



RESEARCH PAPER

Sexual Selection in Black Morph *Girardinus metallicus* (Pisces: Poeciliidae): Females Can Spot a Winner (But We Cannot)

Gita R. Kolluru^{*1}, Crystal Castillo^{*}, Michele Hendrickson^{*}, Meghan Hughes^{*}, Paris Krause^{*}, Krista LePiane^{*}, Colleen McCann^{*}, Emily Pavia^{*}, Colin Porter^{*}, Rodet Rodriguez[†], Tomas Rodriguez-Cabrera[‡], Ellen Scott^{*}, McCall Willrodt^{*} & Susan M. Bertram^{§1}

* Biological Sciences Department, California Polytechnic State University, San Luis Obispo, CA, USA

† Instituto de Ecología y Sistemática, La Habana, Cuba

‡ Jardín Botánico de Cienfuegos, Cienfuegos, Cuba

§ Biology Department, Carleton University, Ottawa, ON, Canada

Correspondence

Susan M. Bertram, Biology Department,
Carleton University, 1125 Colonel By Drive,
Ottawa, Ontario, Canada K1S 5B6.
E-mail: Sue.Bertram@carleton.ca

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¹These authors contributed equally; lead authorship was determined by coin toss.

Abstract

Male–male competition may interfere with the ability of females to choose mates by interrupting courtship or by favoring highly aggressive males who may damage females during mating attempts. Alternatively, females may benefit by mating with dominant males, and female choice and male–male competition may therefore act in unison. The same traits, including aggressiveness, may indicate male quality to females and to rivals. We investigated sexual selection in the black morph of the endemic Cuban poeciliid fish, *Girardinus metallicus*, to ascertain the links between morphological and behavioral traits and success in intra- and intersexual selection. Males conspicuously exhibit their black ventral surface and gonopodium to females during courtship. Dichotomous choice tests revealed female association preferences for certain males, and those same males were more successful in monopolizing access to females when the fish were allowed to directly interact. Dominant males followed, courted, and copulated with females more than subordinate males within a pair, and it appears that females could either assess dominance based on cues we did not measure, or could influence subsequent mating success by their behavior during the dichotomous choice trials. There was an interaction between black status (i.e., whether the male in each pair had more or less ventral black coloration than the other male in that pair) and dominance, such that low-black dominant males courted early and then shifted to following females, whereas high-black dominant males courted far more later in the observation period. These results hint at the importance for sexual selection of the interplay between a static morphological trait (black coloration) and a dynamic behavioral trait (aggressiveness), but the functional significance of the courtship display remains a mystery.

Introduction

Sexual selection operates when members of one sex (typically males) compete for access to the opposite sex by intimidating, deterring, or defeating rivals (intrasexual selection) and when members of one sex

(typically females) select among potential mates (intersexual selection; Darwin 1859, 1871). Male–male competition and female mate choice have historically been viewed as separate, sometimes complementary (Berglund et al. 1996; Wiley & Poston 1996) and sometimes opposing (Moore & Moore 1999), forces

that shape the evolution of secondary sexual traits (Qvarnström & Forsgren 1998).

Several key studies have explored the interaction between male–male competition and female mate choice, and whether these two processes facilitate or compete with each other in their evolutionary effects (e.g., Qvarnström & Forsgren 1998; Wong & Candolin 2005). Wong & Candolin (2005) suggested that mate choice may be facilitated when male–male competition allows females to maximize their fitness potential, for example if dominant males are also high-quality mates. Wong & Candolin (2005) noted, however, that male–male competition may conflict with mate choice when competition results in courtship interference. In addition, dominant males may harm females by aggressively attempting to mate with them (Bierbach et al. 2013). It is therefore important to assess whether male–male competition and female mate choice work in unison to select for elaboration of the same traits, or in opposition (such that one process favors high trait values and the other favors low trait values), or whether they select for different traits altogether (Moore & Moore 1999; Andersson et al. 2002; Bonduriansky & Rowe 2003; Candolin 2003; Wong & Candolin 2005; Hunt et al. 2009).

In poeciliid fishes, coloration and modified fin ornaments often integrate with body size and behavior to produce displays that vary continuously or as part of discrete polymorphisms (Horth et al. 2010; Endler 2011; Culumber et al. 2014). However, many poeciliid species whose males exhibit elaborate displays have not yet been extensively studied. Understanding how integrated morphological and behavioral traits function in sexual selection in such species requires independent tests of female choice and male–male competition. This is especially true in aggressive species, because male–male competition may be costly to females by disrupting female choice (e.g., Fuller 2003; Wong 2004; Hibler & Houde 2006) or harming females due to excessive copulation attempts (Qvarnström & Forsgren 1998). Even in species whose males are not very aggressive, such as guppies (*Poecilia reticulata*), aggressive interactions may influence the ability of females to select mates (Hibler & Houde 2006; Price & Rodd 2006), even to the extent that they circumvent female choice in determining male reproductive success (Kodric-Brown 1992, 1993). Cryptic female choice may nonetheless allow females to exert mate preferences, and it is therefore worth assessing what traits females prefer (Evans et al. 2003; Wang et al. 2015). Fortunately, established methods exist to

examine male–male competition and female choice in poeciliids, and to tease apart the importance of specific traits in each of these contexts (Kodric-Brown 1993; Houde 1997; Fuller 2003; Walling et al. 2010; also see Jeswiet & Godin 2011 for validation of the methods for male choice).

Girardinus metallicus is an endemic Cuban poeciliid polymorphic for male coloration (Lorenzen 1996; Greven 2005; Ponce de León & Rodriguez 2010; Kolluru et al. 2014). Males of the most common ‘colorless’ morph (Fig. 1a) do not appear to exhibit a courtship display (Farr 1980; Lorenzen 1996; Kolluru et al. 2014). Males of the relatively rare black morph (also called the ‘black belly’ or ‘melanic’ morph) exhibit sex-specific black coloration on the ventral surface including the copulatory organ (gonopodium; Fig. 1b). This black coloration appears during development and appears to remain fixed in size and intensity following sexual maturation, as opposed to the facultatively expressed ‘fuzzy black’ spots elsewhere on males of this species and in other poeciliids (e.g., Blows et al. 2003). In contrast to the normal morph, black morph males exhibit a courtship display that involves presenting females with the ventral surface and extended gonopodium (Lorenzen 1996; Greven 2005; Kolluru et al. 2014). The gonopodium itself appears to be involved in this courtship display (Lorenzen 1996; Greven 2005; Kolluru et al. 2014).

In a previous study, we described the relationships between morphology and behavior of black *G. metallicus* (Kolluru et al. 2014). Both sexes are aggressive toward conspecifics in foraging and mating contexts (Farr 1980; Kolluru et al. 2014; Y.A. Akky and G.R. Kolluru, unpublished data). We observed freely interacting fish and found that larger males with longer gonopodia courted more and performed more copulation attempts and that males with greater ventral black coloration were more aggressive (Kolluru et al. 2014). These results suggest that sexual selection favors a multicomponent signal consisting of body size, gonopodium size, and ventral black coloration. However, we could not determine the relative importance of these traits for female choice versus male–male competition, as we did not assess each process independently.

In this study, we tested the hypothesis that body size and ventral black coloration are the targets of sexual selection, with an eye toward understanding which traits are important to different receivers (Møller & Pomiankowski 1993; Andersson et al. 2002; Griffith et al. 2006; reviewed in Candolin 2003; Grether et al. 2004). We employed a dichotomous choice design to first test for female association prefer-

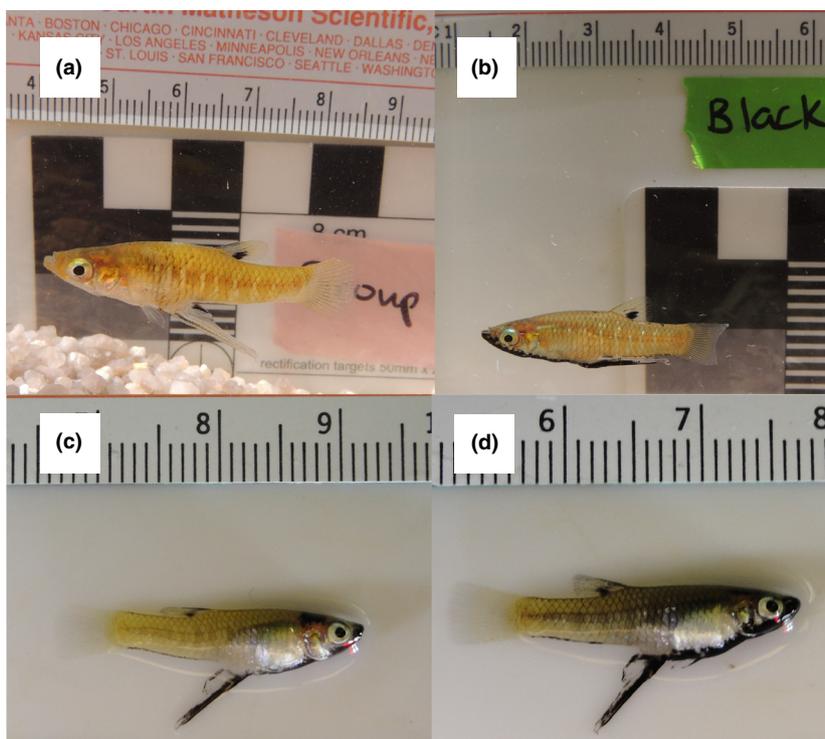


Fig. 1: Wild-caught normal morph (a) and black morph (b) males and a pair of the males used in the mating behavior tests illustrating low black (c) and high black (d) status. Note the images are not all to the same scale.

ences, known to be a predictor of likelihood of mating in poeciliids (Houde 1997; Fuller 2003; Fisher & Rosenthal 2006), and then allowed the same fish to interact directly. We predicted that females would spend more time associating with the male with the greater relative ventral black area within the pair. Because there were small differences in body size within each pair despite our efforts to size-match males, we also predicted that females would spend more time associating with the larger of the two males. We predicted that the same males preferred by females would be more aggressive and have greater access to females when the fish were allowed to interact freely.

Methods

Fish Husbandry

Our fish stocks were descended from a captive population obtained by David N. Reznick at the University of California, Riverside, and maintained at the Kolluru laboratory at California Polytechnic State University. The date of origin and provenance of this population are unknown. The stocks were maintained under controlled temperature ($25 \pm 0.5^\circ\text{C}$) and lighting conditions. Light was provided using a mixture of full-spectrum fluorescent and LED bulbs, on a

12:12 L:D schedule. Fish of unknown age were housed in mixed-sex 38-liter tanks, in which they almost certainly mated, and regularly transferred among tanks to maximize the chances of outbreeding within our colony. Fish were fed high-quality flake food in the stock tanks and frozen brine shrimp when isolated (see below). Fish were individually isolated in 7.5-liter tanks with natural colored gravel and plant material for cover for 3–8 wks prior to the start of the mating behavior trials, to minimize potential bias associated with familiarity and increase the chances that females would be sexually receptive due to deprivation of contact with males. During this time, males were photographed as described below. Also during this time, as part of a larger study whose data are not presented here, each male was individually subjected to a boldness assay in the presence of a natural predator (the Cuban cichlid, *Nandopsis tetracanthus*; Ponce de León & Rodriguez 2010). Males within a pair did not see each other prior to the observations discussed in this study.

Morphological Trait Measurements

Each male was lightly sedated using MS222 (Finquel; Argent Chemical Laboratories), and his right lateral surface photographed using a digital camera with the gonopodium angled away from the body

and with a ruler in view for scale (Fig. 1c, d). We also photographed several wild-caught colorless morph males and a single wild-caught black morph male immediately after capture from their natural habitat, in Guanimar, Cuba (Lat: 22.69805, Lon: -82.65016). These males were photographed in a narrow glass tank with a ruler for scale, and without using sedation (Fig. 1a, b). Using IMAGEJ 1.46r software (Abràmoff et al. 2004), we measured the following morphological traits for the black morph males: standard length (distance from anterior-most portion of the closed mouth to posterior-most portion of caudal peduncle line), gonopodium length (distance from the insertion point of the anterior most portion of the gonopodium to the distal tip of the hook), gonopodium lateral area (outline of the gonopodium from the insertion into the body to the tip of the hook), and ventral black area (black area in the region extending from the anterior dorsal edge of the eye to the tip of the gonopodium). Ventral black area appeared to be generally symmetrical on both sides of the fish, such that measuring one side was adequate to capture variation among males. When pairing males, we attempted to minimize the difference in standard length between males in each pair to minimize the potential effects of body size on aggression. The absolute difference in standard length between males in a pair was (mean \pm SE) 1.0 ± 0.12 mm (range = 0.0–2.4 mm).

General Experimental Design

We performed the study in three rounds over the course of 8 mo (May–December 2014), with 13 pairs tested in round one (over the course of 2 wks), 5 pairs in round two (over the course of 3 d), and 10 pairs in round three (over the course of 2 wks; total $n = 28$ pairs). Trials were performed in a 38-liter glass tank separated into three compartments by clear Plexiglas barriers with a perforated portion at the base that allowed passage of chemical cues (Fig. S1). The tank was situated inside a black-curtained area that minimized external disturbances and was illuminated with full-spectrum fluorescent and LED lights. Between sets of fish, we removed visible algae with cotton wool and performed a partial water change. All behavioral observations were performed blind with respect to the black status of each male (see below), which was not readily apparent to observers with the naked eye, especially because observers were seated slightly away from the tank to minimize disturbance. All trials were video-recorded as backup, but data were scored in real time.

Dichotomous Choice Trials

All fish were fed at least half an hour prior to the first trial. All trials were performed between 11:00 and 16:00. Variation in black markings in the head region and slight differences in body size were used to distinguish the two males within a pair. The males were simultaneously introduced into each of the male compartments, with the sides having been chosen at random. The female was introduced into the acclimation chamber, a food grade clear plastic cylinder, situated at the center of the neutral zone (Fig. S1). Following a 3-min acclimation time during which we ensured that the males and the female were swimming comfortably, we gently removed the acclimation chamber, releasing the female into the neutral zone. During the 10-min trial, we recorded the time spent by the female in each choice zone and in the neutral zone (Fig. S1). Following the first trial, we gently reintroduced the female into the acclimation chamber placed in the center of the test compartment and switched the two males between male compartments. We allowed for another 3-min acclimation time during which the fish could see each other. We then removed the acclimation chamber and performed another 10-min trial as described above.

Open Aquarium Trials

Immediately following the second dichotomous choice trial, we simultaneously moved the two males from the male compartments to the center compartment of the tank, such that all three fish could interact directly. To capture the behavior of both males immediately after the fish were allowed to directly interact, we performed 10-min focal observations on both males simultaneously, during which we recorded the behaviors in Table 1. Following a 5-min intermission during which the fish were not observed, we performed a second 10-min focal observation trial on both males simultaneously. Following the second trial, we sedated all three fish as described above, measured the standard length of the female using digital calipers, and weighed all the fish using a digital balance.

Data Analysis

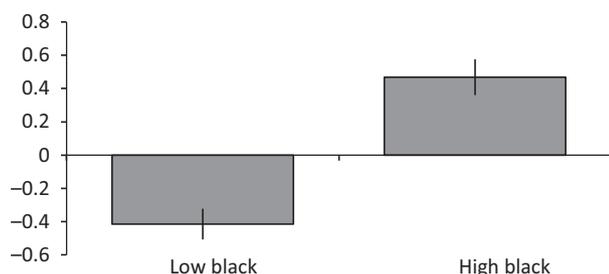
Because the morphological variables were intercorrelated, we used principal component analysis to obtain orthogonal components. We included male standard length, mass, gonopodium length, gonopodium area, body area, and ventral black area, and retained

Table 1: Description of behaviors recorded during open aquarium trials

| Behavior | Description |
|--------------------|--|
| Following duration | Time spent by the focal male following a female without orienting his body in the characteristic display stance described in Lorenzen 1996 and Kolluru et al. 2014 |
| Courtship duration | Time spent by the focal male near a female in the characteristic display stance |
| Copulation attempt | Rotation of the gonopodium forward, such that the male attempts to make physical contact between the tip of the gonopodium and the female gonopore |
| Chase male | Rapid movement by the focal male toward the other male |
| Bite male | Rapid movement by the focal male toward the other male, with visible contact between the mouth of the focal male and a part of the other male |

loadings with an absolute value > 0.75 when interpreting components. We obtained two components: body size (all variables except ventral black area had positive loadings and explained 76% of the observed variation with an eigenvalue of 4.56) and ventral black area (ventral black area was the only variable with a high positive factor loading and explained 13% of the observed variation with an eigenvalue of 0.75). Although body size alone explained a large percentage of the variation in morphology, we elected to retain the ventral black area component based on its importance in our previous study (Kolluru et al. 2014), its apparent role in courtship, and aggression, and because it explained more than 10% of the variation in morphology.

Based on the ventral black area component scores, we assigned males in each pair to either low or high black status, such that the male with greater PC2 ventral black area score within each pair was designated 'high black' and the other male was designated 'low black' (Fig. 2). We categorized black status because each female only saw two males, one with high black and one with low black. Further, categorizing black

**Fig. 2:** Mean ventral black area component scores of males assigned to low or high black status. Bars show least-squared means \pm SE.

status allowed us to look for interactions between black status and dominance status in the open aquarium trials. Using black status in models yielded the same qualitative results for all of the analyses described below as did using the ventral black component scores as a continuous variable.

For the dichotomous choice trials, we performed a mixed model repeated-measures general linear model (GLM) with male nested within pair as a random effect, black status, trial number, and male side as fixed effects and male body size component scores and female standard length as continuous covariates ($n = 28$ pairs). The dependent variable in this model was the time the female spent in the proximity of the male. Round was not significant ($p = 0.052$) and was removed from the analysis. The residuals were normally distributed so we did not transform the data (Shapiro–Wilk test; $W = 0.98$, $p = 0.15$).

We addressed whether the same males preferred by females in the dichotomous choice tests were also more successful at gaining access to females in the open aquarium tests ($n = 28$ pairs), by performing mixed model repeated-measures GLMs, with male nested within pair as a random effect, and time spent with the male across trials and female standard length as continuous covariates. The dependent variables for these models were following duration, courtship duration, and copulation attempts. Round was excluded from these models because it was not significant (all $p > 0.10$). We corrected for three tests using the false discovery rate (FDR, B–Y method; Benjamini et al. 2001), such that alpha-corrected was 0.0273.

We generated a dominance index by summing the chases delivered and received by each male across the two open aquarium trials and then generating a dominance index (Kolluru et al. 2014): $(\text{chases delivered} + \text{bites delivered}) / (\text{chases delivered} + \text{bites delivered} + \text{chases received} + \text{bites received})$. We used the dominance index to designate each male within a pair as either subordinate or dominant, for pairs for which there were no ties for the dominance index value. We were able to assign dominance status to the males in 22 of 28 pairs. Of the remaining six pairs, one had a tie for dominance index scores and the others did not exhibit any chases or bites.

To address whether there was a relationship between morphology and dominance ($n = 22$ pairs), we performed a mixed model repeated-measures GLM with male nested within pair as a random effect, black status as a fixed effect, and body size component scores as a continuous covariate in the model. The

dependent variable in this model was dominance index (a continuous variable). Round was not significant ($p = 0.99$) and was removed from the model. We used a square root of the value plus 0.5 transformation to most closely approximate normality of the residuals.

To examine mating behavior in the open aquarium trials, we performed mixed model repeated-measures GLMs, with male nested within pair as a random effect; trial number, black status, and dominance status as fixed effects; and body size component scores and female standard length as continuous covariates ($n = 22$ pairs). The dependent variables for these analyses were following duration, courtship duration, and copulation attempts. We removed round from these analyses because it was not significant (all $p > 0.20$). The residuals for following duration were normally distributed (Shapiro–Wilk test; $W = 0.98$, $p = 0.11$). We used square root of the value plus 0.5 transformations to most closely approximate normality of residuals for courtship duration and copulation attempts. We corrected for three tests using the false discovery rate as described above, such that alpha-corrected was 0.0273.

Results

Morphological Traits

Black morph male *G. metallicus* exhibited extensive variation in morphology. Standard length ranged from 14 to 24 mm, mass ranged from 49 to 260 mg, gonopodium length ranged from 30 to 46% of standard length, and ventral black ranged from 7 to 19% of body area (Table 2; Fig. 3).

Dichotomous Choice Trials

None of the factors in our model, including black status, influenced female association time (all $p > 0.12$).

Open Aquarium Trials

Males with whom the females associated more in the dichotomous choice tests also followed ($F_{1,53} = 7.39$; $p = 0.009$), courted ($F_{1,53} = 5.91$; $p = 0.019$), and attempted to copulate with females ($F_{1,53} = 7.85$; $p = 0.007$) significantly more in the open aquarium tests (alpha-corrected = 0.0273; Fig. 4). None of the factors in the model examining the relationship between morphology and dominance index were significant (all $p > 0.44$).

When the fish had direct access to each other, dominant males courted and attempted copulations significantly more than subordinate males (alpha-corrected = 0.0273; Table 3; Fig. 5). Males followed females more and attempted to copulate more in trial 2 than in trial 1. There was a significant trial number \times black status interaction for courtship duration, because low black males courted more in trial 1 and high black males courted more in trial 2. There was also a marginally non-significant trial number \times black status \times dominance status interaction for following duration. This complex interaction trend can be summarized as follows, focusing on dominant males because the behavior of dominant males changed more than that of subordinate males across trials: Whereas the following duration of all groups of males was similar in trial 1, the low black, dominant males increased following duration substantially across trials, such that they followed females more than all of the other groups in trial 2. In contrast, the same low black, dominant males dramatically decreased courtship duration across trials, whereas high black, dominant males dramatically increased courtship duration and copulation attempts across trials. Although overall levels of all male–female interaction behaviors were higher in dominant males than in subordinate males, there were also some changes in the subordinate males. Specifically, there were increases in courtship duration and copulation attempts across trials in

| Trait | Mean | SE | Median | Minimum | Maximum | CV |
|---------------------------------------|--------|------|--------|---------|---------|-------|
| Standard length (mm) | 19.40 | 0.32 | 19.54 | 14.32 | 24.27 | 12.51 |
| Body area (mm ²) | 77.89 | 2.48 | 76.88 | 40.99 | 117.18 | 23.79 |
| Mass (mg) | 150.50 | 6.53 | 140.50 | 49.00 | 260.00 | 32.48 |
| Gonopodium length (mm) | 7.21 | 0.11 | 7.27 | 5.44 | 9.56 | 11.67 |
| Relative gonopodium length (%) | 37.33 | 0.44 | 37.34 | 29.98 | 45.98 | 8.87 |
| Ventral black area (mm ²) | 9.62 | 0.30 | 9.53 | 5.74 | 16.25 | 23.32 |
| Relative ventral black area (%) | 12.68 | 0.36 | 13.21 | 7.00 | 19.03 | 21.45 |

Table 2: Descriptive statistics for morphological traits in black *Girardinus metallicus*

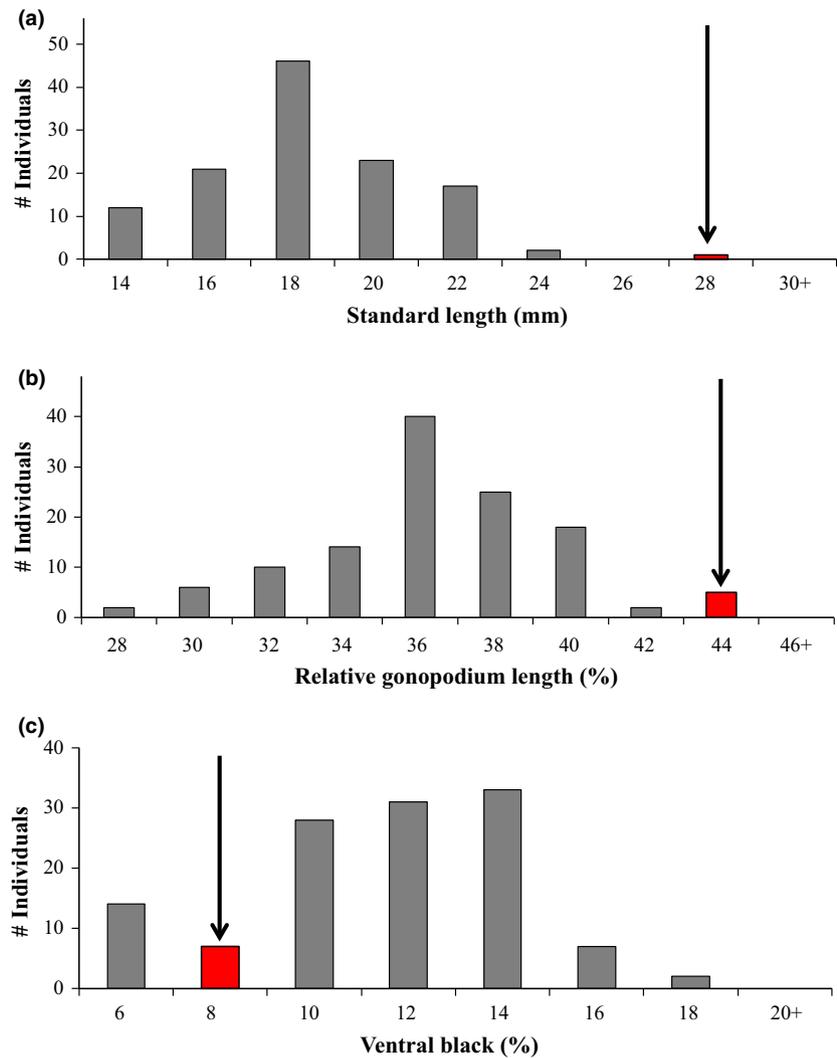


Fig. 3: Frequency distributions of standard length (a), gonopodium length relative to standard length (b), and percent of body covered by ventral black (c). The values for the lone black morph male collected in Cuba are indicated by the arrows.

high black males and in following duration across trials in low black males.

Discussion

Poeciliid fishes are model systems for studying sexually selected traits (Meffe & Snelson 1989; Houde 1997; Pollux et al. 2009; Evans et al. 2011; Kolluru 2014), yet many species have not been studied in detail (Endler 2011). Using the black morph of the endemic Cuban species *G. metallicus*, we demonstrated a correlation between female preference in dichotomous tests and male–male aggression in the open arena, suggesting that females are able to distinguish males who will subsequently be successful at aggressive competition. However, female association during the dichotomous choice tests was unrelated to any morphological trait that we measured, including

body size, gonopodium size, and area of black on the ventral surface of the body—all of which seem to be displayed during courtship (Lorenzen 1996; Kolluru et al. 2014). Instead, dominance status was the greatest predictor of ability to gain access to females in the open aquarium tests, consistent with the situation in the normal ('colorless') morph of this species (Farr 1980). It appears either that females were able to assess the dominance status of males prior to interacting with them or that something about the interactions during the dichotomous choice tests led to the preferred male winning more often in contests in the subsequent open aquarium tests. Because males did not often court females through the partitions during the dichotomous choice tests, females may instead have used chemical cues (Fisher & Rosenthal 2006), or more subtle cues that we did not measure, such as variation in yellow body coloration or swimming

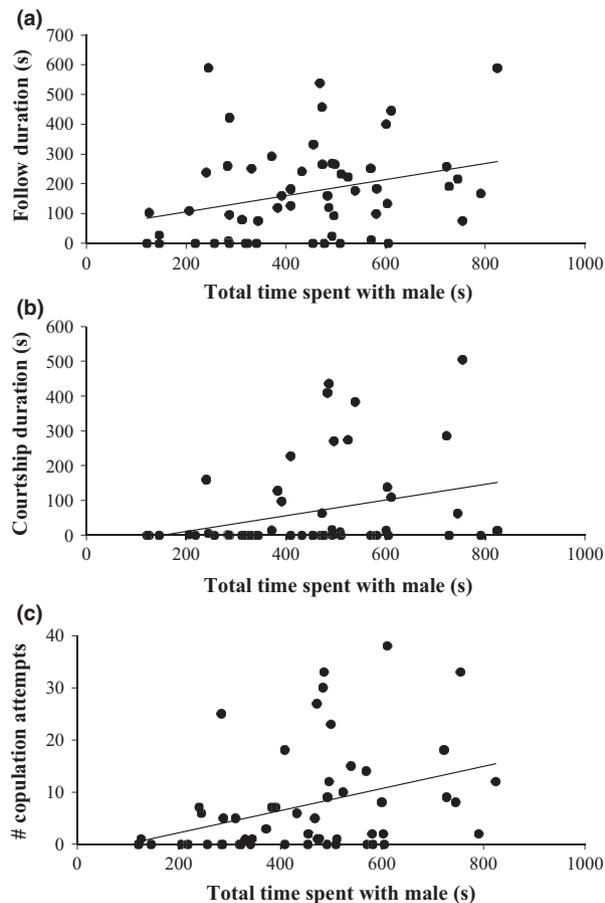


Fig. 4: Relationships between the total amount of time the female spent in a male's choice zone and the behavior of that male in the open aquarium tests. Males that females associated with more in the dichotomous choice tests spent significantly more time following the female (a) and courting the female (b), and attempted to copulate more often (c) (alpha-corrected = 0.0273).

behavior (Bierbach et al. 2013) to assess male dominance status and hence male mate acquisition ability. Traits of dual use (used by both potential mates and potential rivals) occur commonly in other taxa (Berglund et al. 1996). Regardless of the traits, it appears that female choice and male–male competition favor the same males in this system. It is expected that females should benefit by mating with high status males (Bierbach et al. 2013), and preference for contest winners has been demonstrated in poeciliid fishes, even to the extent that females incite competition among males to identify winners (Berglund et al. 1996). However, harassment by dominant males leading to injury during excessive copulation attempts (Wang et al. 2015) may outweigh the benefits of mating with dominant males (Bierbach et al. 2013); to our knowledge, these ideas have not been tested in *G. metallicus*.

Table 3: Results of general linear mixed models

| Behavior | Term | DF | F | p |
|---|---|--------------|--------|-------------------|
| Follows female | Trial Number | 40 | 23.802 | <0.0001 |
| | Black Status | 38 | 0.518 | 0.4763 |
| | Trial Number × Black Status | 40 | 1.416 | 0.2411 |
| | Dominance | 38 | 1.239 | 0.2726 |
| | Trial Number × Dominance | 40 | 1.774 | 0.1904 |
| | Black Status × Dominance | 38 | 0.230 | 0.6345 |
| | Trial Number × Black Status × Dominance | 40 | 4.245 | 0.0459 |
| | Male PC1 Size | 38 | 0.412 | 0.5246 |
| | Female Standard Length (mm) | 38 | 0.592 | 0.4465 |
| | Courtship duration | Trial Number | 40 | 0.336 |
| Black Status | | 38 | 0.966 | 0.3320 |
| Trial Number × Black Status | | 40 | 6.255 | 0.0166 |
| Dominance | | 38 | 7.273 | 0.0104 |
| Trial Number × Dominance | | 40 | 0.132 | 0.7187 |
| Black Status × Dominance | | 38 | 1.192 | 0.2818 |
| Trial Number × Black Status × Dominance | | 40 | 1.494 | 0.2288 |
| Male PC1 Size | | 38 | 1.487 | 0.2303 |
| Female Standard Length (mm) | | 38 | 0.085 | 0.7721 |
| Copulation attempts | | Trial Number | 40 | 5.580 |
| | Black Status | 38 | 1.176 | 0.2851 |
| | Trial Number × Black Status | 40 | 1.966 | 0.1685 |
| | Dominance | 38 | 6.223 | 0.0171 |
| | Trial Number × Dominance | 40 | 0.792 | 0.3788 |
| | Black Status × Dominance | 38 | 0.159 | 0.6927 |
| | Trial Number × Black Status × Dominance | 40 | 0.218 | 0.6433 |
| | Male PC1 Size | 38 | 0.565 | 0.4568 |
| | Female Standard Length (mm) | 38 | 0.068 | 0.7963 |

Boldface p-values indicate significance after correction for multiple tests (alpha-corrected = 0.0273).

We performed the open aquarium trials immediately following the dichotomous choice trials rather than allowing for some acclimation time, because we did not want to potentially miss important behavioral interactions that may have occurred as soon as the fish could directly interact. Although there was no main effect of black status on mating behavior or female preference, there were complex patterns of association between black status and dominance status, which shifted across the two open aquarium trials (10-min trials separated by a 5-min intermission). Within the dominant males, low black males courted and attempted copulations far more than high black males in the first trial and shifted to following females far more than high black males in the second trial. If following females is a mate-guarding behavior, this suggests that low black males (so long as they were dominant) mated with females early on and shifted to guarding them in trial 2, whereas high black males (so long as they were dominant) mated more in trial 2.

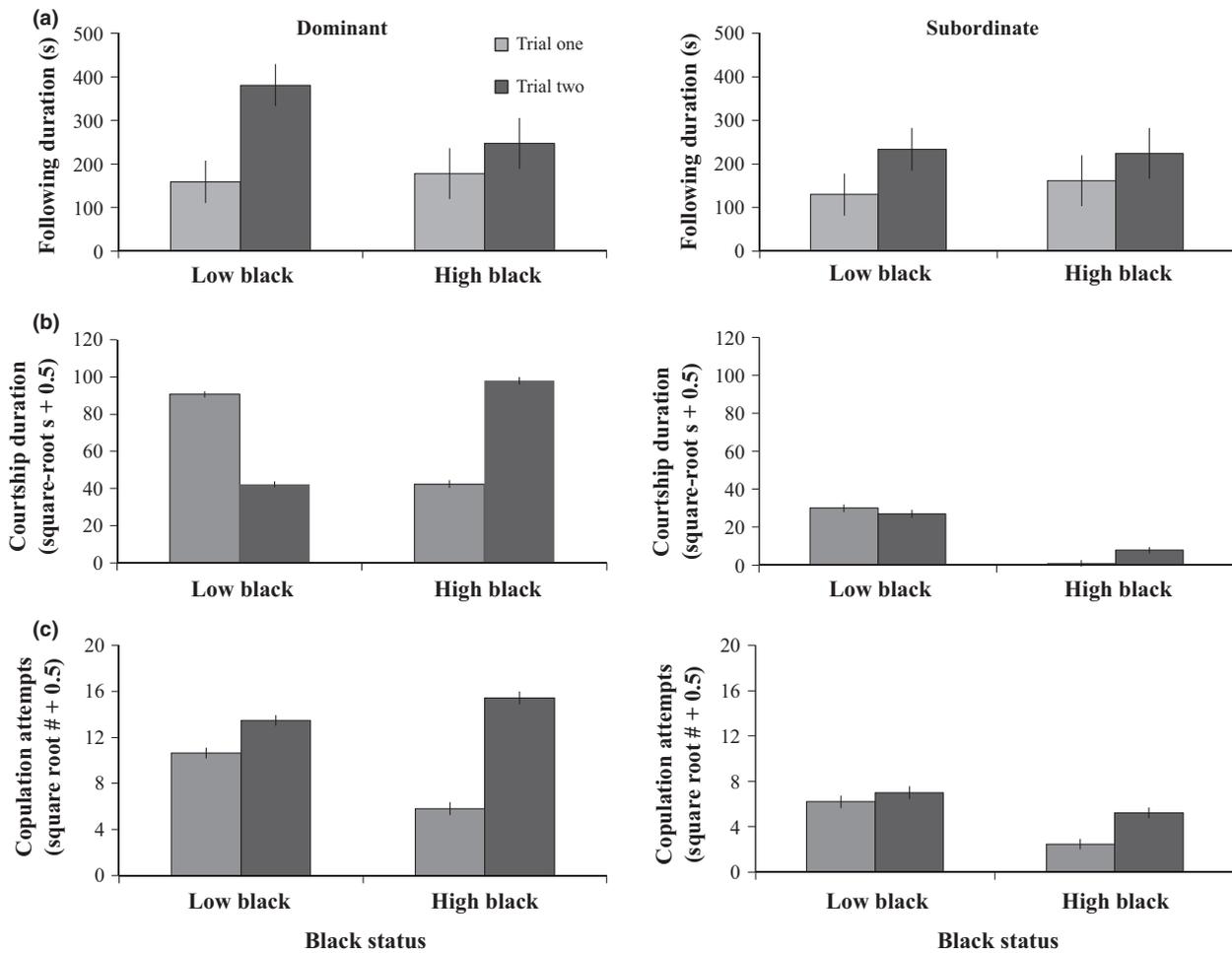


Fig. 5: The influence of dominance status, black status, and trial number on time spent following the female (a), time spent courting the female (b), and number of copulation attempts (c). Bars show least-squared means \pm SE.

This pattern may be explained by females associating with low black males early on, when stress levels due to being placed in the tank together may have been high, because low black males are less likely to attract predators (Lorenzen 1996; but see Horth et al. 2010). *Girardinus metallicus* is known to inhabit the middle of the water column in brightly lit areas in bodies of water containing visually orienting predators such as *N. tetraodon* (Ponce de León & Rodriguez 2013). Females may switch to associating with high black males later because the females became more comfortable with the surroundings, because they could increase the diversity of mating partners or because they could ensure that high black males sired their offspring due to last male sperm precedence. Alternatively, high black males may have increased their reproductive effort in the second trial because they were more comfortable or because they had time to

assess and suppress their rivals. In contrast to all of these patterns, the behavior of subordinate males did not change much across trials, regardless of the black status of the males. Therefore, although dominance status appears to be key to securing access to mates across morphs of *G. metallicus* (Farr 1980), ventral black coloration also contributes to male mating behavior. This is a complex example of a dynamic trait (aggressive behavior) interacting with a static trait (ventral black coloration) in determining mating success (Hill et al. 1999).

The experimental design of the present study is in contrast to our previous work (Kolluru et al. 2014), in which we observed fish after males and females had physically interacted for 18–22 h. In that study, we found that dominant males had greater ventral black coloration than subordinate males, whereas in the present study, we found no such association. It is

possible that coloration becomes important in aggressive interactions after females have had time to exercise choice and hence influence intermale interactions. Indeed, even in the present study, there was a trend toward all behaviors being more common in trial 2 than in trial 1, representing increasing male–female interactions and aggression over the course of just 25 min. In a pilot study of staged contests over a defensible food source, the male with more ventral black (as gauged by eye by observers blind to the goals of the study) more aggressively defended the food source from the other male (Poore H., Weiner D. & Kolluru G.R., unpublished data), suggesting a role for ventral black coloration in aggressive interactions in contexts other than mating. Similarly, black coloration may also become more important in female choice over time. Positive female reactions to males may encourage males with greater ventral black coloration to more aggressively defend the females with whom they are trying to mate.

Additionally, in the present study, we used a male-biased sex ratio (two males and one female), whereas in Kolluru et al. (2014), the sex ratio was even (two males and two females). Under a male-biased sex ratio, females may have had less opportunity to exert pre-copulatory mate choice (e.g., Hibler & Houde 2006), and the effect of female response on the motivation of more black males to be more aggressive may therefore have been diminished. In the field, *G. metallicus* tends to exhibit female-biased sex ratios, which is consistent with operational sex ratios in other poeciliids (e.g., Arendt et al. 2014).

Finally, in the present study, males were exposed to a (confined) predator several days prior to mating trials, as part of another study, and this was not the case in Kolluru et al. (2014). Ventral black coloration may make males more visible to predators, and more black males may therefore have been more reluctant to be aggressive. While this is unlikely to be the case because at least a few days elapsed between predator exposure and mating trials, it is worthy of consideration.

Our results beg the question of why males display the prominent ventral black coloration and gonopodium to females during courtship if those traits are not correlated with female association preferences. Several possibilities emerge. The display could be an indicator of morph (analogous to a species indicator) rather than an indicator of male quality, such that large differences between morphs are important to females, but small differences in traits within the black morph are not. The black morph co-occurs with the normal morph in the same bodies of water in Cuba (pers. obs.), albeit at much lower frequencies.

Laboratory populations of mixed morphs exhibit a stable polymorphism with a strong majority of normal morph males (Lorenzen 1996; Greven 2005). Normal morph males force copulations without first displaying (Farr 1980), and females may prefer displaying black morph males over non-displaying normal morph males. Our recent pilot study of three wild-caught females in a dichotomous choice setup with a single pair of size-matched normal morph and black morph wild-caught males lends support to this idea (Fig. 6). In Kolluru et al. (2014), we provided a link to video of the courtship display of a laboratory-reared male. Here (<http://bit.ly/1EYNp2j>), we provide a video of the courtship display of the wild-caught black morph male. Our video also shows a normal morph wild-caught male following the wild-caught females without displaying.

Alternatively, the ventral black area could be an amplifier of a preferred trait. Amplifiers are not necessarily direct targets of female choice, but rather func-

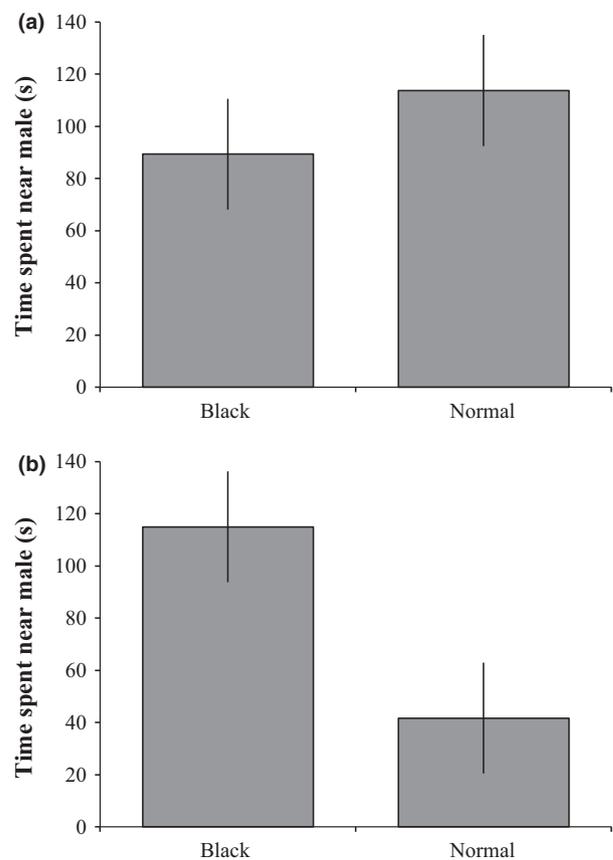


Fig. 6: Results from our pilot study in Cuba exploring how wild female association time in a dichotomous choice test was influenced by the interaction between male morph (black versus normal) and trial number (a) trial 1, (b) trial 2. Bars show least-squared means \pm SE.

tion to enhance the ability of females to perceive the preferred trait (reviewed in Grether et al. 2004). For example, melanin-based black coloration often surrounds the strongly sexually selected orange spots of male guppies, but the black is not itself a target of female choice (Brooks 1996). Rather, by outlining the orange spots, black coloration may serve to enhance the ability of females to assess male quality based on orange spots (Brooks 1996). Brooks (1996) supported this idea by showing that reducing the black coloration caused males to become less attractive to females. Black morph *G. metallicus* males are distinctly yellow in the areas that are not black (Fig. 1), and this yellow is more pronounced in males than in females (Lorenzen 1996). Black may be an amplifier of this yellow coloration, which is likely to be condition-dependent due to the presence of carotenoid pigments (Kemp et al. 2011).

In summary, we have shown that the same males are favored by intrasexual and intersexual selection, such that the same traits may be important in both contexts. However, although a complex interaction between ventral black coloration and dominance status confers the ability to monopolize access to females, we could not identify exactly which traits are the targets of female choice. *Girardinus metallicus* is polymorphic for male coloration, and only the relatively rare black morph exhibits a courtship display, making it likely that courtship in the black morph evolved from the non-courtship colorless morph. In poeciliids in general, courtship displays may evolve from aggressive displays (reviewed in Wang et al. 2015), such that the same traits may be important in both (Berglund et al. 1996). We have seen black morph males extending their gonopodia (but not tilting their chins) during prolonged aggressive battles, and the courtship display may have evolved from these movements. A broader investigation, perhaps using more natural female-biased sex ratios, may enable us to ascertain exactly which traits are important to females. It is possible that females may prefer displaying black morph males over non-displaying normal morph males, or that the yellow coloration is amplified by the ventral black area, and that these coloration traits interact with body and gonopodium size and courtship display behavior to influence mating success in a multicomponent sexual display (Møller & Pomiankowski 1993; Candolin 2003).

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Supporting Information

Additional supporting information may be found in the online version of this article:

Figure S1. Observation tank, showing the dotted lines in the center compartment which denote the choice zones for the dichotomous choice tests.