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Original Article

Multiple stressors lead to complex responses in reproductive behaviors in an African cichlid

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Abstract

Exposure to multiple environmental stressors is a common occurrence that can affect organisms in predictable or unpredictable ways. Hypoxia and turbidity in aquatic environments are 2 stressors that can affect reproductive behaviors by altering energy availability and the visual environment, respectively. Here we examine the relative effects of population and the rearing environment (oxygen concentration and turbidity) on reproductive behaviors. We reared cichlid fish (the Egyptian mouthbrooder, *Pseudocrenilabrus multicolor*) from 2 populations (a swamp and river) until sexual maturity, in a full factorial design (hypoxic/normoxic × clear/turbid) and then quantified male competitive and courtship behaviors and female preference under their respective rearing conditions. Overall, we found that the rearing environment was more important than population for determining behavior, indicating there were few heritable differences in reproductive behavior between the 2 populations. Unexpectedly, males in the hypoxic rearing treatment performed more competitive and courtship behaviors. Under turbid conditions, males performed fewer competitive and courtship behaviors. We predicted that females would prefer males from their own population. However, under the hypoxic and turbid combination females from both populations preferred males from the other population. Our results suggest that reproductive behaviors are affected by interactions among male traits, female preferences, and environmental conditions.

Key words: cichlid, competition, hypoxia, mate choice, turbidity.

Environmental stressors can affect behaviors associated with mate choice and impact reproductive success in animals (Candolin 2019; Candolin and Wong 2019). For example, anthropogenic stressors can alter the visual environment in aquatic ecosystems and affect the ability of animals, such as fish, to receive information about their environment (Seehausen et al. 1997; Heuschele et al. 2009; van der Sluijs et al. 2011). This can affect mate choice and have repercussions for the mating success of individuals and influence population dynamics (Candolin 2019). Yet, animals face multiple environmental stressors simultaneously that can vary from population to population. The interactions between these effects can either be classified as additive (linear), synergistic (detrimental, non-linear), or antagonistic (beneficial, non-linear), which makes it difficult to predict the long-term effects of multiple environmental stressors (Todgham and Stillman 2013; Côte et al. 2016). By considering the effects of multiple stressors on male and female behaviors associated with mate choice, we can better understand how aquatic animals like fish will cope with human-induced environmental changes like increasing turbidity or hypoxia (Diaz and Rosenberg 2008; Gray 2016).

Hypoxia (low-dissolved oxygen; DO) and turbidity (suspended particulate matter) are 2 examples of environmental stressors in aquatic environments that have wide-ranging effects on the physiology and behavior of fishes, as well as specific effects on male and female reproduction (Farrell and Richards 2009; Gray et al. 2012; Chapman 2015; Gray 2016). For example, in some species of fish, males will cease performing reproductive behaviors completely (Wu 2009) or engage in fewer reproductive behaviors under hypoxic conditions relative to fish acclimated to normoxic conditions (Gotanda et al. 2011). Hypoxic conditions also often require fish to allocate more time to aquatic surface respiration, where fish ventilate their gills at the surface where the oxygen concentration is higher (Chapman 2015). This behavior reduces time allocated toward reproduction and foraging and increases exposure to predation risk. Lastly, fish adapted to chronic hypoxia are often characterized by low metabolic rates, which could reduce the energy available for other processes (Chapman 2015). Turbidity also alters the time spent on reproductive behavior in males. Even slightly turbid conditions can increase aggressive behavior in males of an African cichlid (Egyptian mouthbrooder, *Pseudocrenilabrus multicolor*) in comparison to the same males' behavior under clear conditions (Gray et al. 2012). Alternatively, rock-dwelling cichlids in Lake Malawi reallocate time between aggressiveness toward males and courtship to foraging under turbid conditions (Gray et al. 2011b). The combined effects of hypoxia and turbidity on reproductive behaviors could be more detrimental than their individual effects by reducing both the time and energy available to engage in reproductive behaviors and by reducing the effectiveness of visual cues, respectively. Alternatively, because the primary targets of hypoxia and turbidity differ (e.g., aerobic metabolism versus vision), there may be no interaction between the 2 stressors.

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Females also alter how they evaluate potential mates under hypoxic and turbid conditions. For example, in the common goby, *Pomatoschistus microps*, females preferred males with nests that already contained eggs under normoxic conditions, but under hypoxic conditions, this preference was reversed (Reynolds and Jones 1999). This is presumably because males must increase ventilation under hypoxic conditions and the energetic costs of caring for more than one clutch in hypoxic conditions would be prohibitive. Additionally, in turbid environments, female sticklebacks shifted from using primarily visual cues to using primarily olfactory cues in a mate choice context (Heuschele et al. 2009). Because the consequences of multiple environmental stressors on mate choice outcomes depend on both male and female behaviors, studying both males and females is necessary to fully appreciate how environmental stressors will affect reproduction and population-level processes.

Within a species, populations can vary in their expression of and preference for particular traits (Endler and Houde 1995). For example, in mate choice experiments using guppies, *Poecilia reticulata*, females preferred males from their own population, and different populations of guppies used different combinations of criteria to evaluate mates such as color, body size, and tail size (Endler and Houde 1995). These population differences in preference could be due to female preference and male traits diverging simultaneously (Endler and Houde 1995). Similarly, mate choice decisions in the Panamanian bishop fish, *Brachyrhapis episcopi*, also varied by population. Specifically, populations without predators were choosier in bright light, while populations with predators were choosier in dim light when predation risk would be lower (Simcox et al. 2005). Additionally, in Kentish plovers, *Charadrius alexandrinus*, the size and intensity of ornaments were predicted by whether a population was polygamous or monogamous and the amount of rainfall experienced by each population (Argüelles-Ticó et al. 2016). Polygamous populations had darker, smaller ornaments while monogamous populations had lighter, larger ornaments (Argüelles-Ticó et al. 2016). Because populations across a landscape are likely to experience different selective pressures, it is important to consider within-species, population variation to predict a species potential to adapt to long-term environmental change.

Pseudocrenilabrus multicolor (Seegers 1990), an African cichlid found throughout the Nile River Basin, is an ideal species to test the effects of multiple stressors on reproductive behaviors like male courtship, male competition, and female mate choice and how those may vary across populations. *P. multicolor* is commonly found across sites that vary widely in DO concentration and turbidity. It is often the dominant species found in hypoxic, clear *Miscanthidium* swamps, and is also found in high densities in normoxic, turbid rivers (Chapman et al. 2008b; Crispo and Chapman 2008; Gray et al. 2012). This species has been extensively studied; a combination of laboratory-rearing experiments and field studies have highlighted the role of hypoxia and turbidity in influencing traits like behavior (e.g., boldness, courtship, competitive, and foraging behaviors (Gotanda et al. 2011; Gray et al. 2012; Oldham et al. 2019)), body shape (Crispo and Chapman 2010b), gill size (Crispo and Chapman 2010a), brain size (Crispo and Chapman 2010a), brooding period (Reardon and Chapman 2010), egg size and number (Reardon and Chapman 2009), metabolic rate (Reardon and Chapman 2010), and nuptial coloration (McNeil et al. 2016; Atkinson and Gray 2022). Divergence in many of these traits has consistently been found among swamp and river sites in at least 2 different drainages. Although *P. multicolor* survives in habitats that are characterized by varying DO and turbidity levels, there are still costs associated with reproducing under hypoxic and/or turbid conditions, and differences in physiology and behavior have been detected among *P. multicolor* populations. For example, fish in hypoxic environments have a lower standard metabolic rate and a shorter brooding period than fish acclimated to normoxia (Reardon and Chapman 2010), and turbidity can alter male aggression and visual performance (Gray et al. 2012; Oldham 2018). Considering human-induced rapid environmental change and loss of biodiversity, it is critical to understand how ecologically realistic multiple stressor scenarios will affect an organism's ability to survive and reproduce (Sih et al. 2011; Wong and Candolin 2015; Candolin 2019; Candolin and Wong 2019).

Our overall objective was to understand the relative effects of hypoxia and turbidity on reproductive behaviors and how those may differ across populations. To accomplish this, we reared fish from 7 days old until sexual maturity in a full factorial design experiment (hypoxic/normoxic by clear/turbid) and tested the behavioral responses of males and females under their treatment-rearing condition. Because responses to hypoxia and turbidity may differ between populations that naturally experience different extremes of these stressors due to underlying genetic differences, we reared fish whose parents were collected from both a swamp (hypoxic, clear) and a river (normoxic, turbid) population. Our first specific objective was to determine the relevant influences of rearing under hypoxia and turbidity on male reproductive behaviors including male competition and courtship. We predicted that males in hypoxia would decrease their overall activity and the number of courtship and competitive behaviors. Based on previous work in this species (Gray et al. 2012), we predicted that males in turbid conditions would also decrease their overall activity and the number of courtship displays, but be more aggressive. Our second objective was to determine the direct effects of rearing under hypoxia and turbidity on female reproductive behaviors, including the time spent inspecting males and preference for males from their own population. We predicted that females would spend more time inspecting males in normoxic and clear environments and that females would prefer males from their own population, though turbidity could disrupt this preference by masking visual cues. Differences in male traits that are a result of the rearing environment should consequently affect female preference, so we also sought to determine the indirect effects of hypoxia and turbidity on female preference. We predicted that hypoxia and turbidity would affect female mate choice directly (by altering female behavior) and indirectly (by altering male traits that are assessed by females). We used structural equation modeling (SEM), which is well suited to model systems with multiple potential pathways (Grace 2008), to assess these direct and indirect drivers of mate choice outcomes in *P. multicolor*.

Materials and Methods

Rearing experiment

Fish were collected from one swamp site (Lwamunda) and one river site (Ndyabusole) in the Lake Nabugabo region of Uganda in May 2018. The swamp site is characterized by hypoxic (0.79 ± 0.1 mg/L, mean ± SE point in time measurements) and clear (2.02 ± 0.3 NTU) conditions, whereas the river site is normoxic (6.84 ± 0.2 mg/L) and moderately turbid (18.81 ± 1.1 NTU; mean ± SE); measurements were taken between June and August in 2015, 2016, and 2017 from Oldham (2018). The wild-caught fish were transported to the laboratory for a full-sibling split-brood rearing experiment. The 2 populations were held separately and allowed to breed until they produced 10 independent broods (10 different male–female pairs) from each population. To ensure that each brood was produced by different parents, 1 male and 3 females from a single population were isolated until the first brood was observed. *Pseudocrenilabrus multicolor* are maternal mouthbrooders. After a female was determined to be holding a brood (distended jaw, refusal of food, and anti-social behavior), she was isolated to continue brooding. When the female released her brood (approximately 2 to 3 weeks), the young were housed in separate aquaria for 1 week then the brood was split randomly in a full-factorial design with 2 factors (oxygen treatment and turbidity treatment) with each having 2 levels (hypoxic or normoxic and clear or turbid, respectively). Broods from each treatment (oxygen and turbidity) and population (swamp or river) were spread evenly across 80, 19-L aquaria to account for minor differences in temperature and light availability (10 tanks per population/treatment). Weekly water changes were conducted in each tank to maintain water quality.

In the hypoxic tanks, oxygen was gradually reduced over a period of 1 week by bubbling nitrogen gas into the water and placing bubble wrap over the surface of the water. To maintain normoxic (mean ± SE: 7.52 ± 0.001 mg/L O_2) and hypoxic conditions (mean ± SE: 2.27 ± 0.01 mg/L O_2), DO concentrations were measured once a week in normoxic tanks and 3 to 7 times a week in hypoxic tanks using a YSI Pro2030 multimeter probe (see Supplementary File 1 for full summary of rearing conditions). DO was adjusted as needed by bubbling ambient air or nitrogen gas into the tanks. In the turbid tanks, we gradually increased turbidity over a period of 1 week by adding ~1.5 mL of bentonite clay solution (70 g in 700 mL water) each day. To maintain clear (mean ± SE: 1.31 ± 0.03 NTU) and turbid (mean ± SE: 16.6 ± 0.14 NTU) conditions, turbidity was measured once a week in clear tanks and 1 to 2 times a week in turbid conditions using a Hach 2100Q portable turbidimeter (Supplementary File 1). Turbidity was then adjusted as needed during weekly water changes, or for turbid tanks, by adding bentonite clay solution. Temperature was consistent across all tanks (mean ± SE: 24.9 ± 0.02°C).

The number of fish in each brood ranged from 27 to 105 (mean ± SE: 42.25 ± 4.65), so the initial number of fish in each tank ranged from 6 to 25 fish. Fish from each brood were randomly divided between treatments. Note that, at 1 week old, we could not determine sex, so sex ratios varied across tanks. To decrease and homogenize density, the number of fish in each tank was reduced to 10 at 6 weeks and further reduced to 6 fish at 20 weeks post-release. The fish were reared under their respective treatment conditions until they were ~1.5 years old when they were used for the behavioral experiments outlined below. At the time of the behavioral experiments, all fish were sexually mature; for example, males had red spots on anal fin, blue lips, and yellow coloration on ventral surface.

Male–male competition

To test the effects of oxygen, turbidity, and population on aggression, we conducted male–male competition trials. Male competition trials were conducted in the same environmental conditions that the fish were raised under (summary of trial rearing conditions; Supplementary File 2). Hypoxic conditions were achieved by bubbling nitrogen gas into the trial tanks and placing bubble wrap over each compartment to help maintain low oxygen conditions, whereas normoxic tanks had air stones to maintain their respective treatment conditions. Bentonite clay solution was added to turbid trial tanks to increase turbidity. Males were size-matched (less than 8% difference by standard length; distance from snout to caudal peduncle, range 4.4 to 6.3 cm; mean ± SD: 5.13 ± 0.46 cm) within trials and were from different broods of the same population so that siblings were not used in a trial together. DO, turbidity, and temperature measurements were taken from the center of the tank at the start of acclimation

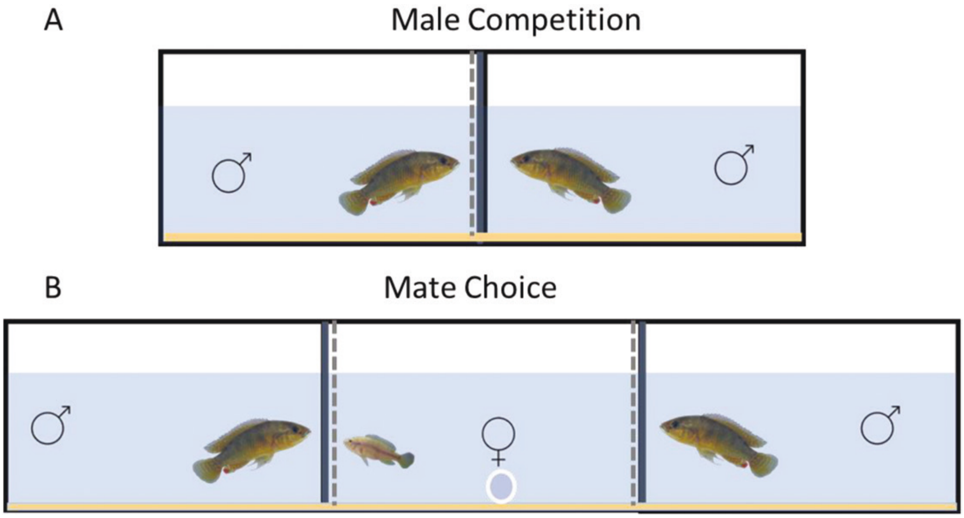

Figure 1. Diagram of male competition (A) and mate choice (B) trial tanks (not to scale). During acclimation, an opaque barrier (gray solid lines) was put in place so that the fish could not see one another. During the trial, the opaque barrier was replaced with a clear one (gray dashed lines), so that fish could interact with each other.

and immediately after the trial (for conceptual diagram, see Figure 1A; for snapshot from trials, see Supplementary Figure 1). An opaque barrier was placed between the 2 males (barrier likely did not block olfactory cues) during an overnight acclimation (18 to 22 h). Note that because sex ratios varied between tanks, our pre-experimentation housing was not standardized. Accordingly, social status of males and prior experience of males and females likely differed across trials. To initiate the trial, the opaque barrier was replaced with a clear one so that the males could interact without direct contact to prevent injury, and trials were filmed from the side for 30 min. Full water changes were performed between trials. Trials were only included in the analysis if the males approached each other (*n* = 33 trials analyzed out of 60; 2 to 5 trials per treatment, sample sizes in Supplementary Table 2). Note that although not quantified formally, there was generally higher mortality in the hypoxia turbid combination, particularly in the river population. Therefore, sample sizes in this treatment combination (river/hypoxic/turbid) in the male–male competition trial were low (2 trials) due to a lack of size-matched males that could be tested. We quantified the number of lateral displays (where the male places himself perpendicular to the opposing male), charges (rapid movement toward the opposing male with mouth closed), and bites (rapid movement toward the opposing male with mouth open), as well as the time active (i.e., moving in the water column). All videos for mate choice and male competition were analyzed by one observer using BORIS (Friard and Gamba 2016). The observer was blind to the population of fish in the trials.

Mate choice

To test the effects of oxygen, turbidity, and population on reproductive behaviors in males and females, we compared female preference and male courtship behaviors between fish reared under the 4 different treatment combinations and from the swamp and river populations. Specifically, we conducted mate choice trials on fish under the treatment conditions that the fish was reared under (e.g., a female raised in hypoxic/clear conditions was placed in a mate choice tank with the choice of 2 males, 1 from each population—swamp and river, that were also raised under hypoxic/clear conditions). The male from the same population as the female is referred to as the native male whereas a male from the different population is referred to as non-native male. All fish within a trial were from different broods so that siblings were not used in a trial together. Immediately before the overnight acclimation (18 to 22 h), the fish were photographed to quantify color (see below) and males were size-matched (less than 8% difference by standard length; range 3.9 to 6.1 cm; mean ± SD: 4.98 ± 0.49 cm).

In each trial, the trial tank was divided into 3 compartments (Figure 1B). Sand was included in the trial tanks so that males could build territorial pits overnight. We randomly assigned 2 males from the same rearing treatment but different populations (swamp population and river population) on either end of the trial tank. Males were isolated from each other and not able to physically interact. The female was placed in the center compartment, also physically isolated from both males. Only females that were not mouthbrooding were selected for use in trials. However, we did not record whether females were gravid at the time of the trial, so female receptivity to male courtship likely varied. A piece of PVC pipe in the middle of the tank was provided as a shelter so the female could choose not to interact with either male. The fish were allowed to acclimate to the tank overnight during which time opaque barriers prevented the fish from seeing each other (barriers likely did not prevent olfactory cues). After acclimation, the opaque barriers were removed (leaving clear barriers), and the fish were filmed from the side for 30 min to record all movement and interactions. Some males were reused, but they were given a least one week in between testing, and males never encountered another male more than twice. Only trials where females left the hiding spot were included in the analysis (*n* = 81 trials analyzed out of 105; 10 to 11 females per treatment/population Supplementary Table 2).

The treatment conditions were created and maintained as in competition trials (described above). Videos were recorded from the side and analyzed using BORIS by one observer who was blind to the population. In each videotaped trial, we recorded the amount of time a female spent within 7 cm (~1.5 body lengths) of each male. For males, we quantified the number of lateral displays and quivers (shaking movement of the whole body), as well as time spent active.

Male color

Male color was calculated using a previously established protocol (Maan et al. 2004). Briefly, before overnight acclimation, males were placed in a small, clear box (i.e., photo cuvette) filled with water and gently pressed against the front of the box using a gray board to be photographed (Canon PowerShot SX740 set to automatic). Using Adobe Photoshop, the photos were white balance corrected using a white standard placed on the front of the cuvette. The area of the fish (excluding fins and eyes) containing red (hue = 0 to 26 and 232 to 255, saturation = 40% to 97%) and yellow (hue = 27 to 45, saturation = 40% to 97%) pixels relative to the total number of pixels of the fish body was quantified. This value was used to calculate the overall % red + % yellow color to evaluate overall differences in red and yellow coloration as these colors typically resulting from carotenoid pigments may be correlated with fitness proxies and female preference in cichlids (Maan et al. 2006; Sefc et al. 2014).

Statistics

All analyses were conducted in R, version 4.3.0 (R Core Team 2019), and we used the lme4 package, version 1.10-35.1 (Bates et al. 2015) for generalized linear mixed models (GLMM) and linear mixed models (LMM). To determine the potential independent and interacting influences of oxygen and turbidity on behavior, we modeled these treatments as separate fixed factors and included an interaction term between oxygen and turbidity in all models. We also included population and standard length in all models. Brood was included as a random effect in all GLMM's and LMM's to account for relatedness between siblings. All data and analyses are available on Dryad.

For the male competition trials, we modeled the number of competitive behaviors (# of lateral displays + charges + bites) and activity (time spent moving through the water column). All models included oxygen, turbidity, the interaction between oxygen and turbidity, and population as fixed factors, standard length as a covariate, and trial and brood as random effects. Due to overdispersion, a GLMM with a negative binomial distribution was used to model the # of competitive behaviors whereas activity (s) was modeled using an LMM.

Color data (% red + % yellow coloration) for males used in the mate choice trials was analyzed using an LMM which included oxygen, turbidity, the interaction between oxygen and turbidity, and population as fixed factors, standard length as a covariate, and brood as random effect. Because some fish were used in more than one trial (90 unique males were used in mate choice trials), we only included the color measurement for the male's first trial in this analysis.

For males in the mate choice trials, we also tested for an effect of oxygen, turbidity, population, and standard length on the number of male courtship behaviors (# of lateral displays + # of quivers) and activity (time spent moving through the water column). Because some males were used in more than one mate choice trial (90 unique males were used in mate choice trials; 8-13 males per population/treatment combination, Supplementary 3), we only analyzed male behavior from each male's first mate choice trial. All models included oxygen, turbidity, the interaction between oxygen and turbidity, and population as fixed factors and standard length as a covariate. A GLMM with a Poisson distribution and a log link was used to model the # of courtship behaviors. Activity was modeled using an LMM.

We quantified female interest in male courtship as the time a female spent within 7 cm (approximately 1.5 body lengths) of either male using an LM with oxygen, turbidity, the interaction between oxygen and turbidity, and population as fixed factors. We also modeled the preference of a female for males from her own population using an LM. Preference was quantified by subtracting the time spent with the non-native male from the time spent with the male from her own population. A positive value indicated that a female spent more time with male from her own population. We calculated a PCA loading factor that combined standard length and the overall % of red + yellow color, as color and size are positively correlated in males of this species. We also calculated relative courtship behavior (# of courtship behaviors by male from native population- # of courtship behaviors by non-native male). A positive value for relative courtship indicated that the male from the local population engaged in more courtship behaviors. The relative differences of the PCA loading factor and time spent in courtship were included in the model of female preference.

We used an SEM to understand the direct and indirect effects of oxygen, turbidity, and population on female preference. We used the results from our GLMM and LMM's on male traits as well as previous studies to develop our a priori model (Figure 2; (Gray et al. 2012; McNeil et al. 2016; Atkinson and Gray 2022)). Our SEM was modeled using Lavaan (Rosseel 2012). To test the assumption of multivariate normality, we examined the χ^2 Q–Q plot from the MVN package (Korkmaz et al. 2014). To improve assumptions of multivariate normality, female preference (time spent with native or non-native male) and male courtship behavior (# of courtship behaviors) were square root transformed. Transformed data met assumptions of

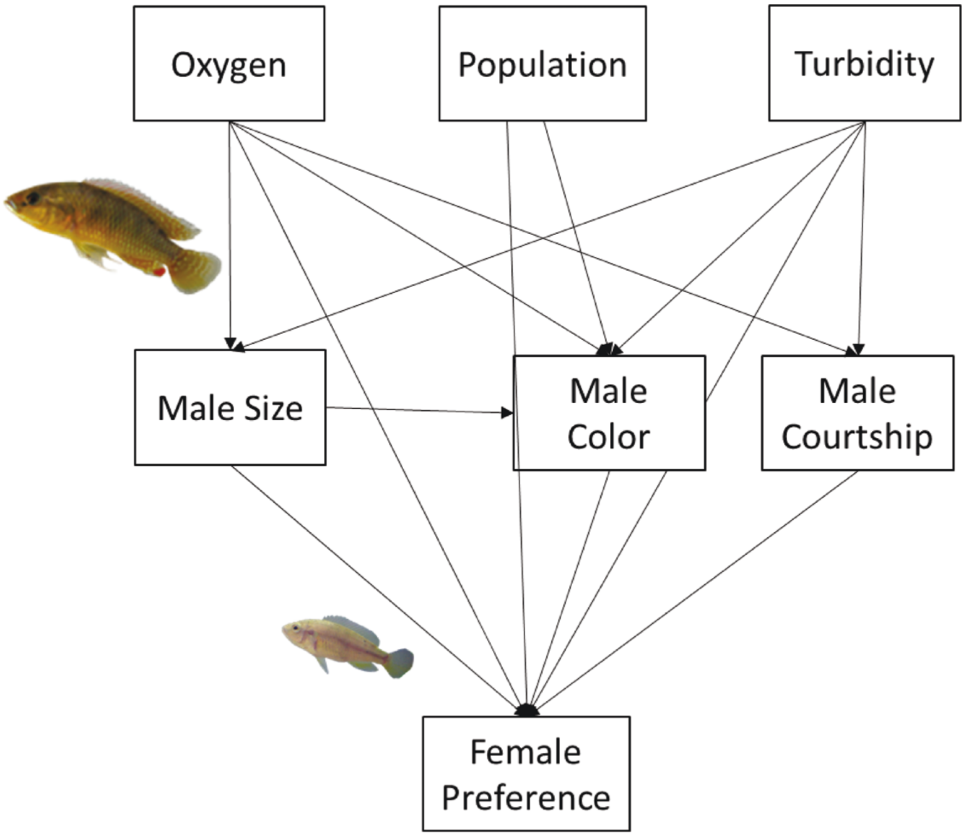

Figure 2. Conceptual diagram of hypotheses underlying the a priori structural equation model informed by the results of the individual models presented in this and prior studies. Rearing treatments were expected to have direct effects on female preference as well as indirect effects due to treatment effects on male traits important for female preference such as size (standard length), color (% red + yellow coloration), and courtship behavior (# of courtship behaviors). Previous studies on wild-caught fish have shown that color differs based on habitat type (McNeil et al. 2016), according to turbidity within a habitat (Atkinson and Gray 2022), and that larger males tend to be more colorful.

multivariate normality. For variables that were expected to be related, but without an obvious causal relationship, we included the covariance between residuals (male courtship, male size, and the time a female spent with either male). Whether our a priori model adequately fit the data was determined by examining the Model χ^2 ($P > 0.05$), and approximate fit indices (comparative fit index > 0.95, standardized root mean square residual < 0.10) (Grace 2020). We did not remove nonsignificant paths from our final model. The model structure is available on Dryad.

Results

Competitive behaviors

The number of competitive behaviors was significantly (P = 0.009) affected by the interaction between the oxygen and turbidity treatments. Specifically, males showed a significantly higher number of competitive behaviors in clear rather than turbid conditions, but only when in hypoxic conditions. Additionally, larger males engaged in more competitive behaviors ($P < 0.001$). Population did not affect the number of competitive behaviors (Figure 3A, Table 1). Activity during male competition trials was significantly lower (P = 0.019) in the turbid treatment, but unaffected by oxygen or population (Figure 3B, Table 1). Additionally, larger males were more active overall during competition trials (P = 0.050).

Male courtship

Oxygen ($P < 0.001$) and turbidity ($P < 0.001$) both affected the number of courtship behaviors performed (Table 1), where males in hypoxic treatments engaged in more courtship behaviors and males in turbid treatments engaged in fewer courtship behaviors (Figure 3C, Table 1). However, the interaction between oxygen and turbidity was not significant nor was the effect of population or standard length on the number of courtship behaviors performed. Oxygen (P = 0.023) and turbidity ($P < 0.001$) both affected overall activity (Table 1) where fish in the hypoxic treatment were more active compared with fish under normoxic conditions and fish in the turbid treatment were less active than fish reared in clear water (Figure 3D). Population, standard length, and the interaction between oxygen and turbidity had no effect on overall activity during mate choice trials (Table 2).

Male color

The first axis of the PCA, which included standard length and % red + yellow coloration, explained 90.1% of the variation and was used as a predictor variable in the female

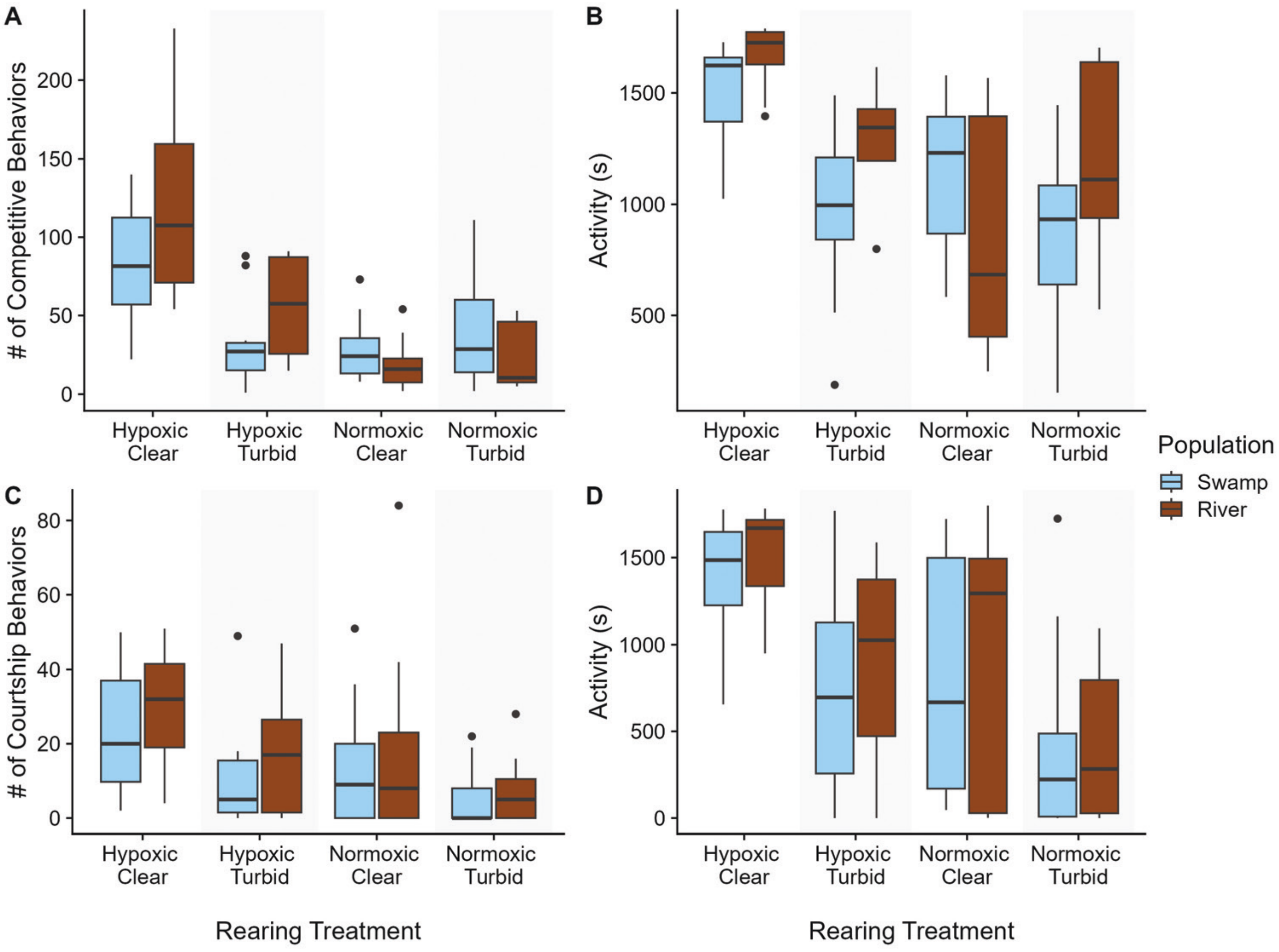

Figure 3. Boxplots of the median # competitive behaviors (A) and activity (B) of males in male competition trials and of courtship behaviors (C), activity (D) of males from the swamp (blue) and river (brown) population in mate choice trials. The tails above and below the boxes represent, respectively, the maximum and minimum values of the sample; the dots represent outlier individuals. Results of statistical analyses are presented in Table 1.

Table 1. Results of the analyses for the number of competitive behaviors (# of lateral displays + charges + bites; negative binomial GLMM) activity during competitive trials (seconds; LMM), the number of courtship behaviors (# of lateral displays + quivers; Poisson GLMM) activity during courtship trials (seconds; LMM), female interest in males (time spent within 7 cm of either male) and the preference for males of the same population native male quantified as the time spent with the native male minus the time spent with the non-native male. Population in the female interest and female preference models refers to the female's population. PCA 1 represents the color and standard length of the male

Variable	Z/t	*P*
# of Competitive behaviors		
Oxygen (normoxia)	–1.862	0.063
Turbidity (clear)	2.243	**0.025**
Population (river)	–1.443	0.149
Oxygen × turbidity	–2.598	**0.009**
Standard length	4.485	**<0.001**
Activity: male–male Competition		
Oxygen (normoxia)	–0.807	0.427
Turbidity (clear)	2.506	**0.019**
Population (river)	0.668	0.514
Oxygen × turbidity	–1.923	0.065
Standard length	2.030	**0.050**
# Of courtship behaviors		
Oxygen (normoxia)	–6.819	**<0.001**
Turbidity (clear)	7.582	**<0.001**
Population (river)	0.155	0.877
Oxygen × turbidity	0.758	0.449
Standard length	1.640	0.101
Activity: mate choice		
Oxygen (normoxia)	–2.312	**0.023**
Turbidity (clear)	3.824	**<0.001**
Population (river)	0.871	0.395
Oxygen × turbidity	–0.925	0.358
Standard length	–0.362	0.718
Female interest		
Oxygen (normoxia)	–2.876	**0.005**
Turbidity (clear)	–1.670	0.099
Population (river)	-0.604	0.555
Oxygen × turbidity	1.607	0.113
Female preference		
Oxygen (normoxia)	2.244	**0.028**
Turbidity (clear)	2.235	**0.029**
Oxygen × turbidity	–1.875	0.065
Population (river)	–2.192	**0.032**
Relative PCA1	1.599	0.114
Relative courtship	2.248	**0.028**

preference models. The average % red + % yellow coloration for all males used in trials was 28.5% (range 2.1% to 58.3%). The % red + % yellow coloration of males used in the trials was positively related to standard length, regardless of population, oxygen or turbidity treatment, or the interaction between oxygen and turbidity (LMM, oxygen: $t = -1.492$, $P = 0.140$, turbidity: $t = -0.037$, $P = 0.971$, population, $t = -1.756$, $P = 0.100$, oxygen × turbidity: $t = 0.563$, $P = 0.575$, standard length: $t = 7.906$, $P < 0.001$).

Female preference

Females in hypoxic trials spent significantly ($P = 0.005$) more time within 7 cm of any male, which was interpreted as time spent investigating potential mates (Table 1, Figure 4A). There was a marginally negative effect of being in the clear treatment on the time a female spent near males ($P = 0.091$). Population and the interaction between oxygen and turbidity were not significant (Table 1).

Females spent significantly more time with males of the same population when in normoxic ($P = 0.028$) and clear ($P = 0.029$) treatments. However, there was a marginally significant interaction suggesting that the effect of turbidity on the time spent with a native male was dependent on the oxygen treatment ($P = 0.065$, Table 1, Figure 4B). Specifically, females spent less time with native males when under the hypoxic and turbid combination. Within a trial, females preferred ($P = 0.028$) the males who engaged in relatively more courtship behaviors (Table 1). Additionally, the female's population had an effect ($P = 0.032$) on which male she spent time with river females spent less time near males from their own population compared with females from the swamp population Table 1). The PCA loading representing size and color did not affect female preference. However, males were size-matched closely, and size is positively correlated with color (Table 1).

Structural equation model

We determined that our a priori SEM fit our model adequately ($\chi = 20.65$, df = 21, $P = 0.481$, comparative fit index = 1.00, standardized root mean square residual = 0.068) and was therefore not rejected (Figure 5). Standardized and unstandardized values are presented in Supplementary 4.

The effects of oxygen and turbidity on individual male traits obtained from the SEM were similar to those from the reported LMM's/GLMM's. Turbidity (clear) had a direct, positive effect on the number of courtship behaviors in both males whereas oxygen (normoxia) had a direct, negative effect. Oxygen (normoxia) also had a direct, positive effect on the standard length of both native and non-native males, whereas turbidity did not affect standard length. The effect of oxygen on the color of both native and non-native males was due to its indirect effect on standard length rather than its direct effect; fish under normoxic conditions were larger and had a larger area of % red and % yellow coloration. Additionally, males from the swamp population had a larger area of % red and % yellow coloration than males from the river population.

The time a female spent with the male from her native population was not as well described ($R^2 = 0.128$) by the model compared with the time a female spent with the non-native male ($R^2 = 0.283$). The time a female spent with a native male was not directly or indirectly (through male traits or behavior) explained by oxygen or turbidity. Rather the time spent with a native male was directly explained by population ($P = 0.013$), such that river females spent less time near males from their own population compared with females from the swamp population (i.e., females spent less time near river males overall). In contrast, the time a female spent with a non-native male was significantly explained by the indirect negative effect of oxygen ($P = 0.005$) on male courtship behavior. Specifically, oxygen affected the time a female spent with a non-native male indirectly by changing male courtship behavior, but oxygen and turbidity also had marginally

Table 2. Summary of standardized indirect, direct, and total (indirect + direct) effects of oxygen, turbidity, and population on the time females spent near males of their native population and time spent with the male of the non-native population

Standardized effects	Time spent with native male			Time spent with non-native		
	Oxygen (Normoxia)	Turbidity (Clear)	Population (River)	Oxygen (Normoxia)	Turbidity (Clear)	Population (River)
Indirect	−0.044 (*P* = 0.386)	0.066 (*P* = 0.133)	0.027 (*P* = 0.290)	−0.183 **(*P* = 0.005)**	0.037 (*P* = 0.430)	−0.002 (*P* = 0.939)
Direct	−0.015 (*P* = 0.892)	0.091 (*P* = 0.407)	−0.263 **(*P* = 0.013)**	−0.185 (*P* = 0.085)	−0.171 (*P* = 0.080)	0.073 (*P* = 0.460)
Total	−0.060 (*P* = 0.575)	0.146 (*P* = 0.170)	−0.236 **(*P* = 0.024)**	−0.368 **(*P* < 0.001)**	−0.134 (*P* = 0.189)	0.071 (*P* = 0.452)

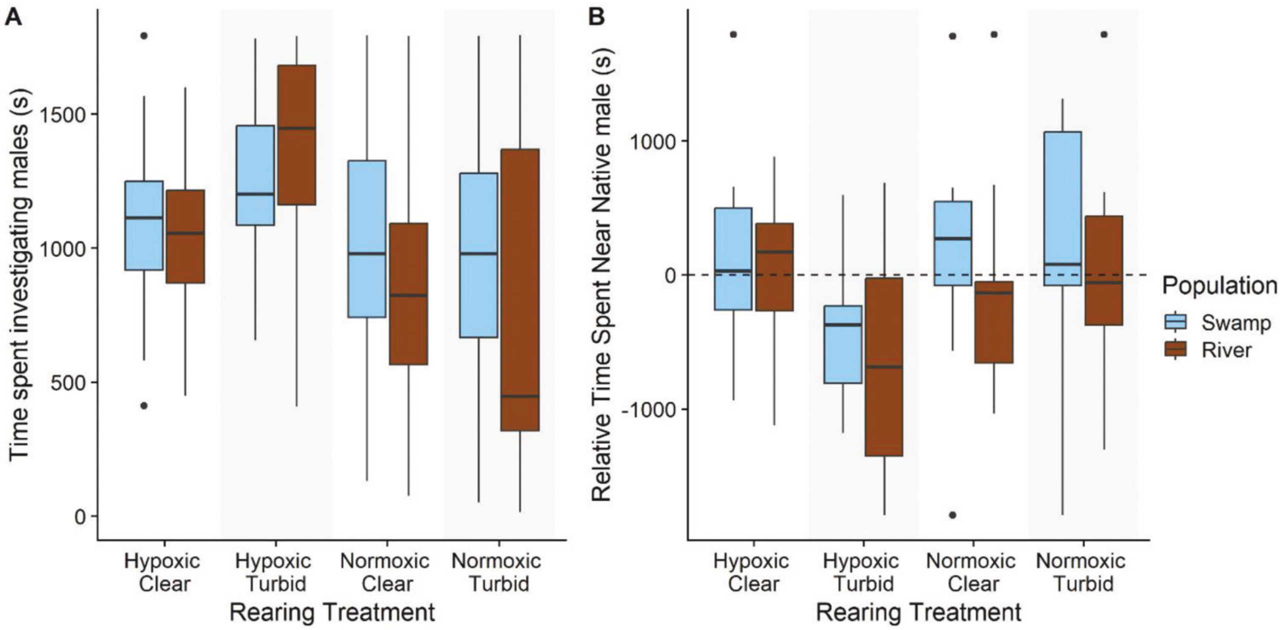

Figure 4. Boxplot of the median time a female spent within 7 cm of either male (A) and the relative time spent near native male (time spent with native male- time spent with non-native male; B). Positive values indicate that females spent more time with their native male than the non-native male while negative values indicate that females spent more time with the non-native male. Population refers to the female's population of origin (blue, swamp population and brown, river population). The tails above and below the boxes represent, respectively, the maximum and minimum values of the sample; the dots represent outlier individuals. Results of statistical analyses are presented in Table 1.

significant direct effects on the time a female spent with the non-native male (oxygen, *P* = 0.085, turbidity *P* = 0.080).

Discussion

Behavioral plasticity is a rapid means through which animals can respond to environmental changes (Wong and Candolin 2015). Overall, we found evidence for plastic behavioral responses to both hypoxia and turbidity in a reproductive context. Additionally, the effects of hypoxia and turbidity did not appear to differ across competitive and courtship contexts. On average, hypoxia tended to increase the number of competitive and courtship behaviors and overall time spent active, whereas fish reared in turbid water were, on average, less active and engaged in fewer competitive and courtship behaviors. In general, male behaviors did not differ between populations. However, females from both populations in hypoxic and turbid conditions preferred males that were not from their native population. Although potential synergistic effects have been emphasized in the ecological literature (Côte et al. 2016), we did not find evidence for a detrimental synergistic interaction of hypoxia and turbidity on male behavior. Our results suggest a complex set of interactions between male traits, female preference, and environmental conditions.

Overall, hypoxia was a strong driver of male courtship and competitive behaviors. Though there were fewer competition trials, particularly in the hypoxic turbid combination, we found that hypoxia affected courtship and competitive behaviors similarly. Typically, hypoxic conditions elicit a reduction in spontaneous swimming activity which reduces energetic demands (Chapman and McKenzie 2009; Chapman 2015). However, surprisingly the males from the hypoxic treatment tended to be more active and engaged in more competitive and courtship behaviors than fish from the normoxic rearing treatments. These results also differ from a previous study on wild-caught *P. multicolor* where a population from a hypoxic environment maintained in laboratory hypoxic conditions engaged in fewer courtship and aggressive displays than fish acclimated to normoxic conditions (Gotanda et al. 2011). Interestingly, a study on

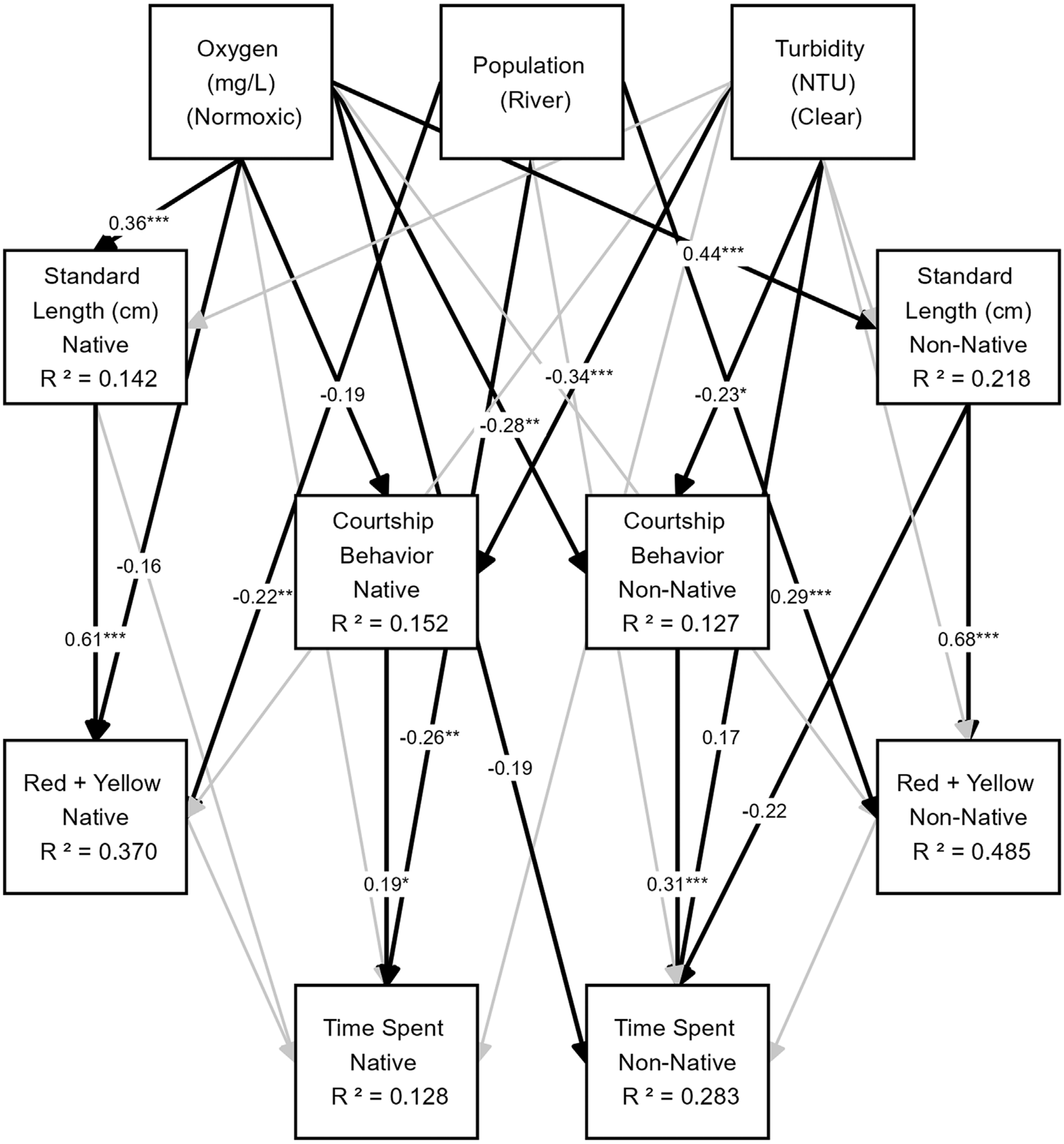

Figure 5. Path diagram for the structural equation model with standardized path coefficients measured in standard deviation units (unstandardized values are presented in Supplementary 4). Gray lines indicate nonsignificant paths. Black lines indicate paths were at least marginally significant ($P < 0.1$). An asterisk was placed after the path coefficients when paths were significant ($P < 0.05$). The covariance between residuals was included in the model but not included in the figure for the path diagram for standard length native/standard length non-native, courtship behavior native/courtship behavior non-native, and time spent native/time spent non-native. R^2 values indicate the amount of variation explained by the arrows leading to that variable. Population refers to the female population.

an undescribed species of cichlid, *Astatotilapia* "wrought-iron," found that when exposed to hypoxia, fish from hypoxic swamps were more aggressive than fish from open water habitats, but as fish began to exhibit aquatic surface respiration, differences in aggression between populations disappeared (Melnychuk and Chapman 2002). An increase in aggressive behaviors toward unknown males is common (Ward and Hart 2003). However, here it might be particularly important for fish from hypoxic conditions to exhibit a higher level of aggressive activity to quickly establish a social hierarchy and reduce the energy expended under hypoxic conditions. We might similarly expect that if our behavioral trials were conducted over a longer period we would have observed reduced activity, and competitive and courtship behaviors under hypoxic conditions. Alternatively, our unexpected results could also be explained by males being relatively unchallenged by our hypoxia treatments. The trial conditions matched the rearing conditions, giving the fish time to develop appropriate traits to establish hypoxia tolerance such as larger gills, and lower routine metabolic rate (Chapman 2007; Chapman et al. 2008a). Overall, behavioral responses to environmental stressors like hypoxia are complex and may have been influenced by our trial design using unfamiliar males as well as the rearing conditions. Clearly, more studies are needed to determine why fish in these conditions were more active under hypoxic conditions.

Turbidity was also a strong driver of courtship and competitive behaviors in males. The number of courtship behaviors and the time males spent active during mate choice trials were lower under turbid conditions, suggesting that turbidity could disrupt the relative importance of behavior as a cue used by females to evaluate males. This disruption could be because turbidity reduces the utility of visual cues, requiring males to reduce their courtship behaviors in favor of other cues. For example, under turbid conditions, guppies, *Poecilia reticulata*, reduced their courtship behaviors but had a higher intensity of carotenoid and UV coloration (Camargo-dos-Santos et al. 2021). Other studies on fish have found both increases (Candolin et al. 2007) and decreases in courtship on exposure to turbidity (Gray et al. 2011b). Our results differ from a previous study on *P. multicolor*, where males in turbid trials were more aggressive than the same males tested in clear trials (Gray et al. 2012). Although we did not find increased aggression during the male competition trials within our study, the turbidity level used in this experiment was higher and we did not use stimulus females in our competition trials, and aggression in males may be higher when females are present (Nagaoka and Karino 2015). Species that rely heavily on visual cues, such as cichlids, will likely be particularly sensitive to disruptions in the visual environment from turbidity (Delhey and Peters 2017).

Our study only included one population from each habitat type which limits our ability to make inferences on the role of hypoxia and turbidity in driving heritable differences in behavior. However, we saw few differences between populations in male reproductive behaviors, suggesting that behavioral differences in these 2 populations were primarily due to plasticity rather than fixed heritable differences. Plasticity can be classified as persistent changes in phenotypic expression in response to the environment experienced during development (i.e., developmental plasticity), or reversible changes in phenotypic expression (i.e., acclimation) (Beaman et al. 2016). Developmental plasticity in the response of this species to hypoxia and/or turbidity has been observed in gills as well as sensory traits like brains and eyes (Chapman et al. 2008a; Tiarks et al. 2024). Although it remains to be tested, such developmental responses to hypoxia and turbidity could underlie the observed behavioral differences. We only tested fish in their rearing environments, so it would be valuable to understand whether the behavioral responses to hypoxia and turbidity are reversible or if behavioral phenotypes are fixed during development. Furthermore, we did not find evidence of heritable differences in the assessed reproductive behaviors in the 2 populations studied, another study on *P. multicolor* found that fish from a swamp site were bolder than fish from a river site when tested under a common environment (Oldham et al. 2019). Heritable differences in boldness could be driven by distinct predation regimes experienced by populations rather than oxygen availability or turbidity concentrations (Oldham et al. 2019). Specifically, swamps have been proposed as a physiological refuge from predation and competition because they exclude hypoxia-intolerant species (Chapman 2021). Overall, we provide evidence that *P. multicolor* maintains plasticity in reproductive behaviors across populations in response to hypoxia and turbidity, but further studies using more populations from each habitat type would be necessary to support this conclusion.

Variation in female preference for males was due to both the direct and indirect effects of the rearing environment that altered female behavior and male traits, respectively. Although there may be non-reproductive reasons explaining why females would spend time associating with males such as reducing predation risk, we interpreted association time as female mating preference because in other species of fish, association time is positively associated with mate choice and females were provided with a refuge (Doughtery 2020). In hypoxic trials, females spent more time associating with males compared with females in normoxic trials, suggesting that DO was directly affecting female behavior. But DO also had an indirect effect on female behavior by increasing the rate of male courtship behavior. As expected based on other fish species (Candolin and Wong 2008), male courtship behavior was particularly important for explaining the time a female spent near each male. Conversely, male color did not influence female preference. In many cichlid species, color is used as a species-specific cue and as an indicator of individual male quality (Seehausen et al. 1997; Sefc et al. 2014; Selz et al. 2014). In many haplochromine species, males will change color patterns very quickly, within minutes of winning or losing a contest. This is not the case in *P. multicolor* where some color change is noticed with stress, major shifts in color pattern happen over much longer time scales (Gray et al. 2011a). Additionally, color and size are strongly correlated in this species and to remove the confounding variable of size, we used size-matched males within trials. It is not clear whether the correlation between size and color is due to the dominance status of larger males or simply more investment in color as males grow. Therefore, our trial design was not ideal for determining the importance of color as a cue. Overall, our results suggest that female preference is affected by the environment directly and indirectly through the environment's effects on male behavior.

Although some females prefer males from their own population (e.g., *Poecilia mexicana* (Tobler et al. 2009) and *Gambusia hubbsi* (Langerhans et al. 2007)), our results suggest that females preferred mating with genetically dissimilar males under certain environmental conditions. We found under the hypoxic/turbid combination females from both populations spent relatively more time with the male from the other population. Mating with males that are genetically dissimilar could provide a fitness advantage through heterozygosity by potentially masking deleterious alleles (Mays and Hill 2004). There is evidence for higher fitness in hybrids of some species (e.g., an alpine butterfly (Gompert et al. 2006), a sunflower (Rieseberg et al. 2008), and spadefoot toads (Chunco et al. 2012)) in extreme or novel environments compared with their parents (Chunco 2014). Although we could not attribute the observed female preference for the population under hypoxic and turbid conditions to the male traits we quantified, some genes like those of the major histocompatibility complex (MHC) can be directly assessed by odors cues (Milinski 2014, 2022). Females in some lizards, fish, mice, and humans can use odor cues to determine which mates would provide offspring with the most complementary number of MHC alleles (Milinski 2014, 2022). Further studies are warranted to determine whether other multiple-stressor scenarios alter female preference and whether such changes in preference are adaptive or maladaptive.

Overall, we found that hypoxia and turbidity were both important drivers of variation in male and female reproductive behaviors. Additionally, in the 2 populations we studied, behavior appeared to be plastic rather than fixed. Behavioral responses are a rapid way that animals can increase their chance of survival when environmental conditions change. Future studies should investigate whether these behavioral responses under hypoxic and turbid conditions are adaptive or maladaptive. For example, in our study we found behavioral responses that could be interpreted as adaptive (e.g., female preference for genetically dissimilar males under multiple stressor conditions) or maladaptive (e.g., males increasing competitive and courtship activity in a chronically hypoxic environment). Such information will be crucial to understand how animals will be affected by complex multi-stressor scenarios.

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Conflict of Interest

The authors have no conflicts of interest to declare.

Authors' Contributions

L.M.P. and S.M.G.: conceptualization, resources, writing - review and editing, supervision, project administration, funding acquisition. B.L.W.: conceptualization, formal analysis, investigation, writing, original draft, writing, review and editing, visualization.

Ethics Statement

Research was conducted under approval from The Ohio State University Institutional Animal Care and Use Committee (2014A00000055-R1). Scientific permits were obtained from the Commissioner of Fisheries Resources Management and Development, Uganda for permission to export fish and from the Uganda National Council for Science and Technology (UNCST) for research permission.

Supplementary Material

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

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