mass + (1\|ID) | -396.58 | 280 | 804.50 | 0.0 | 0.561 |
| Speed + Body Mass + (1\|ID) | -393.86 | 280 | 806.60 | 2.0 | 0.203 |
| Population +Body Mass + (1\|ID) | -396.53 | 280 | 808.00 | 3.4 | 0.101 |
| Sex +Body Mass + (1\|ID) | -396.52 | 280 | 808.00 | 3.5 | 0.100 |
| Sex × Population + Body Mass + (1\|ID) | -395.00 | 280 | 811.10 | 6.5 | 0.022 |
| Speed × Population +Body Mass + (1\|ID) | -391.82 | 280 | 812.10 | 7.6 | 0.013 |
| Speed × Sex × Population +Body Mass + (1\|ID) | -389.71 | 280 | 824.80 | 20.2 | <0.001 |
| Speed × Sex +Body Mass + (1\|ID) | -393.71 | 280 | 940.40 | 135.80 | <0.001 |
| | | 280 | | | |
| *(C) PC3* | | | | | |
| Speed × Population +Body Mass + (1\|ID) | -321.43 | 280 | 674.40 | 0.0 | 0.875 |
| Speed + Body Mass + (1\|ID) | -329.10 | 280 | 678.90 | 4.5 | 0.091 |
| Speed × Sex +Body Mass + (1\|ID) | -324.66 | 280 | 680.90 | 6.5 | 0.034 |

| | | | | | |
|---|---|---|---|---|---|
| Speed × Sex × Population +Body Mass + (1\|ID) | -319.12 | 280 | 688.70 | 14.4 | <0.001 |
| Body Mass + (1\|ID) | -392.61 | 280 | 797.90 | 123.5 | <0.001 |
| Population +Body Mass + (1\|ID) | -392.47 | 280 | 801.80 | 127.4 | <0.001 |
| Sex +Body Mass + (1\|ID) | -392.59 | 280 | 802.10 | 127.7 | <0.001 |
| Sex × Population + Body Mass + (1\|ID) | -392.44 | 280 | 809.00 | 135.6 | <0.001 |

**Table 6: Results of partial least-squares analysis examining covariation between performance variables and body shape for (A) males (B) females.**

| | Dim 1 | Dim 2 | Dim 3 | Dim 4 |
|---|---|---|---|---|
| *(A) Males* | | | | |
| Burst Speed PC1 | 0.380 | -0.512 | 0.311 | 0.705 |
| Burst Speed PC2 | 0.210 | 0.122 | 0.879 | -0.411 |
| $U_{crit}$ | 0.277 | 0.847 | 0.029 | 0.453 |
| Gill Ventilation Frequency | 0.858 | -0.076 | -0.361 | -0.359 |
| Singular Value | 0.663 | 0.270 | 0.052 | 0.015 |
| Correlation | 0.508 | 0.335 | 0.525 | 0.278 |
| $P$ | 0 .150 | 0.847 | 0.334 | 0.900 |
| | | | | |
| *(B) Females* | | | | |
| Burst Speed PC1 | 0.479 | -0.434 | -0.550 | 0.529 |
| Burst Speed PC2 | 0.450 | 0.840 | 0.022 | 0.304 |
| $U_{crit}$ | -0.493 | -0.033 | 0.359 | 0.792 |
| Gill Ventilation Frequency | 0.570 | -0.325 | 0.754 | <0.001 |
| Singular Value | 0.9667 | 0.993 | 0.998 | 1.000000 |
| Correlation | 0.675 | 0.379 | 0.280 | 0.328 |
| $P$ | 0.001 | 0.526 | 0.823 | 0.468 |

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