THE IMPORTANCE OF THE MULTICOMPONENT DISPLAY IN SEXUAL SELECTION OF BLACK MORPH *GIRARDINUS METALLICUS* (PISCES: POECILIIDAE)

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ABSTRACT

The Importance of the Multicomponent Display in Sexual Selection of Black Morph *Girardinus metallicus* (Pisces: Poeciliidae)

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Multicomponent displays are composed of traits, such as coloration, structural ornaments, and behavior, that become integrated and signal information to conspecifics. Estimation of multicomponent displays in fishes often involves measurement of color traits. Fish color measurements are often obtained following immobilization via chemical anesthesia; however, the anesthetics may alter the resulting measurements, for example by darkening the skin. *Girardinus metallicus*, a poeciliid fish endemic to Cuba, has a multicomponent courtship and aggressive display. Black morph males exhibit black ventral coloration including the gonopodium (copulatory organ) and yellow in the non-black areas of their bodies. I investigated the effects of common anesthetics on coloration measurements of *G. metallicus*. I measured the hue, saturation, and brightness of the anterior dorsal, posterior dorsal, posterior ventral, and caudal body regions, from digital images of the same males obtained without using anesthetic and anesthetized using tricaine methane sulfonate (MS222) and eugenol (clove oil). Because multicomponent displays are intriguing with respect to sexual selection, I investigated the importance of size and coloration traits in sexual selection via female choice and male-male competition in *G. metallicus*.

I found that saturation and hue did not differ significantly across treatments (anesthetization using MS222, anesthetization using clove oil, and without anesthetic in a small glass chamber containing water). However, brightness was greater under the anesthetics, possibly due to photographing the fish behind water and glass in the Non-anesthetic treatment or due to reflectivity differences of the iridophores. The body regions varied in hue, saturation, and brightness. Most importantly, I found differences in the responses of different body regions to the anesthetic treatments, suggesting that anesthetics may affect coloration in unpredictable ways, and that multiple regions of fish should be measured when assessing overall coloration. My results suggest that photographing fish in a glass chamber without anesthetic may be an effective way to obtain digital images for color analysis without using anesthetics that may influence coloration.

Having determined a good method for color measurement, I then investigated the role of the multicomponent display in sexual selection. Through direct interaction tests, I found that dominant males had brighter and more saturated yellow coloration than subordinate males, and that dominant males courted more than subordinate males. Within high yellow males, dominant males attempted more copulations than subordinate males. Interestingly, low yellow, subordinate males attempted more copulations than low yellow, dominant males, suggesting that subordinate males invested time into attempting copulations rather than engaging in potentially risky aggressive behavior. I observed a greater difference in body size between the males in pairs to which I could assign dominance status than pairs to which I could not assign dominance status, suggesting the importance of standard length in aggression in this species. I found that yellow saturation may serve to signal status without

the males resorting to aggressive interactions due to only half the pairs exhibiting aggression. Because aggression is key to mating success in *G. metallicus*, my findings that yellow coloration is correlated with aggression, in concert with previous studies showing the importance of ventral black area and body size for aggression, reinforce the idea that these males exhibit a multicomponent signal to conspecifics in the context of sexual selection.

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GENERAL INTRODUCTION

Honest Signaling

Coloration, structural ornaments, and behavior are often integrated into multicomponent mating and aggressive displays (Møller & Pomiankowski 1993; Candolin 2003; Hebets & Papaj 2005; Bro-Jørgensen 2010). Display traits advertise an individual's quality as a potential mate or competitor (e.g., Houde 1987, Grether 2000, Grether et al. 2004b, Price et al. 2008). Coloration, structural ornaments, and behavior can be an honest signal (Zahavi 1975; Hasson 1990; Grether et al. 2004a), emphasizing signaler quality through the amount of energy needed to produce the signal or through tradeoffs between signal intensity and health (Svensson & Wong 2011). Honest signals are expensive to produce and the honesty is maintained through developmental, physiological, or social costs, thus ensuring that the signal often reflects signaler quality (Zahavi 1975).

Coloration in Fishes

Coloration in fish results from different wavelengths of light being absorbed by pigments in chromatophores, which consist of three layers: xanthophores, iridophores, and melanophores (Bagnara & Hadley 1973; Fox 1976; Endler 1980; Grether et al. 2004a). The three layers interact to produce a colored area from absorbed and reflected light through direct or indirect regulation of pigment-containing organelles (Grether et al. 2004a). For example, the darkening of skin is due to the dispersion of melanosomes inside the melanophore layer (Bagnara & Hadley 1973; Grether et al. 2004a; Price at al. 2008), which increases light absorption in melanophores and can the color intensity of a xanthophore patch (Grether et al. 2004a).

Color measurement techniques commonly involve using either reflectance spectrophotometry of the animal itself (e.g., Stevens et al. 2007, Bergman & Beehner 2008, Kiere et al. 2009) or computer-based measurements taken using digital images of the animal (Stevens et al. 2007; Pike 2011). Although some digital cameras may have a bias towards particular wavelengths of light or the need of standard lighting conditions, they allow researchers to obtain data more quickly and inexpensively than spectrophotometers (Stevens et al. 2007; Bergman & Beehner 2008).

When using either reflectance spectrophotometry or digital images, anesthetics such as tricaine methane sulfonate (MS222; Finquel; Argent Chemical Laboratories) or eugenol (clove oil) are used to immobilize fish (Coyle et al. 2004; Ross & Ross 1999; Yasir & Qin 2009; Küçük 2010; Popovic et al. 2012). However, common anesthetics can change the saturation and hue of color patches (Gray et al. 2011). Specifically, MS222 has been known to increase color expression (Gumm et al. 2011) and clove oil can result in darkening due to the dispersion of pigment in melanosomes in fish skin (Metz et al. 2006; Price et al. 2008; Grether et al. 2004a; Sheets et al. 2007; Svensson & Sköld 2011; Kottler et al. 2014).

The Study System

Girardinus metallicus, a poeciliid fish endemic to Cuba (Farr 1980; Lorenzen 1996), is polymorphic for male coloration (Lorenzen 1996; Ponce de Léon & Rodriguez 2010; Kolluru et al. 2014). Two male morphs, normal and black, occur in the wild, and the normal morph is the most common (G.R. Kolluru, pers. comm.). Normal morph males are drably colored

(Farr 1980; Greven 2005) and coercively mate without courting (Farr 1980; Lorenzen 1996). Black morph males are sexually dimorphic with a yellow body and a black ventral surface including the gonopodium (copulatory organ), and black patches occasionally occurring elsewhere on the body (see Fig. 4.8; Lorenzen 1996; Greven 2005), and appear to always court females before attempting copulations (Kolluru et al. 2014, 2015). The multicomponent mating display of the black morph involves a dorsal fin spread, holding the gonopodium perpendicular to the body, and angling the body such that the head is raised and the yellow body coloration and ventral black coloration are visible (Lorenzen 1996; Kolluru et al. 2014).

Previously, Kolluru et al. (2014, 2015) found a complex interaction among black coloration, dominance status and time on courtship and aggression in black morph males. Ventral black coloration and dominance status may together influence the ability of males to gain access to females, with the latter being most important (Kolluru et al. 2015). However, none of the previous studies have addressed another striking aspect of *G. metallicus* coloration: black morph males exhibit sexually dimorphic golden yellow skin coloration in the non-black areas of their bodies (Lorenzen 1996; Greven 2005), which fundamentally differs from that of males of the normal morph.

Here I describe two complementary studies: Chapter 1 focuses on the effect of anesthetics on the hue, saturation, and brightness measurements obtained from digital images of male *G. metallicus*. I used this study to determine whether photographing fish in a glass chamber without anesthetic is a suitable method to obtain color measurements. This photography technique described in Chapter 1 is used in Chapter 2, which focuses on sexual selection of

the multicomponent mating and aggressive display of male black morph *G. metallicus*. Major tables and figures are included within the chapters, and I provide additional information and figures in an Appendix for reference.

1. Effect of Anesthetics on Color Measurements Obtained from Digital Images of Male *Girardinus metallicus* (Pisces: Poeciliidae)

1.1 Introduction

Coloration is intriguing in the context of sexual selection because it is particularly useful in advertising an individual's quality as a potential mate or competitor (e.g., Houde 1987, Grether 2000; Grether et al. 2004b, Price et al. 2008). Because coloration is common in sexually selected displays, the characteristics of color traits are frequently measured. Anesthetics are typically required for immobilization when studying fish coloration; therefore I investigated whether the use of anesthetics affects the measurement of hue, saturation, and brightness in the poeciliid fish, *Girardinus metallicus*.

Determining the best techniques to capture inter-individual variation in color displays is important because coloration can act as an honest signal of quality (Zahavi 1975; Hasson 1990; Grether et al. 2004a) through the amount of energy needed to produce the signal (Zahavi 1975; Kodric-Brown & Brown 1984) or through tradeoffs between signal intensity and health (Lozano 1994; Svensson & Wong 2011). For example, the saturation (chroma) of orange spots reflects an ornament-health tradeoff in male guppies (*Poecilia reticulata*; Endler 1980; Kodric-Brown 1989; Houde 1987, 1997; Grether 2000; Grether et al. 2004b; Svensson & Wong 2011), and that combined with female preference for a specific hue leads to intense sexual selection on male coloration (Endler 1984; Houde 1987, 1997; Grether 2001).

Coloration in fish results from different wavelengths of light being absorbed by pigments inside chromatophores, which consist of three layers: xanthophores (containing carotenoid

and pteridine pigments responsible for yellow and red), iridophores (containing crystalline platelets that diffract light and produce structural colors responsible for iridescence), and melanophores (containing melanin pigments that result in brown and black) (Bagnara & Hadley 1973; Fox 1976; Endler 1980; Grether et al. 2004a). The three layers interact to produce a colored area from absorbed and reflected light through direct or indirect regulation of pigment-containing organelles (Grether et al. 2004a). For example, the darkening of skin is due to the dispersion of melanosomes inside the melanophore layer (Bagnara & Hadley 1973; Grether et al. 2004a; Price at al. 2008). The dispersion increases light absorption in melanophores and can decrease the color intensity of a xanthophore patch (Grether et al. 2004a). Dispersion and aggregation of the pigment-containing organelles in any layer can result in color change (Ali 1980; Price et al. 2008; Grether et al. 2004a; Svensson & Sköld 2011).

Color measurement techniques commonly involve using either reflectance spectrophotometry of the animal itself (e.g., Stevens et al. 2007, Bergman & Beehner 2008, Kiere et al. 2009) or computer-based measurements taken using digital images of the animal (Stevens et al. 2007; Pike 2011). Although it produces accurate measurements, spectrophotometry requires the subject to be placed within a few millimeters of the probe, and positioned exactly the same as other individuals; thus it is extremely stressful for the animal. For this reason, digital images may be preferable to spectrophotometry when minimally invasive sampling is required, such as when color measurements are taken prior to behavioral observations of the same animals (Stevens et al. 2007; Bergman & Beehner 2008; Pike 2011). Some drawbacks associated with the digital image technique, such as a bias towards particular wavelengths of light or the

need for standard lighting conditions, may be mitigated by using high-specification cameras and standardized lighting conditions. Typically, digital photography is followed by the use of computer software to calculate hue, saturation, and brightness (HSB; described below) or red, green, and blue (RGB) values (Stevens et al. 2007; Wollenberg et al. 2008; Yasir & Qin 2009).

Quantifying inter-individual differences in coloration in fish typically requires immobilization. Well-established techniques for fish immobilization involve using either the dissolved anesthetic tricaine methane sulfonate (MS222; Finquel; Argent Chemical Laboratories) or eugenol (clove oil). Both of these anesthetics are used to immobilize fish for the measurement of traits including coloration (Coyle et al. 2004; Ross & Ross 1999; Yasir & Qin 2009; Küçük 2010; Popovic et al. 2012) and to reduce transport stress and facilitate handling (Pramod et al. 2010; Popovic et al. 2012). Although anesthesia enables measurements that would otherwise be prohibitively difficult to obtain, anesthetic use can be problematic for measuring color (Price et al. 2008; Gumm et al. 2011). Common anesthetics can change the saturation and hue of color patches (Gray et al. 2011). Specifically, MS222 has been known to increase color expression (Gumm et al. 2011) and clove oil can result in darkening due to the dispersion of pigment in melanosomes (Metz et al. 2006; Price et al. 2008; Grether et al. 2004a; Sheets et al. 2007; Svensson & Sköld 2011; Kottler et al. 2014), an effect which intensifies the longer the fish is anesthetized (Marshall et al. 2003). Given that an altered state of the pigment-containing organelles can affect color measurements (Ali 1980), techniques that are less likely to cause melanosome movement may lead to color measurements that more accurately reflect what is seen by receivers in nature.

Here I compared coloration measurements of homologous points on the same animals under three randomly ordered treatments: anesthetization using a weak concentration of MS222, anesthetization using clove oil, and without anesthetic in a small glass chamber containing water. I obtained digital images of mature *G. metallicus* males photographed in each of these treatments and compared the HSB values of homologous regions on each fish in all three treatments. Coloration can be described using the HSB model (Künzler & Bakker 2001; Wedekind et al. 2008; Yasir & Qin 2009; Montoya et al. 2014). Hue refers to the degree on a color wheel and ranges from 0° to 360°. The color red is defined as 0° and 360° whereas green is 120° and blue is 240° (See Fig. 4.1). Saturation is the purity of the hue (Yasir & Qin 2009). For example, a red feather with 0% saturation appears gray, a red feather with 50% saturation may appear as a brick red color, and a red feather with 100% saturation would appear as fire engine red (for details see https://www.xrite.com/documents/literature/en/l10-001_understand_color_en.pdf). Brightness refers to the relative lightness or darkness of a hue. At 0% brightness, any hue will appear black and at 100% brightness, any hue will appear white (Yasir & Qin 2009). Increased light absorption by melanophores reduces the saturation of the xanthophore layer of that area, changing the saturation measurement (Grether et al. 2004a). Regardless of the measurement technique, darkening under anesthesia may result in lower saturation measurements (Svensson & Sköld 2011). The darkening from melanosome dispersal may also result in an increase in hue (Goda & Fujii 2001; Logan et al. 2006) and a decrease in brightness (Wucherer & Michiels 2014; Harant et al. 2016).

Males of the poeciliid fish, *Girardinus metallicus*, have been described as polymorphic with

3 morphs: normal, black, and yellow (Kolluru et al. 2014). Black morph males are yellow with black coloration on their ventral surface and gonopodium (copulatory organ; Lorenzen 1996; Kolluru et al. 2014). The yellow morph has more intense yellow, rather than black, ventral coloration (Kolluru et al. 2014). For the present study, I used a mixed-morph population of males, which enabled me to also examine the coloration of hybrids. I hypothesized that the commonly used anesthetics MS222 and clove oil influence *G. metallicus* coloration measurements. I predicted that hue would be higher and brightness lower in the two anesthetic-involved treatments. The predicted effects on brightness could be due to the dispersion of pigments within melanosomes over the iridophores, such that they cover the crystalline platelets and thereby lower the reflectance (Bagnara et al. 1968; Harant et al. 2016). The lowering of reflectance would decrease the overall brightness measurement obtained from the fish by obscuring the light reflecting capacity of the iridophores (Bagnara et al. 1968). I also predicted that saturation would be higher in the two anesthesia-involved treatments because anesthetics can maximize the expression of dispersed melanin pigments, darkening the body coloration and leading to higher saturation (Svensson & Sköld 2011).

1.2 Methods

1.2.1 Animal Husbandry

Fish stocks were maintained at the Kolluru laboratory at California Polytechnic State University. The fish were originally obtained from the lab of David N. Reznick at the University of California, Riverside, and have been housed in captivity for many generations. I fed the fish high quality flake food and housed them in a mixed sex 10-gallon stock tank under controlled temperature (25 ± 0.5 °C) and a 12:12 L:D lighting schedule using a mixture

of full-spectrum fluorescent and LED bulbs. The stock tank contained hybrids of black morph males and yellow morph males (Kolluru et al. 2014); however, for this study I aimed to use males with black ventral coloration, such that they most closely resembled black morph males found in the wild (Kolluru et al. 2014). On the day of photography, I individually isolated fish in 2-gallon tanks and returned to the same stock tank after all images were obtained.

1.2.2 Digital Imagery

I photographed 11 fish under three treatment conditions: a Non-anesthetic treatment in an 8 × 8 × 2cm glass chamber (see below for details), anesthetization using MS222 (200mg/L of water; Finquel; Argent Chemical Laboratories), and anesthetization using a clove oil solution made of a 9:1 95% ethanol: clove oil (100 mg/mL of water; MCB; Neiffer & Stamper 2009; Fig. 4.4). I administered the treatments in a randomized order to each fish during an eight-hour period. Two of the eleven fish were not photographed in the Non-anesthetic treatment because one died following a prior treatment and one was mistakenly returned prematurely to the stock tank.

I obtained RAW digital images with a Nikon D800 camera using Adobe RGB color space. I used a NIKKOR AF 24-70mm f/2.8G ED AIS lens with a 24 mm extension tube for photography of the Non-anesthetic treatment and a NIKKOR AF 105mm f/2.8G Micro AIS lens for photography of the Anesthetic treatments. In photography of both the Non-anesthetic and Anesthetic treatments, the white balance was set to “Automatic” under four 14 W natural daylight color (5000 K) LED bulbs (Philips, 1500 lumen 100 W replacement), and the

"mirror up" mode was used with a remote release to minimize camera vibration. I placed the fish on a stage located in the middle of four securely fastened lamps (Fig. 4.2). The camera was stationed directly above the stage with a tripod during photography of the Anesthetic treatments and placed on a stationary ball head mount directly across from the stage during photography of the Non-anesthetic treatment (Fig. 4.2). For each fish, I allowed a 15-minute acclimation period in a home tank containing clean water, between treatments.

For the Non-anesthetic treatment, I photographed each male in an 8 × 8 × 2cm glass chamber filled with pH-buffered water. I used glass spacers to minimize chamber size and reduce the depth of field. An 18% photography gray scale card with a ruler attached was placed in the chamber and used as the background of each image. I netted each male into the chamber and situated it on the photography stage. Following an acclimation period, the image was taken (Fig. 4.3). I used a sterile cotton swab and plastic pipet to gently nudge the male into position as needed. The left sides of all but one male were photographed. The remaining male could not be nudged into position so I photographed his right side. I added new water to the chamber for each fish.

For the Anesthetic treatments, I immersed the male in either the MS222 or the clove oil solution until it listed to one side. I then removed the fish and placed him on an 18% gray scale card with ruler. I photographed the left lateral surface with the same lighting setup and camera settings described above (Fig. 4.3). In these treatments, there was no water or glass between the camera lens and the fish, in contrast to the Non-anesthetic treatment.

1.2.3 Image Analysis

I save images in RAW format and analyzed them using Photoshop CS6 (Version 13.0.6). I measured the standard length of each fish as the distance from the tip of the snout to the caudal peduncle line. Lines were drawn to divide each fish into equal sections (see Fig. 4.4), and using the color sampler tool, a point sample of HSB values was recorded for fifteen homologous points from four body regions: four anterior dorsal measurements, four posterior dorsal measurements, four posterior ventral measurements, and three caudal peduncle measurements (Fig. 4.4A,B,C,D). The body regions varied in the yellow coloration such that the anterior and posterior body regions were thought to be more yellow to the human eye. The posterior ventral and caudal body regions were thought to be less yellow for they appeared to have a whiter coloration tint. A 5 × 5 average pixel measurement of the 18% gray scale was taken from each image as a control across images.

To obtain homologous points to measure, I did the following. Starting in each body region near the center of the fish, I designated the first whole scale closest to the vertical guideline as "point one". Moving laterally along this line of scales towards the head or tail, I obtained measurements from the center of each scale, skipping every two scales until four different point measurements were taken (Fig. 4.4A,B,C). If a black scale was encountered, I measured the scale preceding the black area. In addition to these body measurements, I obtained three homologous caudal peduncle measurements from the opaque region directly posterior to the caudal peduncle line (Fig. 4.4D). The first point was placed in the middle of the caudal region, establishing equal distances above and below the point. Two more points were then measured, one superior to and one inferior to the first point.

1.2.4 Data Analysis

All tests were performed using JMP Pro 12.1.0 (SAS Institute, Inc. 2014). I averaged the values for the four points within each body region resulting in four averages (one anterior dorsal, one posterior dorsal, one posterior ventral, and one caudal). I performed a generalized linear mixed model (GLMM) with individual as the random effect, treatment and body region as fixed effects, and standard length as the covariate. HSB values were the dependent variables. I tested the residuals for normality using the Shapiro-Wilk goodness-of-fit test. Saturation ($W = 0.98$, $P = 0.34$) and brightness ($W = 0.98$, $P = 0.21$) were normally distributed and hue was Box-Cox transformed such that the residuals most closely approximated normality ($W = 0.97$, $P = 0.01$). I corrected for three tests using the false discovery rate (FDR, B-Y method; Bejamini & Yekutieli 2001), yielding an alpha corrected of 0.027.

1.3 Results

Variation among individuals represented 2.1% of hue, 32.7% of saturation, and 53.6% of brightness. There was no significant effect of treatment on hue ($F_{2,105} = 1.7$; $P = 0.18$; Fig. 4.5; Table 3.1) or saturation ($F_{2,103} = 2.4$; $P = 0.093$; Fig. 4.6; Table 3.1). Brightness differed significantly among treatments ($F_{2,102} = 63.3$; $P < 0.0001$; Fig. 4.7; Table 3.1), such that the fish in the Non-anesthetic treatment were the least bright and the fish under the clove oil treatment were the brightest. Fish under MS222 treatment exhibited intermediate values (post-hoc Tukey's HSD test, $P < 0.05$). There were significant differences among body regions in hue ($F_{3,102} = 25.5$; $P < 0.0001$; Fig. 4.5), saturation ($F_{3,102} = 46.6$; $P < 0.0001$; Fig.

4.6), and brightness ($F_{3,102} = 28.7$; $P < 0.0001$; Fig. 4.7; Table 3.1). The posterior ventral and caudal body regions exhibited significantly higher hue and lower saturation than the anterior and posterior dorsal body regions (post-hoc Tukey's HSD test, $P < 0.05$). The posterior and anterior dorsal body regions also differed significantly in saturation from each other, such that anterior dorsal body regions had a higher saturation value (post-hoc Tukey's HSD test, $P < 0.05$). The posterior ventral body region was significantly higher in brightness compared to the other regions (post-hoc Tukey's HSD test, $P < 0.05$). There was a significant body region × treatment interaction for saturation ($F_{6,102} = 3.9$; $P = 0.0013$; Fig. 4.6), such that the differences in saturation of body regions were less pronounced in the Non-anesthetic treatment. This is largely driven by the two anesthetics causing a greater increase in saturation in the anterior dorsal body region and a greater decrease in saturation in caudal regions. There was also a significant body region × treatment interaction for brightness ($F_{6,102} = 4.6$; $P = 0.0003$; Fig. 4.7), which was largely driven by the posterior ventral body region being brighter in the two anesthetic treatments, compared to the other body regions. There were no significant relationships between standard length and the three variables measured (Table 3.1).

1.4 Discussion

Anesthetics can be essential in obtaining digital images of fishes for the purpose of color measurements; however, concerns have been raised about the effects of anesthesia on fish coloration (e.g., Price et al. 2008, Gray et al. 2011). To my knowledge the effect of anesthetics on fish coloration measured via analysis of digital images has not been formally addressed. The HSB values of male poeciliid displays have all been shown to be important in

female choice (e.g., Endler 1984, Grether et al. 2001). However, hue and saturation are well-studied (e.g., Houde 1987, Kodric-Brown 1989, 1993), most likely because they are more directly affected by pigment content than brightness (Grether et al. 2004a). I showed that neither eugenol (clove oil), nor tricaine methane sulfonate (MS222), affected the overall hue and saturation measurements of several body regions of *G. metallicus* males. However, I found that there were differential effects of anesthetics on the saturation and brightness of different body regions, suggesting that anesthetics may adversely affect color measurements of some areas, and that it is unpredictable how different anesthetics will affect the HSB of different areas (see Price et al. 2008 Fig. 1 for images showing differential darkening under MS222 in another poeciliid).

Brightness values were higher when obtained from images of fish under anesthesia than from images of the same fish taken in a small chamber with water and glass between the camera lens and the fish. This may be explained by what was between the camera lens and the fish, rather than the influence of anesthetics *per se*. In the Non-anesthetic treatment, glass and water separated the fish from the camera lens, unlike in the Anesthetic treatments. Unfortunately, this potential confound was unavoidable, because immersing the anesthetized fish in water would have caused the fish to come out of anesthesia too quickly, whereas immersion in anesthetic solutions would have resulted in excessive anesthesia, potentially causing increased darkening (Marshall et al. 2003) and risk to fish health (Carter et al. 2011). The brightness differences among treatments may also be explained by differences in the reflectivity of the guanine crystalline platelets in the iridophore layer. The color of the reflected light is determined by the thickness, spacing and refractive index of the crystalline

platelets (Fujii 1993; Herring1994; Grether et al. 2004a). However, the position of the crystalline stacks can cause variation in where the light is reflected on the platelets, and if the platelets are not parallel to the body the reflectivity can either increase or decrease depending on the amount of light and viewing angle (Land 1972; Rowe & Denton 1997). Images in which the fish exhibited substantial yaw or roll were excluded to minimize effects due to position relative to the camera lens. However, when fish were placed on the stage for the Anesthetic treatments the crystalline stacks may have been angled differently, reflecting a different amount of light, than in the Non-anesthetic treatment. In any case, the objective of my study was to examine the effects of commonly used fish photography techniques, which typically involve anesthetized animals being photographed outside of water (e.g., Grether et al. 2001, Kodric-Brown & Johnson 2002, Marshall et al. 2003).

G. metallicus black morph males are uniformly "lemon yellow" (Greven 2005) and black (Kolluru et al. 2014). For this study I examined the yellow regions of males from a stock that was hybridized with another morph (the yellow morph; Kolluru et al. 2014), and therefore varied in coloration from pure black morph males. Because I photographed the same individuals under all three treatments I did not consider it problematic that I made use of mixed-morph males. The two morphs have been described (Kolluru et al. 2014), but to my knowledge the coloration of hybrids has not been described outside of the hobbyist literature. I found that the anterior dorsal, posterior dorsal, posterior ventral, and caudal body regions differed in HSB values. The anterior dorsal region had the highest saturation value compared to the other regions, possibly due to countershading (Norman & Greenwood 1963). The posterior ventral and caudal regions had lower saturation and were a more greenish yellow

hue than the anterior and posterior dorsal regions, which were less greenish and more yellowish.

Unstandardized light conditions are known to influence color measurements of digital images (Stevens et al. 2007; Bergman & Beehner 2008; Pike 2011). In this study, the camera and lights were identically positioned across treatments, to minimize differences in the amount of light captured in each image. The Non-anesthetic lens had an extra-low dispersion glass element, which controlled for chromatic aberrations that occur when lenses have different refractive angles from various wavelengths of light (Boult & Wolberg 1992). A nano crystal lens coating also enhanced light transmission and reduced glare (http://www.tucsoncamerarepair.com/lenses/#/nikon-af-s-nikkor-24-70mm-f28g-ed-lens/).

The use of digital images to obtain color measurements is common, but the technique is also criticized when the camera's mechanical response to varying light conditions is not controlled for (Stevens et al. 2007; Bergman & Beehner 2008; Pike 2011). The HSB measurements of color standards and color patches obtained from the images should demonstrate a linear relationship when plotted against the light reflectance value, which is the measure of visible and usable light that is reflected from a surface when illuminated (Lauziére et al. 1999; Stevens et al. 2007). Stevens et al. (2007) noted that variation in light intensity might cause color measurements to differ among images, resulting in the nonlinearity of color models such as HSB and others, including RGB. In the present study, I used a high quality camera and standardized light conditions across all images. This resulted in consistent HSB values of the 18% gray color standard, and therefore no attempt to

linearize the relationship was made. The camera's ability to manually change white balance and photograph in high resolution meant that I attained a more "natural color balance" (Stevens et al. 2007) and depicted more details in smaller color patches than a camera with a nonlinear response. I used RAW images because they do not degrade in quality when copied and because they display a wider variety of color than JPEG and TIFF image files (Stevens et al. 2007). The lens I used corrected for chromatic aberrations and glare (as stated above). The aperture was constant across all photographs and allowed me to lower the depth of field while photographing the focal fish.

In summary, my results suggest that photographing fish in a glass chamber without anesthetics may be an effective way to obtain digital images for color analysis, especially when hue and saturation are likely to be the variables of interest. Photographing without anesthetic under standardized light conditions is a minimally invasive process that can be used to obtain measurements of animals prior to behavioral observations and may give a more accurate measurement of natural color based on how fish appear to conspecifics. Future studies of fish coloration should involve sampling multiple body regions even if the fish appear uniformly colored. Additionally, my results encourage either obtaining measurements without the use of anesthetics or the use of a camera lens submerged in water when using anesthetics, to accurately assess brightness.

2. Aggressive Males Get Lucky: The Importance of the Multicomponent Display of the Black Morph *Girardinus metallicus* (Pisces: Poeciliidae)

2.1 Introduction

Courtship and aggressive displays can include coloration, structural ornaments, and behavior, which are typically integrated into multicomponent displays (Møller & Pomiankowski 1993; Candolin 2003; Hebets & Papaj 2005; Bro-Jørgensen 2010). When various traits are combined in a multifaceted display, selection can coevolve to enhance expression of each other (Endler 1992; Rosenthal et al. 1996; Gumm & Mendelson 2011). In contrast, the selection of morphological traits and behavior can be uncoupled (Gumm & Mendelson 2011), such that the traits are independently used to signal information about signaler quality (Suk & Choe 2008). Inter- and intrasexual selection may simultaneously operate on multicomponent displays (Miller & Svensson 2014), by selecting for different traits (Wong & Candolin 2005; Pryke et al. 2001a; Andersson et al. 2002; Johnson & Fuller 2014) or working in unison to favor the same traits, such that the traits signal quality to potential mates and rivals (Berglund et al. 1996; Hunt et al. 2009; Miller & Svensson 2014). Several hypotheses have been put forth to explain the evolution of multicomponent displays, including that they send multiple messages, such that each trait signifies a different aspect of quality to receivers (Møller & Pomiankowski 1993), that they are redundant signals, such that traits function together to signal quality (Møller & Pomiankowski 1993), or that different traits signal to different receivers (e.g., potential mates and rivals; Pryke et al. 2001a; Andersson et al. 2002). Here I investigated the multicomponent display of male black morph *G. metallicus* by examining the importance of individual traits in the context of female choice and male-male competition.

Male-male aggression can disrupt female choice when it promotes forced copulations, reduces courtship, or decreases the ability of females to view courtship displays (Wong & Candolin 2005; Miller & Svensson 2014; Wang et al. 2015). Furthermore, preferred males may be less successful in male-male competition, which can reduce their access to females (Jennions & Petrie 1997; Miller & Svensson 2014). If competition hampers female choice, females may rely on multicomponent displays to identify preferred high quality mates (Wong & Candolin 2005; Miller & Svensson 2014; Candolin & Reynolds 2001; Suk & Choe 2008) and prioritize different signals to focus on different aspects of mate quality (reviewed in Jennions and Petrie 1997; Candolin 2003).

Poeciliid species range from those whose males use only coercive mating attempts to those whose males exhibit a courtship display before mating attempts (reviewed in Cummings & Ramsey 2015). In species with only coercive males, such as *Gambusia holbrooki* (Bisazza et al. 2001; Cummings 2015), mating success is largely influenced by male-male competition (Farr 1984, 1989; Bisazza 1993); however, recent studies have shown that females may demonstrate choice based on size and familiarity in such species (Kahn et al. 2012; Vega-Trejo et al. 2014). In species with displaying males, such as *Poecilia reticulata*, the interaction between behavioral displays and morphology can influence the traits that are preferred by females (Kodric-Brown & Nicoletto 2001; Wong et al. 2011). Females can then assess quality of potential mates and benefit from mating with the high quality male (Wang et al. 2015).

Girardinus metallicus, a poeciliid fish endemic to Cuba, is polymorphic for male coloration and behavior (Lorenzen 1996; Ponce de Léon & Rodriguez 2010; Kolluru et al. 2014). Two male morphs, normal and black, occur in the wild, and the normal morph is the most common (G.R. Kolluru, pers. comm.). Normal morph males occur at a predictably high frequency in mixed-morph captive populations, suggesting a balanced genetic polymorphism (Lorenzen 1996). Normal morph males are drably colored (Farr 1980; Greven 2005) and coercively mate without courting (Farr 1980; Lorenzen 1996). Black morph males are sexually dimorphic with a yellow body and a black ventral surface including the gonopodium (copulatory organ), and black patches occasionally occurring elsewhere on the body (see Fig. 4.8; Lorenzen 1996; Greven 2005). Black morph males appear to always court females before attempting copulations (Kolluru et al. 2014, 2015) by swimming behind, beside, and below the female, while holding the gonopodium away from the body and raising the head (Lorenzen 1996; Kolluru et al. 2014).

Although it is known that black morph males display to females, previous studies have not been able to determine the traits that females use to assess male quality. Kolluru et al. (2014) found that male size and gonopodium length are positively correlated with mating activity. Males with greater ventral black area were more aggressive, and more aggressive males had greater mating activity (Kolluru et al. 2014). Under conditions likely to generate more competition, Kolluru et al. (2015) found that although dominant males had greater mating activity, longer interaction time may allow females to influence intermale interactions or dominance relationships may have already become established. Interestingly, Kolluru et al. (2015) found a complex interaction among black coloration, dominance status and time spent

on courtship and aggression, suggesting that females were either able to assess dominance status based on cues they didn't measure and associate with dominant males, or that females influenced male dominance status. Alternatively, cues exchanged between males during the dichotomous choice tests contributed to dominance status establishment prior to direct interactions. In general, dominance status appears to be key to gaining access to females in both the black and normal morphs (Farr 1980; Kolluru et al. 2014, 2015).

G. metallicus multicomponent mating display involves a dorsal fin spread, holding the gonopodium perpendicular to the body, and angling the body such that the head is raised and the yellow body coloration and ventral black coloration are visible (Lorenzen 1996; Kolluru et al. 2014). However, previous studies have not addressed the importance yellow body coloration in sexual selection of the black morph *G. metallicus* (Lorenzen 1996; Greven 2005). The intensity of yellow coloration varies among males (Fig. 4.8A,B) and possibly also temporally (H.M. Neldner, pers. comm.). As in other poeciliids (Endler 1984), yellow may be produced using carotenoid pigments, which can be honest signals of male quality (see Appendix; Endler 1980; Goodwin 1984). The yellow and black coloration of black morph males may therefore be sexually selected independently or because black amplifies yellow (Hasson 1989, 1990), facilitating the assessment of quality by enhancing the visibility of the yellow coloration to conspecifics (Hasson 1989, 1990; Grether et al. 2004a).

Thus, given the lack of information about how morphological traits and behavior serve as a multicomponent display, I investigated the multicomponent mating and aggressive display of male black morph *G. metallicus* by examining how integrated traits influence female choice

and male-male competition. I paired size-matched, low and high yellow males and subjected them to an aggressive context over a defensible food source to establish baseline dominance status relationships. The same pairs were then tested in dichotomous choice and direct interaction tests with females, to investigate sexual selection on behavioral and morphological traits. Under the hypothesis that female choice and male-male competition favor the same traits, I predicted that yellow body saturation, ventral black area, body size, and gonopodium size are targets of sexual selection. Specifically, I predicted that when presented with size-matched males in dichotomous choice tests, females would spend more time associating with males with greater values of these morphological traits, and that these same males would be more likely to be dominant and exhibit greater mating activity in the direct interaction tests.

2.2 Methods

2.2.1 Animal Husbandry

G. metallicus stocks have been maintained in captivity for many generations in the Kolluru laboratory at California Polytechnic State University. I housed the fish in mixed-sex 38-liter stock tanks under controlled temperature (25 ± 0.5 °C) and a 12:12 L:D lighting schedule using a mixture of full-spectrum fluorescent and LED bulbs. I fed the fish high-quality flake food (TetraMin Plus Tropical Flakes ®, Tetra, Spectrum Brands, Inc.) before the experiment and frozen brine shrimp (*Artemia* sp.) during isolation. I isolated males (n = 48) in 7.5-liter tanks for between 2 and 25 days before photography (see below for details). Home tanks contained gravel and plant material and were visually separated from each other. I isolated females in similar tanks for 11 to 49 days prior to the start of behavior trials.

2.2.2 General Overview

Isolation, photography, and behavior trials occurred over a seven-week period in 2015. All trials were performed in a black-curtained area lit by full-spectrum fluorescent and LED lights, and new conditioned water was added to all observation tanks after each pair was tested. I covered three sides of the observation tanks in brown paper to provide a uniform background and to decrease the chance of distractions. Observers within the curtained area called out behaviors to recorders sitting outside the curtain area. Trials were performed blind with respect to the yellow saturation status of the males. Video recordings were used as a backup in cases where behavior was not readily visible in real time.

2.2.3 Photography and Image Analysis

I photographed males over a three-day period in an 8 × 8 × 2cm glass chamber filled with fresh pH-buffered water (see Chapter 1 for details). Each image contained a 90% white color standard as the background, a ruler for scale, and an 18% gray and yellow color standard to ensure that lighting and camera conditions were consistent among photographs.

I obtained RAW digital images using Adobe RGB color space with a Nikon D800 camera. I used a NIKKOR AF 24-70mm f/2.8G ED AIS lens with a 24 mm extension tube while the white balance was set to "Automatic" under four 14 W natural daylight color (5000 K) LED bulbs (Philips, 1500 lumen 100 W replacement). The "mirror up" mode was used with a remote release to minimize camera vibration. I netted each male into the chamber and situated it on the photography stage located in the middle of four securely fastened lamps.

The camera was placed on a stationary ball head mount directly across from the stage. I used a sterile cotton swab to gently nudge the male into position and the image was taken. Each male was photographed again, this time under sedation using tricaine methane sulfonate (MS222; 200 mg/L of water; Finquel; Argent Chemical Laboratories), to allow for positioning of the gonopodium and body for morphological measurements. I placed males on a stage with a ruler for scale, and photographed them on their right lateral surface with the gonopodium angled away from the body.

I saved the images in RAW format and determined the HSB values using Photoshop CS6 (Version 13.0.6). I drew two lines, one parallel and one perpendicular to the length of the body, to divide each fish into equal sections (see Figure 4.4A,B,C,D). Using the color sampler tool, I recorded a point sample of hue, saturation, and brightness (or HSB) values for fifteen homologous points for each body region of the lateral surface of the fish: four anterior dorsal measurements, four posterior dorsal measurements, four posterior ventral measurements, and three caudal peduncle measurements (see below; Fig. 4.4A,B,C,D). I took a 3 × 3 average pixel point measurement of the yellow color standard from each image as a control across images. The HSB values of the yellow color standard were consistent among images; therefore, the data obtained from the color standard measurements were not used in analysis.

I obtained homologous points to measure by starting in each body region near the center of the fish and designating the first whole scale closest to the vertical guideline as “point one”. Moving laterally along this line of scales towards the head or tail, I obtained measurements

from the center of each scale, skipping every two scales until four different point measurements were obtained. If a black scale was encountered, I measured the scale immediately anterior to the black area. In addition to these body measurements, I obtained three homologous caudal peduncle measurements from the opaque region directly posterior to the caudal peduncle line. The first point was placed in the middle of the caudal region, establishing equal distances above and below the point. Two more points were then measured, one superior to and one inferior to the first point.

I used Image J software (1.49v) to measure body area, standard length, ventral black area, gonopodium black area, gonopodium length, and gonopodium area (previously described in Kolluru et al. 2014, 2015; see Table 3.2 for details).

I used a factor analysis to extract latent factors from the morphological data. I included standard length, gonopodium length, gonopodium area, body area, ventral black area, mass, and average hue, average saturation, and average brightness of the anterior dorsal, posterior dorsal, posterior ventral, and caudal body regions. I obtained three components with eigenvalue > 1 and interpreted loadings with an absolute value > 0.50 (Table 3.3). Based on the saturation factor scores, I assigned males a "yellow saturation status" such that males with the higher saturation value within a pair were classified as high yellow and males with the lower saturation value were classified as low yellow (Fig. 4.9). Males were paired such that I maximized the difference in yellow saturation for the two males in each pair (range = 0.56 - 2.78 saturation factor scores) and minimized the differences in standard length (range

= 0.06 – 1.8 mm). I assigned females to a pair based on a body size estimate. All three paired test fish came from different housing tanks.

2.2.4 Foraging Context

To evaluate a dominance status of each male prior to female exposure, I performed contest competition tests in which each pair of males competed over a defensible food source (Tetra Veggie Algae Wafers ®, Spectrum Brands, Inc.). To increase hunger levels, the morning feeding was withheld on the day of testing. After I was certain I could individually identify each male within a pair based on subtle differences in black coloration, I gently netted both males into a 19-liter tank at the same time. Following a three-minute acclimation period, I dropped an algae wafer in the center of the tank and let it sink to the bottom. I then performed a five-minute trial, which was started when at least one of the males pecked either at the wafer or pecked within a two-cm radius around the wafer (hereafter referred to as "the wafer zone"). I scored chases, bites, and pecks in the wafer zone or on the wafer itself (Table 3.4). For the first 10 pairs tested, pecks at the wafer were rare and males displayed some behaviors not listed above. Therefore, I amended the protocol for the subsequent 13 pairs to a 20-minute trial, during which the observer noted gonopodial jabs, follow duration and male-male display, in addition to the behaviors listed above (all described in Table 3.4). Following the foraging context, males were returned to their home tanks, fed flake food (TetraMin Plus Tropical Flakes ®, Tetra, Spectrum Brands, Inc.), and allowed to acclimate for 20-30 minutes before the dichotomous choice tests.

2.2.5 Dichotomous Choice Tests

I used dichotomous choice tests to measure the association time of females with each male in a pair. The testing arena consisted of three separate, rimless aquaria, to exclude chemical and tactile cues among fish (Fig. 4.10; Houde 1997; Jeswiet & Godin 2011). The female was in a 38-liter glass aquarium with choice and neutral zones demarcated. Males were in 19-liter glass aquaria situated directly against the back of the female aquaria, creating a "U" shape (Fig. 4.10). My arena had a larger neutral zone relative to the choice zones, compared to typical dichotomous choice arenas (e.g., Plath et al. 2008, Jeswiet & Godin 2011, Kolluru et al. 2014, 2015). This increased the chances that a female's time in a choice zone represented a choice favoring that male. All aquaria contained gravel and were covered with brown butcher paper, ensuring that the two males could not see each other. This arena excluded male-male communication and consequently male-male competition, and allowed females to associate with males based solely on visual cues (Gowaty et al. 2003; Moore et al. 2003).

Males were randomly assigned to each aquarium for the first trial. I netted the two males and female into their aquaria and allowed a five-minute acclimation period during which all three fish could swim freely. I ensured that the female appeared to see each male before the start of each trial; occasionally this required extension of the acclimation period to 10 minutes.

Following the acclimation period, I gently herded the female into a cylindrical, clear plastic acclimation chamber and placed the chamber in the middle of the neutral zone. The trial began upon the release of the female and removal of the chamber. I recorded time spent by the female in the choice and neutral zones for 10 minutes. Immediately following the first

trial, the males were switched between aquaria, the female was herded into the acclimation chamber, and the process was repeated for another 10-minute trial.

2.2.6 Direct Interaction Tests

Dichotomous choice tests may only indirectly measure mate choice and female preferences may be different when the sexes are allowed to interact (Shackleton et al. 2005). Therefore, I performed direct interaction tests, where all three fish could interact, to assess female choice when chemical and tactile cues were present, and to examine male-male competition simultaneously. Immediately following the dichotomous choice tests, the two males were netted into the female aquarium. I allowed a five-minute acclimation period during which all three fish could swim freely. I then performed a 10-minute focal observation on both males simultaneously, during which I recorded the behaviors described below (Table 3.5). After this trial, I allowed for a 20-minute intermission, during which the fish continued to interact and the observers left the curtained area. Following the intermission the observers reentered the curtained area and a second acclimation and trial were performed as described above. The fish were then lightly sedated with MS222, weighed to the nearest 0.1g, and returned to their home tanks.

2.2.7 Data Analysis

All statistical tests were performed using JMP Pro 12.1.0 (SAS Institute, Inc. 2014). For direct interaction tests, I corrected for four tests using the false discovery rate (FDR B-Y method; Bejamini & Yekutieli 2001), yielding an alpha-corrected of 0.024.

To address the relationship between aggression and morphology in the foraging context, I performed a generalized mixed model (GLMM) analysis of covariance (ANCOVA) with male nested within pair as a random effect, yellow saturation status as a fixed effect, and the yellow brightness and body size and ventral black factor scores as continuous covariates in the model. I summed chases, bites, and gonopodial jabs to obtain a composite aggression score, which was used as the dependent variable. I transformed composite aggression score by taking the square root (X + 0.5) because this most closely approximated normality of residuals.

For dichotomous choice trials, I performed a GLMM ANCOVA with male nested within pair as a random effect and yellow saturation status, trial number, and side (referring to which aquaria the male was in) as fixed effects. The body size and ventral black area factor score, yellow brightness factor score, and female mass were continuous covariates. The dependent variable was female association time with each male. I took the square root of the dependent variable to normalize residuals.

To address whether the males the female associated with more in the dichotomous choice tests were also more successful at gaining access to females in the direct interaction tests, I performed a GLMM ANCOVA with male nested within pair as a random effect and association time and female mass as continuous covariates. The dependent variables were following duration, courtship duration, copulation attempts with contact, and copulation attempts without contact. Following duration, courtship duration, copulation attempt with contact, and copulation attempt without contact were Box-Cox transformed to closely

approximate normality of residuals.

I calculated dominance index scores from behaviors in the direct interaction tests, following Kolluru et al. (2014, 2015) as (chases delivered + bites delivered) / (chases delivered + bites delivered + chases received + bites received). I used the dominance index scores to designate each male within a pair as subordinate or dominant, such that the male with the higher dominance index score was assigned as the dominant male. I assigned dominance status to males in 12 of 23 pairs. The remaining 11 pairs did not exhibit any chases or bites.

To determine whether pairs that exhibited aggression (12/23) differed from pairs that did not (11/23) I compared standard length differences and saturation factor score differences within a pair and the ability to assign dominance status. I used these variables because they were the morphological variables used to pair males initially. I performed two paired t tests with the ability to assign dominance status as the independent categorical variable and standard length differences or saturation factor score differences as continuous dependent variables.

To ascertain the relationship between morphology and dominance index scores in direct interaction test, I performed a GLMM ANCOVA with male nested within pair as a random effect, yellow saturation status as a fixed effect, and yellow brightness, body size and ventral black factor scores as continuous covariates in the model. Dominance index, a continuous variable, was the dependent variable. I Box-Cox transformed dominance index because this most closely approximated normality of residuals.

To examine mating behavior in the direct interaction tests, I performed a GLMM ANCOVA with male nested within pair as a random effect and trial number, yellow saturation status, and dominance status as fixed effects. The yellow brightness factor score, body size and ventral black factor score, and female mass were continuous covariates. The dependent variables were following duration, courtship duration, copulation attempt with female with contact, and copulation attempt with female without contact. Courtship display and copulation attempt without contact were transformed using $\log_{10}(X + 0.5)$, and following duration was Box-Cox transformed, such that the residuals of all three variables were normally distributed. A Box-Cox transformation was used to approximate normality of residuals of copulation attempt with contact.

The relationship between aggression composite scores and mating behavior in the direct interaction test was determined using a GLMM ANCOVA with male nested within pair as a random effect and yellow saturation status and dominance status as fixed effects. The yellow brightness factor score, body size and ventral black factor score, composite aggression score from the foraging context, and female mass were continuous covariates. The dependent variables were following duration, courtship duration, copulation attempts with contact, and copulation attempts without contact. The residuals of follow duration, courtship duration, and copulation attempts with contact were normally distributed and copulation attempts without contact was Box-Cox transformed to normalize the residuals for that variable.

2.3 Results

2.3.1 Foraging Context

Yellow saturation status, the yellow brightness factor score, and the body size and ventral black factor score did not influence the composite aggression scores (all $P > 0.26$). Furthermore, there was no relationship between composite aggression scores and mating behaviors in the direct interaction tests (all $P > 0.21$).

2.3.2 Dichotomous Choice Tests

Female association time was not dependent on yellow saturation status, trial number, side (referring to which aquaria the male was in), the body size and ventral black area factor score, the yellow brightness factor score, or female mass (all $P > 0.16$).

2.3.3 Direct Interaction Tests

Pairs that engaged in aggression, such that were assigned a dominance status, had a greater difference in standard length between the males than pairs that were not assigned a dominance status, due to a lack of aggression (mean ± SE = able to assign status: 0.68 ± 0.1, unable to assign status: 0.42 ± 0.1; $t = -1.84$, $P = 0.08$). Differences in saturation factor scores between males within each pair had no effect on that ability to assign dominance status (mean ± SE = able to assign status: 1.56 ± 0.18, unable to assign status: 1.3 ± 0.18; $t = -1.02$, $P = 0.32$).

Males with a high yellow saturation had a higher average dominance index scores than low yellow saturation males (Fig. 4.11; $F_{1,20} = 6.4$, $P = 0.019$), suggesting more saturated males

instigated a greater proportion of aggression within a pair. There was a positive relationship between dominance index scores and yellow brightness factor scores (Fig. 4.12; $F_{1,20} = 10.4$, $P = 0.004$). Therefore, males with brighter, more saturated yellow skin were more likely to be dominant within pairs.

Males that the female associated with more in the dichotomous choice tests did not follow ($F_{1,63} = 2.4$, $P = 0.13$), court ($F_{1,73} = 1.4$, $P = 0.24$), attempt copulations with contact ($F_{1,66} = 0.7$, $P = 0.4$) or attempt copulations without contact ($F_{1,58} = 1.5$, $P = 0.23$) more than their rivals.

Dominant males courted significantly more than subordinate males (Fig. 4.13; Table 3.6; $F_{1,17} = 10$, $P = 0.006$). The relationship between dominance status and the number of copulation attempts without contact was marginally nonsignificant when corrected for multiple tests (Table 3.6; $F_{1,17} = 5.3$, $P = 0.035$); however, there was a trend towards more dominant males attempting copulations without contact more often than subordinate males (mean ± SE = dominant: 0.81 ± 0.17, subordinate: 0.16 ± 0.18). Body size and ventral black area were also positively related to the number of copulation attempts without contact (Table 3.6; $F_{1,17} = 6.5$, $P = 0.02$). There was a significant yellow saturation status × dominance status interaction for copulation attempts with contact, such that high yellow, dominant males attempted more copulations with contact than high yellow, subordinate males (Fig. 4.14; Table 3.6; $F_{1,17} = 8.23$, $P = 0.011$, post-hoc Tukey's HSD test). The opposite effect was seen in low yellow males, such that low yellow, subordinate males attempted more copulations than low yellow,

dominant males (Fig. 4.14). None of the factors in the model explained follow duration (all $P > 0.14$).

2.4 Discussion

G. metallicus black morph males are "lemon yellow" and black (Greven 2005), and exhibit the ventral surface and gonopodium as part of a multicomponent mating and aggressive display (Lorenzen 1996; Kolluru et al. 2014, 2015). I showed that males with brighter and more saturated yellow coloration instigated a greater proportion of aggressive interactions, consistent with studies on a range of taxa showing that dominance status is communicated through color ornaments (e.g., Kodric-Brown 1993, Pryke et al. 2001b, Senar 2006). Consistent with previous studies on this system (Kolluru et al. 2014, 2015), I also found that dominant males courted females more frequently than subordinate males. Although yellow saturation was not directly correlated with mating activity, my results suggest that yellow saturation may nonetheless be involved in sexual selection, because of the interaction between yellow saturation status and dominance on copulation attempts with contact. Within the high yellow males, dominant males attempted more copulations with contact than subordinate males. Within low yellow males, however, subordinate males attempted more copulations with contact than dominant males, albeit with less of a difference than in the high yellow males. Low yellow males who were dominant may have gained more access to females through courtship, but invested more time into being aggressive at the expense of attempting copulations. Low yellow, subordinate males performed less courtship, but may have attempted more copulations rather than engaging in potentially risky aggressive behavior.

Inter- and intrasexual selection can simultaneously operate on multicomponent displays (Miller & Svensson 2014) and may favor the same individual traits (Berglund et al. 1996; Hunt et al. 2009; Miller & Svensson 2014) or different individual traits (Pryke et al. 2001a; Andersson et al. 2002; Johnson & Fuller 2014) within the multifaceted phenotype. I found no evidence that females use yellowness to assess quality, but I did find that it is related to aggression, suggesting that yellow coloration is primarily used as a signal to other males. Although neither Kolluru et al. (2015) nor I found evidence for female choice favoring specific male traits, those authors found a positive relationship between female preference for a male and his subsequent mating activity. In contrast, in the present study I found no such relationship. In other words, females were not able to "spot a winner" in the dichotomous choice tests as they did in Kolluru et al. (2015). The difference in results is likely because my dichotomous choice test arena did not allow the males to see or chemically signal to each other, or chemically signal to the female, during the trials, as they could in Kolluru et al. (2015). My results indicate that female preference and male-male competition select for different traits within the multicomponent display.

Despite the findings of the present study and Kolluru et al. (2014, 2015), the significance of the courtship display has yet to be elucidated. Females do not appear to directly prefer any of the traits we have measured thus far (Kolluru et al. 2014, 2015; present study). It is possible that the display serves to signal morph rather than individual quality to females, because black morph males do not appear to force copulations, as do normal morph males (Farr 1980; Lorenzen 1996). In some species, coercion is selected against because it decreases both male

and female fecundity (Parker 1979, 2006; Wang et al. 2015). Langerhans (2011) suggested that actively displaying the gonopodium during courtship might be a signal used by females. Females may prefer courtship displays because males can adjust the rate or intensity of the display in response to a female's reaction, which can avoid startling females and thereby enhance mating success (Patricelli et a. 2006, 2016). Although males of other poeciliids commonly display by placing the lateral body surface in front of the female (Endler 1984; Farr 1989; reviewed in Plath et al. 2007), black morph *G. metallicus* males display by orienting themselves below females, tilting up the chin and lowering the gonopodium (described in Lorenzen 1996 and Kolluru et al. 2014). Previous authors have suggested that the display served to make the gonopodium visible (Lorenzen 1996; Kolluru et al. 2014, 2015; Dadda 2015); however if the courtship display serves to pacify females wary of coercive mating attempts, then males may angle themselves beneath the female so that the gonopodium is hidden (M.E. Cummings, pers. comm.). Kolluru et al. (2014) showed that gonopodium size was important in female choice; however, that finding could be due to its function in male-male aggression. Indeed, the lowering of the gonopodium may simultaneously hide the gonopodium from females and signal quality to other males. Studies in which the two morphs (black and normal) are pitted against each other in tests investigating female choice and male-male competition are essential in identifying the functional significance of the black morph courtship display.

Potential rivals use traits that communicate resource holding potential (RHP) to estimate fighting capacity (Parker 1974) and thereby settle disputes without direct physical contest (Rohwer 1975; Barlow & Wallach 1976). I found that yellow is correlated with aggression,

suggesting males may use this trait to in assessing RHP. Interestingly, Kolluru et al. (2015) size-matched males without consideration for yellow coloration and found higher levels of aggression, possibly because the males were more closely matched in yellow saturation and yellow brightness than in the present study.

Body size is a predictor of dominance status in a variety of taxa (Qvarnström & Forsgren 1998) and usually influences the outcome of both male-male competition and female choice (Hunt et al. 2009). Whereas larger males have been shown to be more dominant in some poeciliids (*Belonesox belizanus* and *G. falcatus*; Bisazza et al. 1996), Farr (1980) found that in the normal morph of *G. metallicus*, smaller males were more aggressive. He demonstrated that this occurred because aggressive juvenile males matured earlier, and were consequently smaller but more aggressive as adults. Whereas Kolluru et al. (2014) found that larger, blacker males attempted more copulations, I found that smaller, less black males attempted more copulations (albeit without contact). My results are consistent with Farr (1980), suggesting that smaller males are more sexually vigorous than larger males. Farr (1980) also found that if males were isolated and reintroduced to each other, juvenile social status had no effect on dominance status; furthermore, the larger males were dominant to the smaller males, a reversal of what happens when they are reared together. Therefore, the relationship between male body size and aggression is complex in this species. Despite size matching, I observed a greater difference in standard length between the males in pairs to which I could assign dominance status, due to sufficient aggression in mating tests, than pairs to which I could not assign dominance status, due to lack of aggression in mating tests. This result suggests that the relationship between body size and dominance may be fueled by complex

interactions between the rearing environment and the test environment. I did not collect information on dominance status of my fish in the rearing environment. When two novel males are placed in a test environment, the social hierarchy dynamic may be challenged due to each male's developmental history and experience. Prior exposure in foraging contexts and isolation before mating tests may have affected hormone levels of each male. The varying hormone levels may have altered aggression levels, and caused males in pairs that were less size matched to reassess the importance of size differences on dominance status, during the mating tests. The dominance hierarchy may have been shifting more between males in pairs with greater size differences than males in pairs with smaller size differences, resulting in the sufficient amount of aggression used to assign dominance status.

Although both sexes of *G. metallicus* are aggressive over food and mates (Farr 1980; Y.J. Akky & G.R. Kolluru, unpublished data; Kolluru et al. 2014, 2015), I observed low levels of aggression both contexts. In the food context, this may have resulted because the food source used in the trials (algae wafer) was not a sufficiently valuable resource (prior to the trials, males were fed frozen brine shrimp). I chose to use a fixed design approach, such that all males experienced a foraging context prior to mating tests, to ensure that the males had similar aggressive experiences. This approach may have introduced order effects (Bell 2012), which could have led to the reduced aggression in the mating context trials because the males had already interacted with each other.

Though low levels of aggression occurred, males participated in aggressive behavior involving the gonopodium, suggesting it may function as a weapon. I observed a novel,

aggressive behavior, termed gonopodial jab, which involves physical contact between a male's gonopodium and another male. Interestingly, *G. metallicus* aggressive encounters also sometimes involve biting the rival's gonopodium (J.M. Budke, pers. comm.). In addition to reinforcing or resolving dominance hierarchies (Bailey & Zuk 2009) by influencing levels of aggression (Lane et al. 2016), gonopodial jabs and bites may increase attractiveness to potential mates (Bierbach et al. 2012). For example, in the Atlantic molly (*P. mexicana*), female preference increases after observing a male initiate a "sexual interaction" with another male, suggesting that females may exhibit a general preference for increased male activity (Bierbach et al. 2012).

In summary, my results are consistent with previous studies in showing that aggression is key to mating success in this species, *G. metallicus* (Farr 1980; Kolluru et al. 2014, 2015). I found that yellow body coloration is positively correlated with dominance status. Manipulating ventral black area and yellow coloration simultaneously will provide us with more information about the role of coloration in sexual selection in the black morph *G. metallicus*. A broader investigation with the two morphs (normal and black) competing against each other may allow researchers to determine the functional significance of the black morph courtship display. It is possible that males may use the display to signal morph so that females can avoid coercive mating attempts (Wang et al. 2015) or that males may be hiding the gonopodium from females (M.E. Cummings, pers. comm.) and aggressively signaling other males while courting, maintaining the courtship display in the black morph.

3. TABLES

Table 3.1. Results of general linear mixed model for HSB variables. Boldface p-values indicate significance after correction for multiple tests (alpha-corrected = 0.027).

Trait	**Term**	**DF**	**F**	**P**
Hue (°)	Treatment	105	1.7	0.18
	Body Region	102	25.5	**< 0.001**
	Standard Length	9	2.8	0.13
	Body Region × Treatment	102	1.6	0.16
Saturation	Treatment	103	2.43	0.093
(%)	Body Region	102	46.6	**< .0001**
	Standard Length	13	1.2	0.29
	Body Region × Treatment	102	3.9	**0.0013**
Brightness	Treatment	102	63.2	**< .0001**
(%)	Body Region	102	28.7	**< .0001**
	Standard Length	18	2.2	0.15
	Body Region × Treatment	102	4.6	**0.0003**

Table 3.2. Description of morphological traits measured in ImageJ.

Behavior	Description
Body area	Outline of the body, excluding the gonopodium
Standard length (mm)	Distance from the tip of the snout to the caudal peduncle line
Ventral black area	Black area starting superior to the middle of the eye or anterior to the edge of the eye to the insertion point of the gonopodium
Gonopodium black area	Black area in the region from the insertion point of the gonopodium to the tip of the palps
Gonopodium length	Distance from the insertion point of the gonopodium to the distal tip of the palps
Gonopodium area	Outline of the gonopodium from the insertion point of the gonopodium to the tip of the palps

Table 3.3. Results of the factor analysis on morphological traits. Factor loadings greater than 0.50, which were used in interpreting the factors, are shown in boldface.

Morphological Traits	Factor 1 Body Size and Ventral Black Area	Factor 2 Saturation	Factor 3 Brightness
Standard Length (mm)	**0.974**	0.173	-0.087
Body Area	**0.977**	0.181	-0.080
Gonopodium Length (mm)	**0.879**	-0.068	-0.107
Ventral Black Area	**0.705**	-0.015	0.087
Gonopodium Area	**0.845**	0.036	-0.008
Male mass (g)	**0.927**	0.173	-0.026
Anterior Dorsal Hue	-0.348	0.324	-0.087
Anterior Dorsal Saturation	0.019	**0.904**	0.007
Anterior Dorsal Brightness	-0.033	-0.066	**0.760**
Posterior Dorsal Hue	-0.423	0.103	0.167
Posterior Dorsal Saturation	0.138	**0.984**	-0.114
Posterior Dorsal Brightness	-0.105	-0.062	**0.993**
Posterior Ventral Hue	-0.249	0.202	0.216
Posterior Ventral Saturation	0.231	**0.740**	0.051
Posterior Ventral Brightness	-0.157	-0.049	**0.827**
Caudal Hue	-0.086	0.133	0.064
Caudal Saturation	0.025	**0.644**	-0.224
Caudal Brightness	0.077	-0.009	**0.614**
Eigenvalue	5.92	3.54	2.93
Percent Explained	32.88	19.67	16.27
Cumulative Percent	32.88	52.55	68.82

Table 3.4. Description of aggressive behaviors recorded in the foraging contest.

Behavior	Description
Chase male	Quick movement by the focal male toward the other male
Bite male	Visible contact between the mouth of the focal male and a part of the other male
Gonopodial jab	Movement of the gonopodium toward the other male, in attempt to make physical contact with a part of the other male aggressively
Male-male display	Following stance of focal male that shows of his gonopodium to other male
Following duration	Time spent by the focal male following the other male, with his gonopodium folded (i.e., not a display)

Table 3.5. Description of behaviors recorded in the direct interaction mating behavior tests.

Behavior	Description
Following duration	Time spent by the focal male following the female without performing a characteristic courtship display
Courtship duration	Time spent courting the female in the characteristic display stance, which involves tilting up of the chin and lowering of the gonopodium (described in Lorenzen 1996 and Kolluru et al. 2014)
Copulation attempt with contact	Movement of gonopodium toward the female, such that the gonopodium makes visible contact with the female
Copulation attempt without contact	Movement of gonopodium toward the female without visible contact with the female
Chase male	Quick movement by the focal male toward the other male
Bite male	Contact between the mouth of the focal male and a part of the other male

Table 3.6. Results of the generalized linear mixed models from the direct interaction tests. Boldface p-values indicate significance (alpha-corrected = 0.024).

Behavior	Term	DF	F	P
Following duration	Trial Number	20	2.17	0.16
	Yellow Saturation Status	17	0.26	0.61
	Trial Number × Yellow Saturation Status	20	0.003	0.96
	Dominance Status	17	1.96	0.18
	Trial Number × Dominance Status	20	0.32	0.58
	Yellow Saturation Status × Dominance Status	17	2.36	0.14
	Trial Number × Yellow Saturation Status × Dominance Status	20	0.81	0.38
	Body Size and Ventral Black Area	17	4.36	0.05
	Brightness	17	0.55	0.47
	Female Mass (g)	17	1.77	0.20
Courtship duration	Trial Number	20	0.13	0.72
	Yellow Saturation Status	17	0.15	0.70
	Trial Number × Yellow Saturation Status	20	0.06	0.81
	Dominance Status	**17**	**9.98**	**0.006**
	Trial Number × Dominance Status	20	0.06	0.81
	Yellow Saturation Status × Dominance Status	17	2.48	0.13
	Trial Number × Yellow Saturation Status × Dominance Status	20	0.03	0.87
	Body Size and Ventral Black Area	17	2.14	0.16
	Brightness	17	0.83	0.37
	Female Mass (g)	17	0.55	0.47
Copulation attempt with contact	Trial Number	20	0.26	0.62
	Yellow Saturation Status	17	0.14	0.71
	Trial Number × Yellow Saturation Status	20	0.79	0.39
	Dominance Status	17	2.94	0.10
	Trial Number × Dominance Status	20	1.40	0.25
	Yellow Saturation Status × Dominance Status	**17**	**8.23**	**0.011**
	Trial Number × Yellow Saturation Status × Dominance Status	20	0.64	0.43
	Body Size and Ventral Black Area	17	1.9	0.19
	Brightness	17	0.97	0.34
	Female Mass (g)	17	2.32	0.15
Copulation attempt without contact	Trial Number	20	3.0	0.09
	Yellow Saturation Status	17	0.08	0.78
	Trial Number × Yellow Saturation Status	20	0.02	0.87
	Dominance Status	17	5.3	0.035
	Trial Number × Dominance Status	20	0.28	0.60
	Yellow Saturation Status × Dominance Status	17	3.1	0.09
	Trial Number × Yellow Saturation Status ×	20	0.25	0.62

Dominance Status			
Body Size and Ventral Black Area	**17**	**6.5**	**0.02**
Brightness	17	0.24	0.63
Female Mass (g)	17	0.29	0.11

4. FIGURES

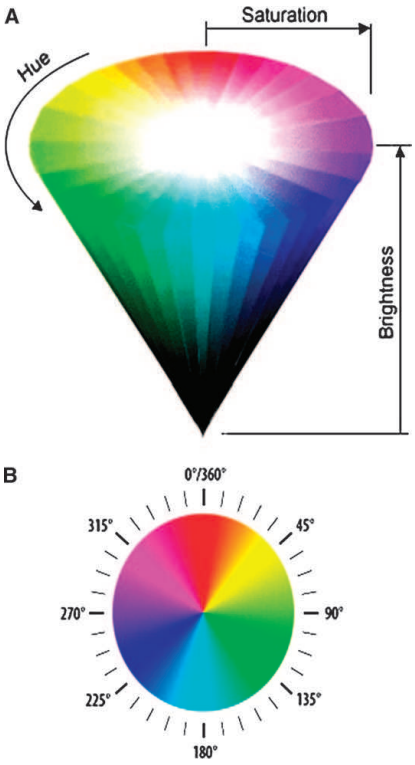

Figure 4.1. A lateral view (A) and top view (B) of the HSB color model which demonstrates the relationship of the three variables (Yasir, I. & Qin, J. G. 2009. Effect of light intensity on color performance of false clownfish, *Amphiprion ocellaris* Cuvier. *Journal of the World Aquaculture Society*, 40, 337-350; Copyright © 2009 by John Wiley Sons, Inc. Reprinted by permission of John Wiley & Sons, Inc.).

Figure 4.2. Males under the Non-anesthetic treatment were photographed with the camera placed on a stationary ball head mount directly across from the stage under securely fastened lamps.

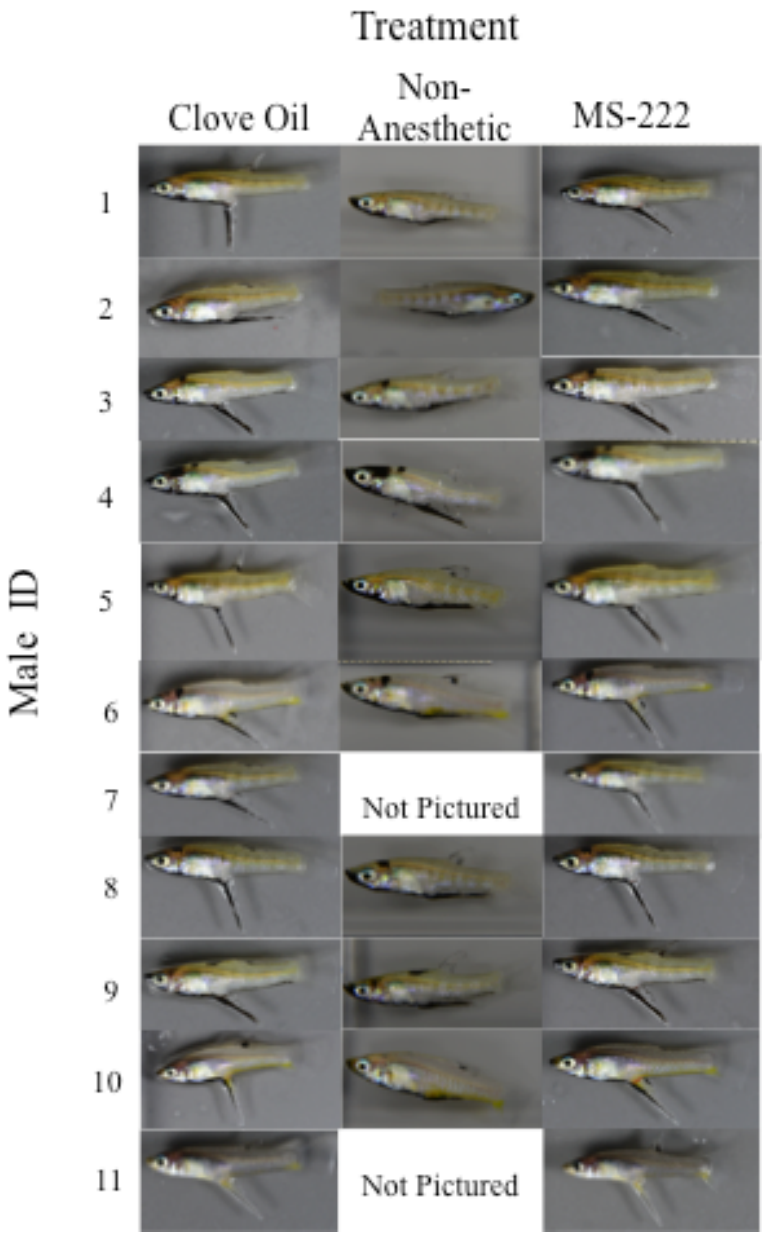

Figure 4.3. The photographs of the eleven male *G. metallicus* in the three treatments: clove oil, Non-anesthetic, and MS222. The left rather than right side of male 2 was accidently photographed, and two males (males 7 and 11) were not photographed in the Non-Anesthetic treatment (see methods for details).

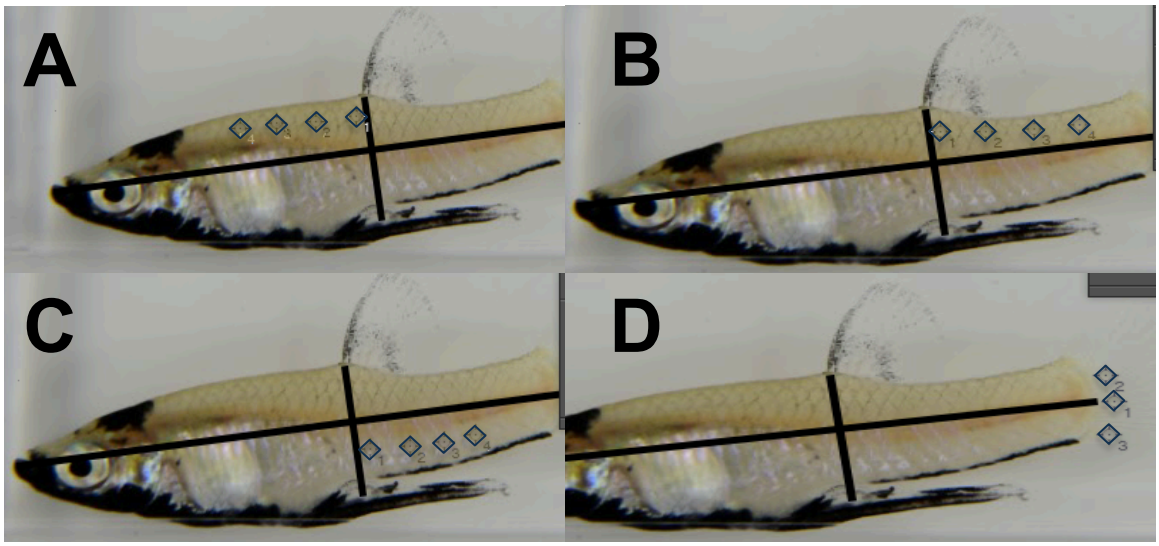

Figure 4.4A,B,C,D. Images of the 15 homologous HSB measurements of the same fish: four anterior dorsal measurements (A), four posterior dorsal measurements (B), four posterior ventral measurements (C), and three caudal peduncle measurements (D). Lines were drawn to divide each fish into equal sections

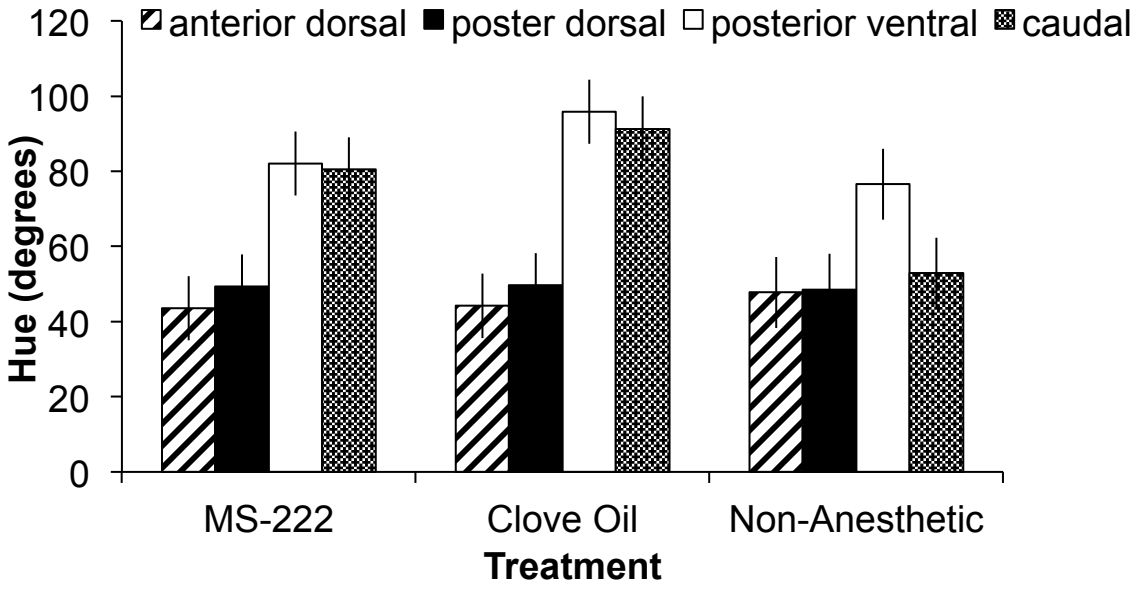

Figure 4.5. The average hue of the four body regions in the three treatments: clove oil, glass, MS222. The graph is showing the untransformed values because they are a more meaningful indication the position on the color wheel. The interaction was not significant. Bars show least-squares means ± SE.

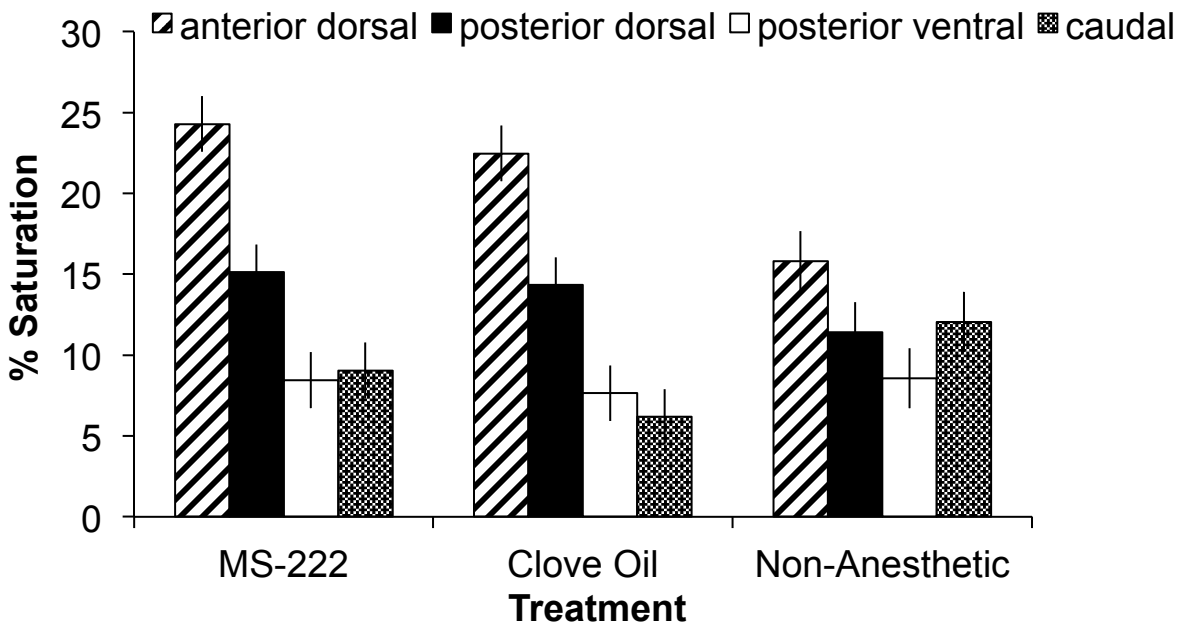

Figure 4.6. The average saturation of the four body regions in the three treatments: clove oil, glass, MS222. The interaction was significant. Bars show least-squares means ± SE.

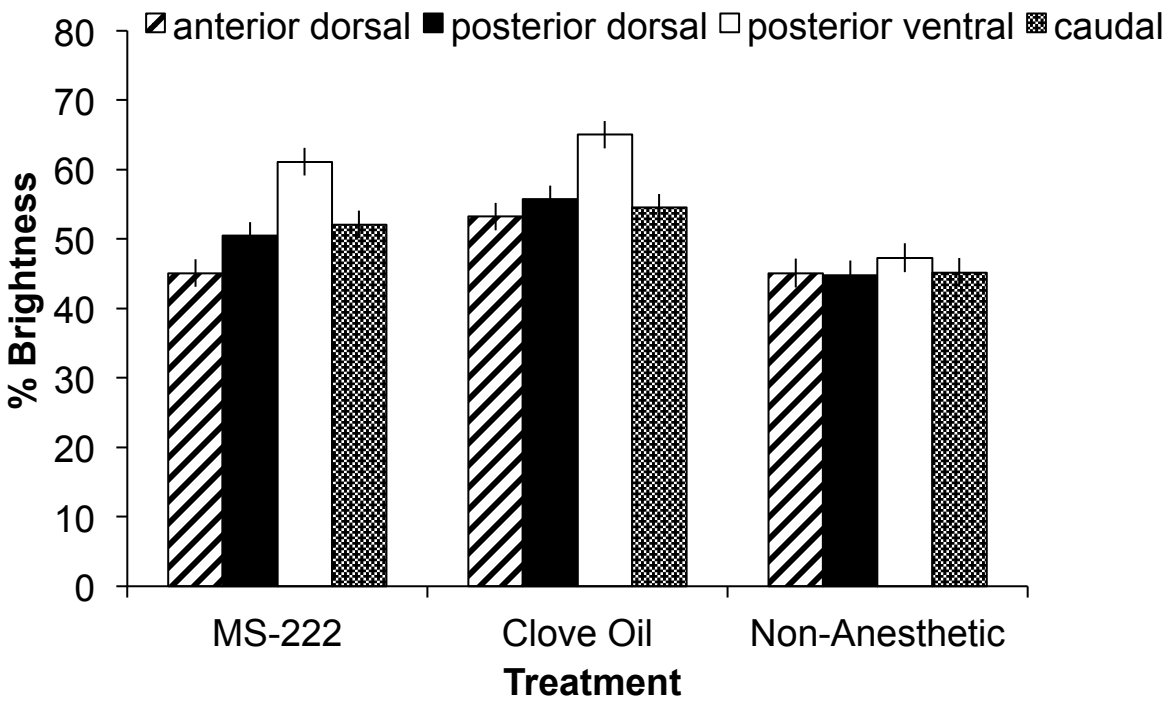

Figure 4.7. The average brightness of the 4 body regions in the three treatments: clove oil, glass, MS222. The interaction was significant. Bars show least-squares means ± SE.

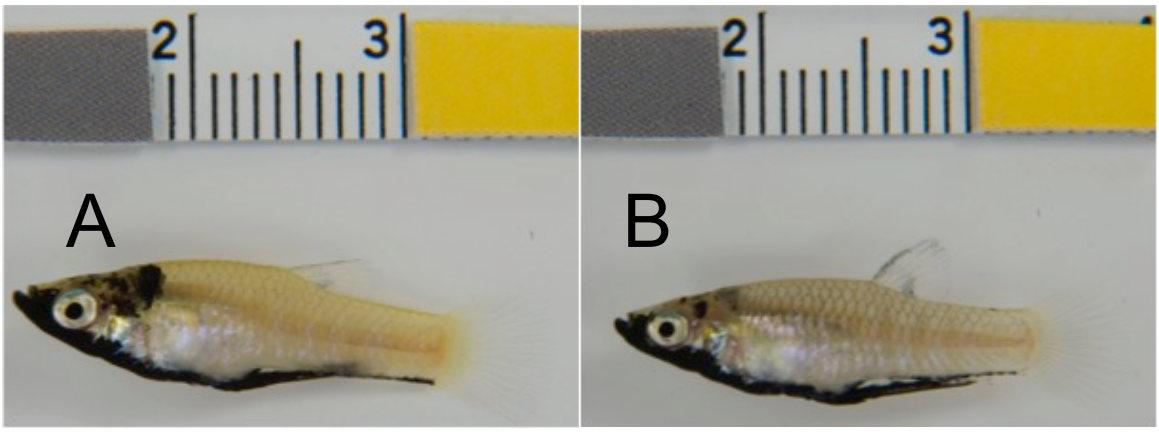

Figure 4.8. Two male black morph *G. metallicus* demonstrating the variance in yellow body coloration: “high yellow” male (A) and “low yellow” male (B). These males were paired for behavioral tests.

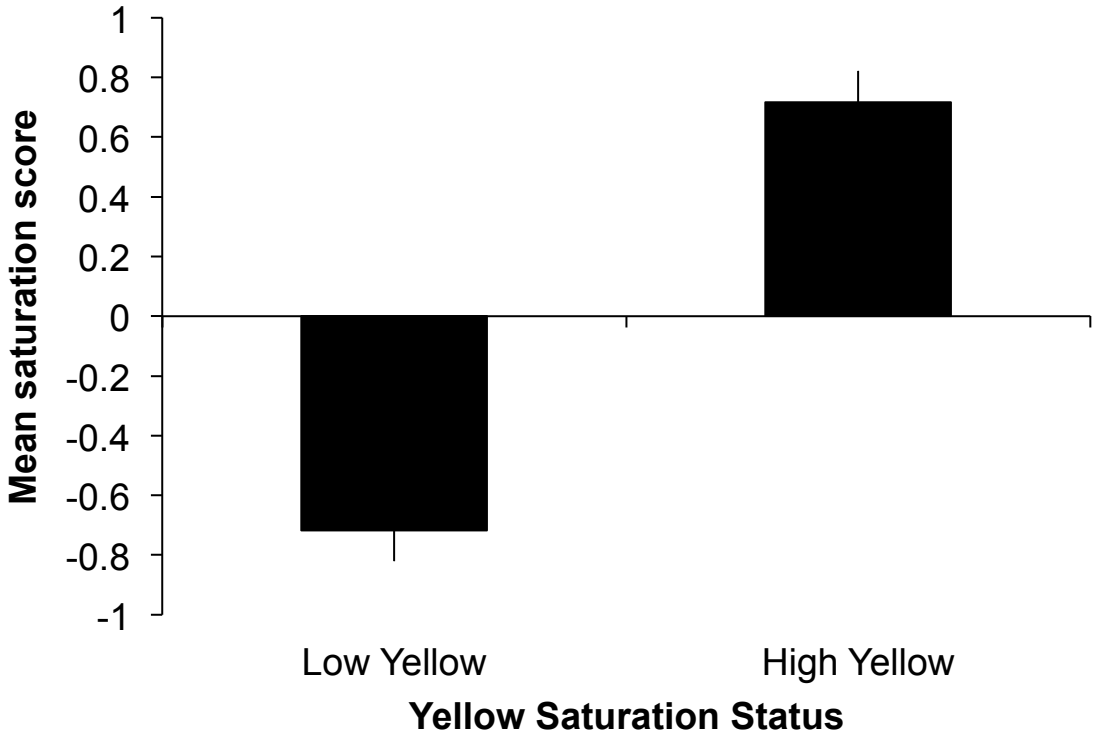

Figure 4.9. Mean saturation factor scores of males assigned to low or high yellow saturation status. Bars show least-squared means ± SE.

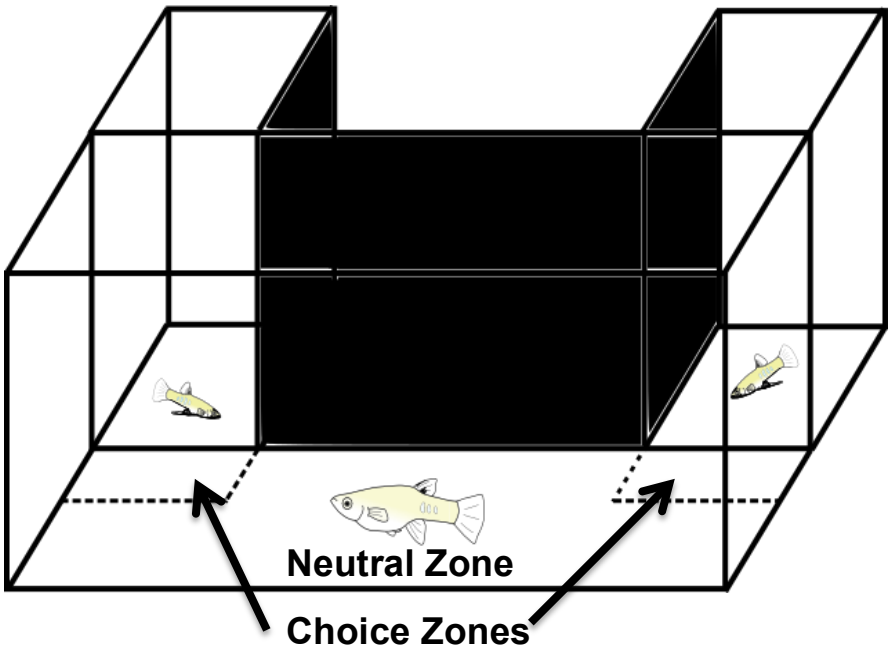

Figure 4.10. Dichotomous choice experimental set up consisting of three separate aquaria. Neutral and no choice zones are demarcated in the female aquarium. Photo: H.M. Neldner.

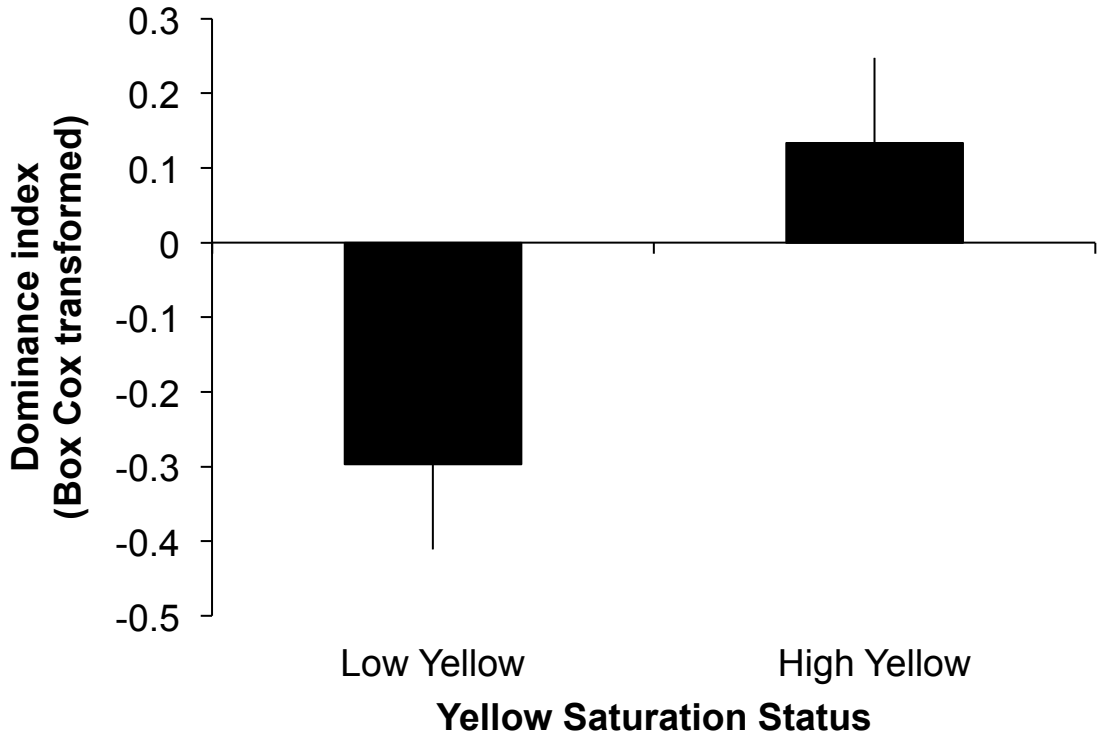

Figure 4.11. The relationship between yellow saturation status and dominance index. Dominance index refers to the proportion of aggressive interactions instigated by a male. Bars show least-squares means ± SE.

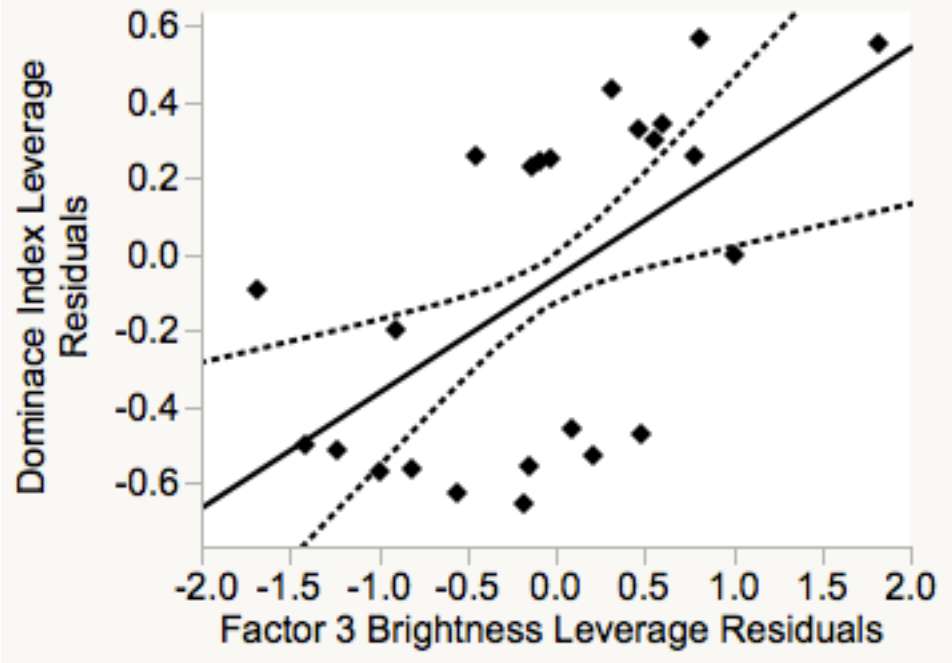

Figure 4.12. The relationship between dominance index and yellow brightness. The relationship is a leverage plot, revealing the impact of adding this effect to the model, given the other effects already in the model.

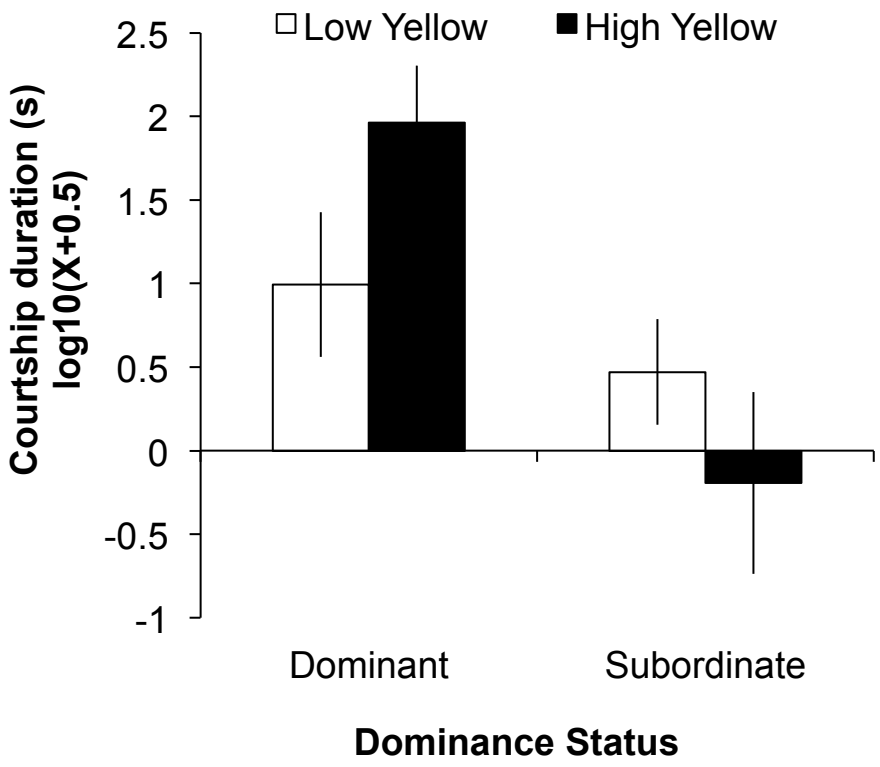

Figure 4.13. The interaction between yellow saturation status and dominance status on courtship. The interaction is not significant. Bars show least-squares means ± SE.

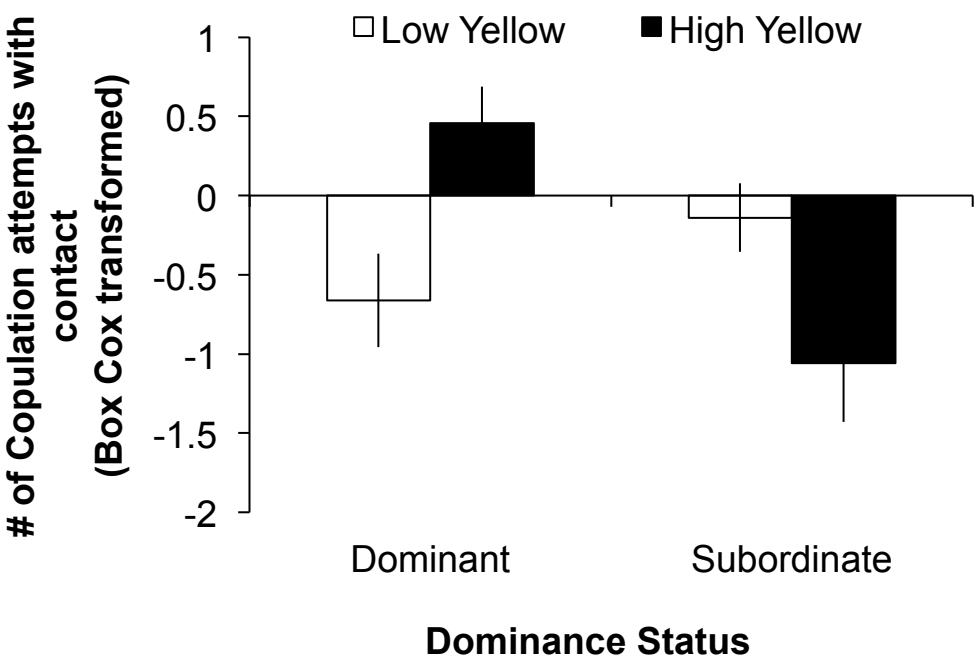

Figure 4.14. The interaction between yellow saturation status and dominance status on copulation attempts with contact. Bars show least-squares means ± SE.

REFERENCES

Ali, M. A. (Ed.). (1980). *Environmental physiology of fishes* (pp. 723). NY and London: Plenum Press.

Andersson, S., Pryke, S. R., Örnborg, J., Lawes, M. J., & Andersson, M. (2002). Multiple receivers, multiple ornaments, and a trade-off between agonistic and epigamic signaling in a widowbird. *The American Naturalist*, *160*, 683-691.

Bagnara, J. T., Taylor, J. D., & Hadley, M. E. (1968). The dermal chromatophore unit. *The Journal of Cell Biology*, *98*, 67-79.

Bagnara, J. T., & Hadley, M. E. (1973). *Chromatophores and colour change: the comparative physiology of animal pigmentation*. Englewood Cliffs, NJ: Prentice Hall.

Bailey, N. W., & Zuk, M. (2009). Same-sex sexual behaviour and evolution. *Trends in Ecology and Evolution*, *24*, 439-446.

Barlow, G. E., & Wallach, S. J. (1976). Colour and levels of aggression in the midas cichlid. *Animal Behaviour*, *24*, 814-817.

Bejamini, Y., & Yekutieli, D. (2001). The control of the false discovery rate in multiple testing under dependency. *The Annals of Statistics*, *29*, 1165-1188.

Bell, A. (2012). Randomized or fixed order for studies of behavioral syndromes? *Behavioral Ecology*, *24*, 16-20.

Berglund, A., Bisazza, A., & Pilastro, A. (1999). Armaments and ornaments: an evolutionary explanation of traits of dual utility. *Biological Journal of the Linnean Society*, *58*, 385-399.

Bergman, T. J., & Beehner, J. C. (2008). A simple method for measuring color in wild animals: validation and use on chest patch color in glades (*Theropithecus gelada*). *Biological Journal of the Linnean Society*, *94*, 231-240.

Bierbach, D., Jung, C. T., Hornung, S., Streit, B., & Plath, M. (2012). Homosexual behavior increases male attractiveness to females. *Biology Letters*, *9*, 20121038.

Bisazza, A. (1993). Male competition, female mate choice and sexual size dimorphism in poeciliid fishes. *Marine & Freshwater Behaviour & Physiology*, *23*, 257-286.

Bisazza, A., Novarini, N., & Pilastro, A. (1996). Male body size and male-male competition: interspecific variation in poeciliid fishes. *Italian Journal of Zoology*, *63*, 365-369.

Bisazza, A., Vaccari, G., & Pilastro, A. (2001). Female mate choice in a mating system dominated by male sexual coercion. *Behavioral Ecology*, *12*, 59-64.

Boult, T. E., & Wolberg, G. (1992). Correcting Chromatic Aberrations using Image Warping. *In Proceedings of the IEEE Conference on Computer Vision and Pattern Recognition*, 684-687.

Britton, G. (1992). Carotenoids. In G. F. Hendry (Ed.), *Natural foods colorants* (pp. 141-148). New York: Blackie & Son.

Bro-Jørgensen, J. (2010). Dynamics of multiple signalling systems: animal communication in a world in flux. *Trends in Ecology and Evolution*, *25*, 292–300.

Candolin, U., & Reynolds, J. D. (2001). Sexual signaling in the European bitterling: females learn the truth by direct inspection of the resource. *Behavioral Ecology*, *12*, 407-411.

Candolin, U. (2003). The use of multiple cues in mate choice. *Biology Review*, *78*, 575-595.

Carter, K. M., Woodley, C. M., & Brown, R. S. (2011). A review of tricaine methane sulfonate for anesthesia of fish. *Review in Fish Biology and Fisheries*, *21*, 51-59.

Coyle, S. D., Durborow, R. M., & Tidwell, J. H. (2004). Anesthetics in aquaculture. Southern Regional Aquaculture Center Publication (SRAC), *3900*, 1-6.

Cummings, M. E. (2015). The mate choice mind: studying mate preference, aversion and social cognition in the female poeciliid brain. *Animal Behaviour*, *103*, 249-258.

Cummings, M. E., & Ramsey, M. E. (2015). Mate choice as social cognition: predicting female behavioral and neural plasticity as a function of alternative male reproductive tactics. *Current Opinion in Behavioral Sciences*, *6*, 125-131.

Dadda, M. (2015). Female social response to male sexual harassment in poeciliid fish: a comparison of sex species. *Frontiers in Psychology*, *6*, 1-9.

Endler, J. A. (1980). Natural selection on color patterns in *Poecilia reticulata*. *Evolution*, *34*, 76-91.

Endler, J. A. (1984). Natural and sexual selection on color patterns in poeciliid fishes. *Environmental Biology of Fishes*, *9*, 173-190.

Endler, J. A. (1992). Signals, signal conditions, and the direction of evolution. *American Naturalist*, S125-S153.

Farr, J. A. (1980). The effects of juvenile social interaction on growth rate, size and age at maturity, and adult social behavior in *Girardinus metallicus* Poey (Pisces: Poeciliidae). *Zeitschrift Fur Tierpsychologie-Journal of Comparative Ethology*, *52*, 247–268.

Farr, J. A. (1984). Premating behavior in the subgenus *Limia* (Pisces: Poeciliidae): sexual selection and the evolution of courtship. *Zeitschrift Fur Tierpsychologie-Journal of Comparative Ethology*, *65*, 152-165.

Farr, J. A. (1989). Sexual selection and secondary sexual differentiation in poeciliids: determinants of male mating success and the evolution of female choice. In G. K. Meffe, & F. F. Snelson (Eds.), *Ecology and Evolution of Livebearing Fishes* (Poeciliidae) (pp. 91-123). Englewood Cliffs, NJ: Prentice Hall.

Fox, D. (1976). *Animal biochromes and structural colors*. Berkeley, CA: University of California Press.

Fujii, R. (1993). Cytophysiology of fish chromatophores. *International Review of Cytology*, *143*, 191–255.

Goda, M., & Fujii, R. (2001). Coloration and chromatophores of the domino damsel, *Dascyllus trimaculatus*. *Zoological Science*, *18*, 165-174.

Goodwin, T. W. (1984). Carotenoid-protein complexes. In T. W. Goodwin (Ed.), *The biochemistry of carotenoids, Vol. II Animals*. London, U.K.: Chapman and Hall.

Gowaty, P. A., Drickamer, L. C., & Schmid-Holmes, S. (2003). Male house mice produce fewer offspring with lower viability and poorer performance when mated with females they do not prefer. *Animal Behaviour*, *65,* 95-103.

Gray, S. M., Hart, F. L., Tremblay, M. E. M., Lisney, T. J., & Hawryshyn, C. W. (2011). The effects of handling time, ambient light, and anesthetic method, on the standardized measurement of fish colouration. *Canadian Journal of Fisheries and Aquatic Sciences*, *68*, 330-342.

Grether, G. F. (2000). Carotenoid limitation and mate preference evolution: A test of the indicator hypothesis in guppies (*Poecilia reticulata*). *Evolution*, *45*, 1712-1724.

Grether, G.F., Hudon, J., & Endler, J. (2001) Carotenoid scarcity, synthetic pteridine pigments and the evolution of sexual coloration in guppies (*Poecilia reticulata*). *Proceedings of the Royal Society B*, *268*, 1245-1253.

Grether, G. F., Kolluru, G. R., & Nersissian, K. (2004a). Individual colour patches as multicomponent signals. *Biology Review, 79*, 583-610.

Grether, G. F., Kasahara, S., Kolluru, G. R., & Cooper, E. L. (2004b). Sex-specific effects of carotenoid intake on the immunological response to allografts in guppies (*Poecilia reticulata*). *Proceedings of the Royal Society London B*, *271*, 45-49.

Greven, H. (2005). Structural and behavioral traits associated with sperm transfer in poeciliidae. In H. J. Grier, & M. C. Uribe (Eds.), *Viviparous Fishes* (pp. 147-165). Homestead, FL: New Life Publications.

Gumm, J. M., & Mendelson, T. C. (2011). The evolution of multi-component visual signals in darters (genus *Etheostoma*). *Current Zoology*, *57*, 125-139.

Harant, U. K., Michiels, N. K., Anthes, N., & Meadows, M. G. (2016). The consistent difference in red fluorescence in fishes across a 15 m depth gradient is triggered by ambient brightness, not by ambient spectrum. *BMC Research Notes*, *9*, 1-10.

Hasson, O. (1989). Amplifiers and the handicap principle in sexual selection: a different emphasis. *Proceeding of the Royal Society London B*, *235*, 383-406.

Hasson, O. (1990). The role of amplifiers in sexual selection: an integration of the amplifying and the Fisherian mechanisms. *Evolutionary Ecology*, *4*, 277-289.

Hebets, E. A., & Papaj, D. R. (2005). Complex signal function: developing a framework of testable hypotheses. *Behavioral Ecology and Sociobiology*, *57*, 197-214.

Herring, P. J. (1994). Reflective systems in aquatic animals. *Comparative Biochemistry and Physiology*, *109A*, 513–546.

Hill, J. A., Enstrom, D. A., Ketterson, E. D., Nolan, Jr., V., & Ziengenfus, C. (1999). Mate choice based on status versus dynamic secondary sexual traits in the dark-eyed junco. *Behavioral Ecology*, *10*, 91-96.

Houde, A. E. (1987). Mate choice based upon naturally occurring color pattern variation in a guppy population. *Evolution*, *41*, 1-10.

Houde, A. E. (1997). *Sex, Color, and Mate Choice in Guppies*. Princeton, NJ: Princeton University Press.

Hunt, J., Breuker, C. J. Sadowski, J. A., & Moore, A. J. (2009). Male-male competition, female mate choice and their interaction: determining total sexual selection. *Journal of Evolutionary Biology*, *22*, 13-26.

Jennions, M. D., & Petrie, M. (1997). Variation in mate choice and mating preferences: a review of causes and consequences. *Biological Reviews of the Cambridge Philosophical Society*, *72*, 283–327.

Jeswiet, S. B., & Godin, J. G. J. (2011). Validation of the method for quantifying male mating preferences in the guppy (*Poecilia reticulata*). *Ethology*, *117*, 442-449.

Johnson, A. M., & Fuller, R. C. (2014). The meaning of melanin, carotenoid, and pterin pigments in the bluefin killifish, *Lucania goodei*. *Behavioral Ecology*, *00*, 1-10.

Kahn, A. T., Livingston, J. D., & Jennions, M. D. (2012). Do females preferentially associate with males given a better start in life? *Biology Letters*, *8*, 362-364.

Kiere, L. M, Hofmann, C. M., Price, J. J., Cronin, T. W., & Omland, K. E. (2009). Discrete evolutionary color changes in caciques suggest different odes of carotenoid evolution between closely related taxa. *Journal of Avian Biology*, *40*, 605-613.

Kodric-Brown, A., & Brown, J. H. (1984). Truth in advertising: the kinds of traits favored by sexual selection. *The American Naturalist*, *124*, 309-323.

Kodric-Brown, A. (1989). Dietary carotenoids and male mating success in the guppy: an environmental component to female choice. *Behavioral Ecology and Sociobiology*, *25*, 393-401.

Kodric-Brown, A. (1993). Female choice of multiple male criteria in guppies: interacting effects of dominance, coloration and courtship. *Behavioral Ecology and Sociobiology*, *32*, 415-420.

Kodric-Brown, A., & Nicoletto, P. (2001). Female choice in the guppy (*Poecilia reticulata*): the interaction between male color and display. *Behavioral Ecology and Sociobiology*, *50*, 346-351.

Kodric-Brown, A., & Johnson, S. C. (2002). Ultraviolet reflectance patterns of male guppies enhance their attractiveness to females. *Animal Behavior*, *63*, 391-396.

Kolluru, G. R., Bertram, S. M., Chin, E. H., Dunmeyer, C. V., & Graves, J. S. (2014). Mating behavior and its morphological correlates in two color morphs of *Girardinus metallicus* (Pisces: Poeciliidae), a species previously thought not to exhibit courtship display. *Behavioral Processes*, *106*, 44-52.

Kolluru, G. R., Castillo, C., Hendrickson, M., Hughes, M., Krause, P., LePiane, K., McCann, C., Pavia, E., Porter, C., Rodriguez, R., Rodriguez-Cabrera, T., Scott, E., Willrodt, M., & Bertram, S. M. (2015). Sexual selection in black morph *Girardinus metallicus* (Pisces: Poeciliidae): Females can spot a winner (but we cannot). *Ethology*, *121*, 1212–1224.

Kottler, V. A., Koch, I., Flötenmeyer, M., Hashimoto, H., Weigel, D., & Dreyer, C. (2014). Multiple pigment cell types contribute to the black, blue, and orange ornaments of male guppies (*Poecilia reticulata*). PLoS ONE 9(1): e85647.

Küçük, S. (2010). Efficacy of tricaine on *Poecilia latipinna* at different temperatures and concentrations. *African Journal of Biotechnology*, *9*, 755–759.

Künzler, R., & Bakker, T. C. M. (2001). Female preferences for single combined traits in computer animated stickleback males. *Behavioral Ecology*, *12*, 681-685.

Land, M. F. (1972). The physics and biology of animal reflectors. *Progress in Biophysics & Molecular Biology*, *24*, 75-106.

Lane, S. M., Haughan, A. E., Evans, D., Tregenza, T. & House, C. M. (2016). Same-sex sexual behavior as a dominance display. *Animal Behavior*,*114*, 113-118.

Langerhans, R. B. (2011). Genital evolution. In: J. P. Evans, A. Pilastro, & I. Schlupp (Eds.). *Ecology and evolution of poeciliid fishes* (pp. 228-240). Chicago, IL: University of Chicago Press.

Lauziére Y. B., Gingras D., & Ferrie F. P. (1999). Color camera characterization with an application to detection under daylight. *Vision Interface Conference*, *99*, 280–287.

Logan, D. W., Burn, S. F., & Jackson, I. J. (2006). Regulation of pigmentation in zebrafish melanophores. *Pigment Cell and Melanoma Research*, *19*, 206-213.

Lorenzen, E. (1996). Polymorphismus Männlicher *Girardinus metallicus* Poey 1854 (Teleostei, Cyprinodontiformes, Poeciliidae). In H. Greven, & R. Riehl (Eds.), *Fortpflanzungsbiologie der Aquarienfische* (pp. 263-266). Birgit Schmettkamp Verlag, Bornheim.

Lozano, G. A. (1994). Carotenoids, parasites, and sexual selection. *Oikos*, *70*, 309-311.

Marshall, N. J., Jennings, K., McFarland, W. N., Loew, E. R., & Losey, G. S. (2003). Visual biology of hawaiian coral reef fishes. II. Colors of hawaiian coral reef fish. *Copeia, 3*, 455-466.

McGraw, K. J., Hudon, J., & Hill, G. E. (2005). A simple and inexpensive chemical test for behavioral ecologists to determine the presence of carotenoid pigments in animal tissues. *Behavioral Ecology and Sociobiology*, *57*, 391-397.

Metz, J. R., Peters, J. M. J., & Flik, G. (2006). Molecular biology and physiology of the melanocortin system in fish: a review. *General and Comparative Endocrinology*, *148*, 150-162.

Miller, C. W., & Svensson, E. I. (2014). Sexual selection in complex environments. *Annual Review of Entomology*, *59*, 427-445.

Møller, A. P., & Pomiankowski, A. (1993). Why have birds got multiple sexual ornaments? *Behavioral Ecology and Sociobiology*, *32*, 167-176.

Montoya, C. M., Vega, F. V., Nolasco, H. S., Espinosa, L. C., Carrillo, O. F., & Olvera, F. U. (2014). Effects of dietary antioxidant of tomato extract and lycopene on *Carassius auratus* and *Xiphophorus maculatus*. *Revista MVZ Córdoba*, *19*, 4059-4071.

Moore, A. J., Gowaty, P. A., & Moore, P. J. (2003). Females avoid manipulative males and live longer. *Journal of Evolutionary Biology*, *16*, 523-530.

Neiffer, D. L., & Stamper, M. A. (2009). Fish Sedation, Anesthesia, Analgesia, and Euthanasia: Considerations, Methods, and Types of Drugs. *Institute for Laboratory Animal Research Journal*, *5*, 343-360.

Norman, J. R., & Greenwood, P. H. (1963). *A history of fishes* (pp. 398). NY: Hill & Wang.

Parker, G. A. (1979). Sexual selection and sexual conflict. In M.S. Blum, & N. A. Blum (Eds.), *Sexual selection and reproductive competition in insects* (pp. 123-166). New York, Academic Press.

Parker, G. A. (1984). Evolutionarily stable strategies. In: J. R. Krebs & N. B. Davies (Eds.). *Behavioural Ecology: An Evolutionary Approach*, 2nd edn (pp. 30-61). Oxford, UK: Blackwell.

Parker, G. A. (2006). Sexual conflict over mating and fertilization: an overview. *Philosophical Transactions of the Royal Society London B*, *361*, 235-259.

Patricelli, G. L., Coleman, S. W., & Borgia, G. (2006). Male satin bowerbird, *Ptilonorhynchus violaceus*, adjust their display intensity in response to female startling: an experiment with robotic females. *Animal Behavior*, *71*, 49-59.

Patricelli, G. L., Krakauer, A. H., & Taff, C. C. (2016). Variable Signals in a Complex World: Shifting Views of Within-Individual Variability in Sexual Display Traits. *Advances in the Study of Behavior*, *48*, 1-67.

Pike, T. W. (2011). Using digital cameras to investigate animal coloration: estimating sensor sensitivity functions. *Behavioral Ecology Sociobiology*, *65*, 849-858.

Plath, M., Makowicz, A. M., Schlupp, I., & Tobler, M. (2007). Sexual harassment in live-bearing fishes (Poeciliidae): comparing courting and noncourting species. *Behavorial Ecology*, *18*, 680-688.

Plath, M., Blum, D., Schlupp, I., & Tidemann, R. (2008). Audience effect alters mating preferences in livebearing fish, the Atlantic molly, *Poecilia mexicana*. *Animal Behaviour*, *75*, 21-29.

Ponce de Léon, J. L., & Rodriguez, R. (2010). *Peces cubanos de la familia Poeciliidae*. Ciudad Habana: Editorial Academia.

Popovic, N. T., Strunjak-Perovic, I., Coz-Rakovac, R., Barisic, J., Jadan, M., Persin Berakovic, A., & Sauerborn Klobucar, R. (2012). Tricaine methane-sulfonate (MS-222) application in fish anesthesia. *Journal of Applied Ichthyology*, *28*, 553-564.

Pramod, P. K., Sajeevan, T. P., Ramachandran, A., Sunesh Thampy, A., & Somnath Pai, S. (2010). Effects of two anesthetics on water quality during simulated transport of a tropical ornamental fish, the Indian tiger barb *Puntius filamentosus*. *North American Journal of Aquaculture*, *72*, 290–297.

Price, A. C., Weadick, C. J., Shim, J., & Rodd, F. H. (2008). Pigments, patterns, and fish behavior. *Zebrafish*, *4*, 297-307.

Pryke, S. R., Andersson, S., & Lawes, M. J. (2001a). Sexual selection of multiple handicaps in red-collared widow- birds: female choice of tail length but not carotenoid display. *Evolution*, *55*, 1452–1463.

Pryke, S. R., Lawes, M. J., & Andersson, S. (2001b). Agonistic carotenoid signaling in male red-collared widowbirds: aggression related to the colour signal of both the territory owner and model intruder. *Animal Behaviour*, *62*, 695-704.

Qvarnström, A., & Forsgren, E. (1998). Should females prefer dominant males? *Trends in Ecology & Evolution*, *13*, 498-501.

Rohwer, S. (1975). The social significance of avian winter plumage variability. *Evolution*, *29*, 593-610.

Rosenthal, G. G., Evans, C. S., & Miller, W. L. (1996). Female preference for dynamic traits in the green swordtail *Xiphophorus helleri*. *Animal Behaviour*, *51*, 811-820.

Ross, L. G., & Ross, B. (1999). *Anesthetic and Sedative Techniques for Aquatic Animals* (2nd Ed). Oxford, U.K.: Blackwell publishing.

Roulin, A. (2016). Condition-dependence, pleiotropy and the handicap principle of sexual selection in melanin-based colouration. *Biological Reviews*, *91*, 328-348.

Rowe, D. M., & Denton, E. J. (1997). The physical basis for reflective communication between fish, with special reference to the horse mackerel, *Trachurus trachurus*. *The Philosophical Transactions of the Royal Society of London B*, *352*, 531-549.

Searcy, W. A., & Nowicki, S. (2005). *The evolution of animal communication: reliability and deception in signaling systems*. Princeton, NJ: Princeton University Press.

Senar, J. C. (2006). Color displays as intrasexual signals of aggression. In: G. E. Hill & K. J. McGraw (Eds.). *Bird coloration, Vol. 2: function and evolution* (pp. 87-136). Cambridge, MA: Harvard University Press.

Sheets, L., Ransom, D. G., Mellgren, E. M., Johnson, S. L., & Schnapp, B. J. (2007). Zebrafish melanophilin facilitates melanosome dispersion by regulating dynein. *Current Biology*, *17*, 1721-1734.

Stevens, M., Párraga, C. A., Cuthill, I. C., Partridge, J. C., & Troscianko, T. S. (2007). Using digital photography to study animal coloration. *Biological Journal of the Linnean Society*, *90*, 211-237.

Suk, H. Y., & Choe, J. C. (2008). Dynamic female preference for multiple signals in *Rhinogobius brunneus*. *Behavioral Ecology and Sociobiology*, *62*, 945-951.

Svensson, P. A., & Sköld, H. N. (2011). Skin biopsies as tools to measure fish coloration and colour change. In: U. Khopkar (Ed.), *Skin Biopsy—Perspectives* (pp. 299-316). Croatia: InTech.

Svensson, P. A., & Wong, B. B. M. (2011). Carotenoid-based signals in behavioural ecology: a review. *Behaviour*, *148*, 131-189.

Vega-Trejo, R., O'Dea, R. E., Jennions, M. D., & Head, M. L. (2014). The effects of familiarity and mating experience on mate choice in mosquitofish, *Gambusia holbrooki*. *Behavioral Ecology*, *25*, 1205-1211.

Wang, S., Cummings, M., & Kirkpatrick, M. (2015). Coevolution of male courtship and sexual conflict characters in mosquitofish. *Behavioral Ecology*, *00*, 1-8.

Wedekind, C., Jacob, A., Evanno, G. Nusslé, S., & Müller, R. (2008). Viability of brown trout embryos positively linked to melanin-based but negatively to carotenoid-based colours of their fathers. *Proceedings of the Royal Society B*, *275*, 1737-1744.

Wollenberg, K. C., Lötters, S., Mora-Ferrer, C., & Veith, M. (2008). Disentangling composite colour patters in a poison frog species. *Biological Journal of the Linnean Society*, *93*, 433-444.

Wong, B., & Candolin, U. (2005). How is female mate choice affected by male competition? *Biological Reviews*, *80*, 559-571.

Wong, R. Y., So. P., & Cummings, M. E. (2011). How female size and male displays influence mate preference in a swordtail. *Animal Behaviour*, *82*, 691-697.

Wucherer, M. F., & Michiels, N. K. (2014). Regulation of red fluorescent light emission in a cryptic marine fish. *Frontiers in Zoology*, *11*, 1-8.

Yasir, I., & Qin, J. G. (2009). Effect of light intensity on color performance of false clownfish, *Amphiprion ocellaris* Cuvier. *Journal of the World Aquaculture Society*, *40*, 337-350.

Zahavi, A. (1975). Mate selection – a selection for a handicap. *Journal of Theoretical Biology, 53*, 205–214.

APPENDIX

A.1 Elucidating the Pigment Basis of Yellow Coloration

I hypothesized that the yellow coloration of black morph males contains carotenoid pigments, given the prevalence of carotenoids in yellowish coloration in other poeciliids (Endler 1984; Goodwin 1984).

I performed a chemical extraction to detect the presence of carotenoid pigments described by McGraw et al. (2005) using a sample of fish separate from those described above. This method uses both a thermochemical extraction technique to free carotenoid pigments from sample tissue and a solvent transfer to confirm the presence of carotenoids in animal tissues. I increased the tissue amount from the recommended 3-5mg to 32.5mg, reduced the recommended amount of DI water from 2mL to 1mL, and added 1mL of TBME to the solution before the 1:1 ratio of hexane: TBME in attempt to obtain a distinct separation. I extracted pigments from the yellow portions of skin of 10 *Girardinus metallicus* males (32.5mg), excluding black areas, fins, and the head. I also used two positive controls known to have carotenoids: one vial with the dissected skin of 10 guppies (49.8mg, *P. reticulata*; Endler 1980) and one vial with shavings of a carrot (85mg; Britton 1992). I extracted lipid-soluble pigments from the skin with heated acidified pyridine and later combined the solution with a hexane: tert-butyl methyl ether (TBME) mixture to address whether coloration could be a result of pteridine pigments instead of carotenoids. If a tissue sample has carotenoid pigments then they will leach out of the tissue and color the heated pyridine solution. Furthermore, carotenoid pigments, unlike pteridine pigments, will transfer to non-polar organic solvents such as hexane (for the non-polar carotenoids) and TBME (for polar

carotenoids), suggesting that if carotenoids are present then when the hexane:TBME is added to the pyridine solution and centrifuged, the hexane: TBME phase will be colored, instead of the pyridine phase.

I found that the heated pyridine solution leached color from the tissue samples of *G. metallicus*, *P. reticulata*, and the carrot, suggesting that carotenoid pigments may be present in all samples (Fig.A1). When performing the additional procedure using hexane:TBME to address whether the coloration is due to carotenoids *per se*, rather than pteridines, my results were not consistent with those outlined by McGraw et al. (2005). The separation between the hexane:TBME layer and the pyridine layer was evident; however, both phases contained pigment, suggesting that the coloration may be due to multiple pigment types (Fig. A1; McGraw et al. 2005). Furthermore, both the pyridine and hexane:TBME phase for both positive controls were only slightly colored, implying that the separation failed, because both guppy skin (Endler 1980) and carrots (Britton 1992) are known to contain concentrated carotenoid pigments. I repeated the extraction three times; however, all attempts resulted in inconclusive results. Regardless, these results suggest that there may be multiple pigments responsible for the coloration and more rigorous tests need to be performed.

The yellow body coloration may be a dynamic trait, changing temporally, as opposed to a static trait, as it was assumed here. The yellow coloration could be affected by the dispersion of melanosomes in melanophores, which can mask the yellow pigment-containing xanthophores and the interaction of light and iridophores, which can change appearance (Grether et al. 2004; Price et al. 2008). Although I was not able to determine the pigmentary

source of the yellow coloration, there is variation in yellow saturation and yellow brightness among males and more detailed tests (outlined in Grether et al. 2001) are needed to determine whether carotenoids are present in *G. metallicus* skin.

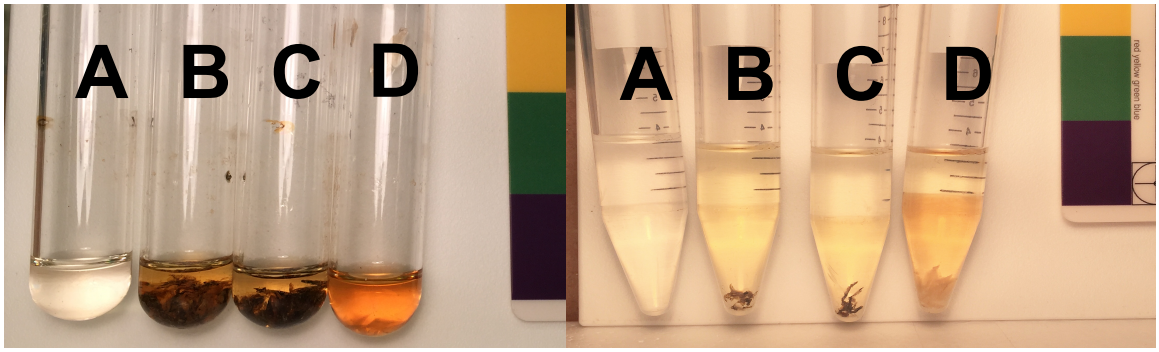

Figure A.1.1 Heated pyridine solution with coloration as a result of pigments leached from tissue samples (left) and separation between the hexane:TBME layer and the pyridine layer (right) of (A) pyridine control (B) black morph *G. metallicus* (C) guppy (*P. reticulata*) and (D) carrot.