ABSTRACT

Title of Thesis:	INSIGHTS IN ECOLOGY, BEHAVIOR, AND REPRODUCTION FROM VISUAL MODELS OF AFRICAN CICHLIDS
	Zeke Martin Gonzalez, Doctor of Philosophy, 2024
Thesis Directed By:	Professor Karen L. Carleton, Department of Biology

Sexual selection has long been proposed to have played an important role in the explosive speciation of east African cichlids. Further, it is known that visual signals are the most salient ones to cichlids when it comes to reproduction. However, studies examining visual signals such as egg spots and size have been historically difficult to conduct due to the relationship between such phenotypes and confounding variables like age. In addition, the results from such studies often conflict and do not highlight clear patterns and hypotheses. In this dissertation, I use a receptor noise limited (RNL) visual model of increasing complexity to examine the discriminability of important visual signals in cichlid ecology, behavior, and evolution. In determining whether cichlid egg spots are truly mimics of cichlids eggs, I quantified fish and egg reflectance and found that two cichlid species are unable to distinguish the colors of eggs and egg spots in the lighting of their natural habitat. In order to bring together these quantitative methodologies with behavioral data, I tested the viability of using virtual stimuli displayed on a

monitor to robustly examine how various visual signals affect conspecific male aggression. I found that although the cichlid *Metriaclima zebra* responds to virtual stimuli with equal aggression as towards live fish, it also responds with equal aggression towards virtual stimuli that differ in egg spot presence, body color, movement, and size. This suggests that virtual stimuli are not useful for behavioral tests in this species. Finally, in order to examine the salience of egg spots and body color in the wild, I calculated chromatic distance as a function of viewing distance for cichlid body colors against biologically-relevant backgrounds, conspecific body colors, and heterospecific body colors. The study shows that *M. zebra* body colors are discriminable from the space light at up to 5 m, but from the rocks at shorter distance, though distances that are comparable to the spacing of male territories. This suggests that males should be able to discriminate potential conspecific rivals on their breeding territories. Additionally, the visual model shows that *M. zebra* is highly discriminable from yellow heterospecifics but not so from blue heterospecifics. This dissertation emphasizes the importance of avoiding human biases in studies of cichlid color vision and behavior.

DESCRIPTION OF SUPPLEMENTARY MATERIALS

Video 1: A .mp4 file that represents an example of the virtual stimulus used for the experiments described in Chapter 3.

INSIGHTS IN ECOLOGY, BEHAVIOR, AND REPRODUCTION FROM VISUAL MODELS OF AFRICAN CICHLIDS

by

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Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2024

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Acknowledgements

Thank you to the brilliant, dedicated, and thoughtful Dr. Karen Carleton. You are my academic knight in shining armor, guiding me through a long and scary process with confidence. You always extended me the freedom to explore my own curiosity, while helping me keep the big picture in mind. It cannot be understated how much I have learned and achieved simply by striving towards your example. Thank you for everything.

Thank you to Tom, Scott, Heidi, and Jens for being a great committee. Whenever I was in a muddle and couldn't decide the best way forward, you offered invaluable guidance and knowledge. My science is all the better for your feedback.

Thank you to my fellows from the cichlid labs through the years. Whether you study fish from lakes or from rivers, or do experiments in the lab or on a computer, my research is better for your help, guidance, and support. I will remember lunches in graduate school as the backbone of both my studies and my well-being. I would especially like to thank Dani, Miranda, Molly, Mara, Jessica, Karen, and Kristen.

Thank you to the undergraduate research volunteers Maurice Leary, Anne-Fleur Winters, Marina Francis, and Nathaniel Hoang. The total sum of videos I had to record, watch, and analyze for this thesis summed more than all of Game of Thrones twice over, so every minute you helped with goes more appreciated than I can say.

Thank you to all the friends with whom I have shared the last seven years. Some of us have a long history, some of us much shorter than that; some of you are here and others are far away. Whether we gather to be merry, watch scary movies, play games, or do nothing at all, you remind me that we are put on this world to care for one another, to laugh, and to tell stories.

Thank you to my family. They say it takes a village to raise a child and this thesis was no different. Thank you to my Mom & Dad for being my port in a storm, for always giving unconditional love and support. Thank you to my siblings Noah, Gus, and Lillie for the joy and laughter to keep me buoyed even during the hardest hours. Thank you to my Grandma & Grandpa for always cheering me on and teaching me all the right signs to explain my science. Thank you for your love and your patience. This thesis was only possible because of you.

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Chapter 1: Introduction

1.1 Introduction

The study of evolution, the process by which organisms change over generational time, has remained one of biology's central pillars since the concept was introduced to scientific society by Darwin in 1859. This robust pillar of biology has encouraged scientists to study all manner of organisms from bacteria to fruit flies and fossilized plants to the world's largest whales. However, one key avenue for investigating evolution is speciation: how one ancestral species becomes two or more species over large periods of evolutionary time. One way to follow this avenue of study is by identifying groups which have experienced explosive radiation: evolving a great number of species in a short period of time. There is one such group of fish that has captured significant scientific attention for exactly this reason: the cichlid fish (Kocher et al. 2004).

Cichlids are one of the most speciose vertebrate groups on the planet (Barlow, 2008; Turner et al., 2001) and the cichlids of the African Rift Valley contain more than a thousand species (Turner et al., 2001). In particular, the following studies focus mainly on the cichlids of Lake Malawi, a clear water habitat which contains more than 700 species (Turner et al., 2001). Although it was once suggested that there was a singular origin of this radiation in Lake Malawi (Moran et al. 1994), new theories have begun to support the hypothesis that multiple invasions of and hybridizations within Lake Malawi by riverine ancestors has led to the current levels of cichlid diversity (Joyce et al. 2011). Identifying the mechanisms and processes which led to

this explosive radiation has been the subject of tremendous study, as it provides an excellent opportunity to delve deep into the mechanics of evolution itself. It is widely believed that sexual selection played an important role in the diversity of these cichlids (Holzberg 1978; Ribbink et al. 1983; Hert 1991; Kornfield & Smith 2000; Salzburger et al. 2005).

Specifically, sexual selection arises from sexual conflict: when the courters and the choosers of a species employ unique optimal fitness strategies which conflict with one another. In cichlids, sexual selection arises mainly from the fact that female cichlids, especially the haplochromine cichlids, are mouthbrooders. While males defend the territories on which they court and mate with females (Holzberg 1978; Ribbink et al. 1983), females pick up their eggs in their mouth and carry the offspring until they are hatched and mobile (Fryer & Iles, 1972). Females are courted by multiple colorful males from who she picks one or more males (Ribbink et al., 1983; Barlow, 1991; Parker & Kornfield, 1996; Kellogg et al., 1998). This means that females are attempting to have their eggs fertilized by the "best" males while males are attempting to simply fertilize as many eggs as possible, generating sexual conflict. It has been demonstrated that males defend and fight for territories on which to mate and that females choose males who have territories, saturated body colors, the correct conspecific patterns, and courtship vigor (Seehausen & van Alphen, 1998; Salzburger et al., 2005; Henning & Meyer, 2012). Although much of the focus in the study of cichlid visual signals has been body color (Dominey, 1984; Schluter, 2000; Seehausen, 2000; Jordan et al., 2003; Carleton et al., 2005; Maan et al., 2006;

Salzburger et al., 2009), there is another phenotype that has been identified as playing a role in sexual selection: the egg spot (Salzburger et al. 2005).

1.2 Cichlid egg spots

The haplochromine egg spot is a small area, most often a circle, of yellow, orange, or red color on the anal fin of a male fish which is surrounded by a translucent ring (Wickler, 1963). This phenotype is well-documented across both the scientific literature and in photographs of haplochromines in their natural habitat, demonstrating variation in the number, color, and size of egg spots on different species (Goldschmidt 1991; Hert 1989; Hert 1991; Theis et al., 2012; Theis et al. 2017). Egg spots first appear in the literature as "egg-dummies" which are hypothesized to mimic eggs to take advantage of the sensory bias for egg-shaped and egg-colored objects of mouthbrooding females and thereby increase fertilization (Wickler, 1963). Although the phrase "egg dummies" and "egg spots" have remained prevalent in the literature, this hypothesis has not been definitively assessed. Further, this hypothesis has been the course of disagreement in the literature – although some have viewed the egg spot as a phenotype likely to play a role in sexual selection to the natural variation in the wild across species and individuals, others have long disagreed with this assessment.

The earliest source of disagreement with Wickler's egg mimicy hypothesis came when scientists measured the size and color of both egg spots and eggs across a number of Lake Malawi haplochromines and determined that both the size and color of the two objects were notably different in several species (Jackson & Ribbink, 1975). A later study disagreed, finding similarity between the egg spots and eggs in

several Lake Victoria haplochromines (Goldschmidt, 1991). However, it is worth noting that all these early studies came at this question from a human perspective, measuring color by the human eye and not interrogating the salience of egg spots as a visual signal to the fish themselves. For example, it is possible that egg spots draw on a sensory bias that doesn't require egg spots and eggs to be the same size to get a behavioral reaction from the animal. Further, egg spot number is a heritable trait, with offspring of parents with more egg spots having a greater number of egg spots themselves (Leighton & Meyer 2011). In conjunction with the natural variation of egg spots in the wild, this introduces the possibility that these egg spot characteristics, particularly number, either now or in the evolutionary past could act as a signal within- and between-species in the east African rift lakes. Due to their hypothesized role in sexual selection, this phenotype could be a significant factor in haplochromine speciation.

It is worth noting two of the alternative explanations for the role that egg spots play in haplochromine reproduction. Although alternatives are not discussed in the literature that I am aware of, it is possible that egg spots played an important role in fertilization while the haplochromine clade was emerging but has since become part of a fixed action pattern (circling during courtship). It is also possible that the act of the female pecking on the egg spots themselves that are the trigger for male haplochromines to release sperm, rather than playing a role in sexual selection. Studies that investigate these and other alternative roles for the egg spot would be valuable avenues for future research. However, sexual selection is currently the most well-supported hypothesis for the role of this unique phenotype. As such, scientists

have attempted to clarify the role that egg spots may play in female choice or male-male competition.

With regards to female choice, it has been shown that while females of some species prefer males with more or larger egg spots (Hert, 1991; Couldridge, 2002; Couldridge & Alexander, 2002; Egger, 2011), females of other species mate indiscriminately with regards to egg spots (Henning & Meyer, 2012; Theis et al., 2012; 2015). On the other side of sexual selection, the role of egg spots is equally muddy. There is some evidence that egg spots predict aggression or the outcomes of male fights (Theis et al., 2012; 2015) and evidence that egg spots aren't important in male-male competition (Henning & Meyer, 2012). It is important to note that whether studying female choice or male-male competition, it can be difficult to separate the effects of dominance, size, and age from the effects of egg spots in traditional behavioral assays. Additionally, no study has yet accounted for the differences between human and cichlid vision while studying haplochromine egg spots and its effect on reproduction or behavior. In summary, there have been a number of studies conducted on the role of egg spots in cichlid sexual selection, but no clear patterns have yet emerged.

1.3 Goals of Research

This thesis will focus on understanding the role of egg spots in the reproductive behavior of haplochromine cichlids, though it also considers the relevance of other important visual signals used by haplochromine cichlids. It is the first research to consider cichlid visual sensitivities and color discrimination abilities to assess whether or not there is quantitative support for the persistent early hypotheses

regarding the egg spot's role in sexual selection. These studies bring together quantitative and behavioral methods with data from both the wild and the laboratory to examine haplochromine egg spots in the context of cichlid color vision.

In the following studies I aim to develop a robust visual model to better understand the role of important visual signals to the ecology, reproduction, and behavior of African cichlids. In Chapter 2, I quantify the reflectance of eggs, egg spots, and anal fins which I use in a simple visual model to assess a historical hypothesis on the function of egg spots. In Chapter 3, I use visual modeling to develop salient virtual stimuli that allow me to perform robust behavioral experiments examining the role of egg spots, color, movement, and size in male-male competition. In Chapter 4, I introduce viewing distance into the visual model to examine the conspicuousness and discriminability of cichlid body colors against one another and biologically relevant backgrounds. In Chapter 5, I synthesize the main results from prior chapters and suggest fruitful avenues of future research based on the results of this research. Results from this work provide additional clarity and context to our historical understanding of cichlid vision and behavior and shed light on how these important factors contribute to the recent and rapid evolution of African cichlids.

Chapter 2: Testing Wickler's Hypothesis: Cichlids are unable to distinguish eggs from egg spots in the wild

Previously published under: Gonzalez, Z. M., Juntti, S. A., & Carleton, K. L. (2023). Testing Wickler's hypothesis: Cichlids are unable to distinguish eggs from egg spots in the wild. Hydrobiologia, 850(10), 2355–2369. https://doi.org/10.1007/s10750-022-05113-2

2.1 Abstract

Cichlid fish have undergone explosive radiation in a process aided by sexual selection. Thus, phenotypes that contribute to reproduction may partially explain the rate of radiation. One such phenotype is the presence of circular spots on the anal fin of the haplochromine cichlids. In 1963, Wickler postulated this phenotype acts as an egg mimic which enhances male fertilization success. However, it remained unclear whether cichlids can distinguish between eggs and egg spots in their natural environment. Further, studies have found differing effects of egg spot characteristics in female mate preference. In this study, we quantified the color of eggs, egg spots, and anal fins of two haplochromine species (*Metriaclima benetos* and *Astatotilapia burtoni*). Using a receptor noise limited (RNL) visual model, we found that these species are unable to distinguish the colors of eggs and egg spots in the lighting of their natural habitat. Further, only *M. benetos* could distinguish the color of egg spots from anal fins. This study supports Wickler's egg spot mimicry hypothesis and potentially explains species differences in the roles of egg spots as sexually selected

traits. It further points out the importance of avoiding human biases in quantifying what colors fish can and cannot distinguish.

2.2 Background

Cichlid fish are one of the most speciose vertebrate groups on the planet, with more than a thousand cichlid species in the African Rift Valley alone (Barlow, 2008; Turner et al., 2001). In particular, the lacustrine cichlids of Lake Tanganyika, Lake Victoria, and Lake Malawi and the riverine cichlids of the surrounding landscape represent an explosive radiation which makes them a useful model for studying evolution (Kocher, 2004). In fact, these three lakes alone are estimated to be home to more than 1,650 unique species (Turner et al., 2001). Sexual selection likely played an important role leading to this diversity in the African Rift Valley (Holzberg, 1978; Ribbink et al., 1983; Hert, 1991; Kornfield & Smith, 2000; Wagner et al., 2012). Salzburger et al., (2005) have identified three key innovations which may have been targets for selection: female mouthbrooding, male egg spots, and sexual dimorphism in color pattern.

Within the Cichlidae of east Africa, one group is useful for studying these key innovations: the haplochromine cichlids, which include cichlids of Lake Malawi, Lake Victoria, the riverine *Astatotilapia*, and the Tropheini from Lake Tanganyika (Salzburger et al., 2005). Haplochromine adults are typically sexually dimorphic, with males being larger and brightly colored whereas females are smaller and possess more cryptic coloration (Ribbink et al., 1983). The females swim above the lek-like male territories (Rogers & Barlow, 1991) and are courted by nearby males, from

which females choose their mates (Parker & Kornfield, 1996; Kellogg et al., 1998). Females then lay their eggs on substrate before picking them up into their mouths, where they carry them for two or more weeks until the fry are free-swimming (Fryer & Iles, 1972). The only parental care offered by the males is fertilization of the eggs and a place to mate: their heavily defended male territory (Holzberg, 1978; Ribbink et al., 1983). This imbalance in contribution by males and females creates sexual conflict and results in sexual selection on sexually dimorphic traits, including male color pattern phenotypes (Deutsch, 1997; Hert, 1991; Allender et al., 2003; Carleton et al., 2005). In this case, females choose males with bright hues, species-specific patterns, body size, and frequent courtship behaviors (Seehausen & van Alphen, 1998; Henning & Meyer, 2012). This pattern and coloration include another distinctive trait and key-innovation of the haplochromine cichlids: the egg spot (Salzburger et al., 2005).

There is a great deal of natural variation in the number, size, and appearance of egg spots both within and between species (Goldschmidt, 1991; Hert, 1989; Hert, 1991; Theis et al., 2012; Theis et al., 2017). Egg spots are an area of yellow, orange, or red coloration on the anal fin of a male fish which are typically surrounded by a translucent ring (Figure 2.1, Wickler, 1963, Lehtonen & Meyer, 2011; Hert, 1989; Hert, 1991; Theis et al., 2012). Further, it is suggested that egg spot number is heritable (Lehtonen & Meyer, 2011), which raises the possibility that egg spot characteristics, particularly number, could act as a signal within and between species in the east African rift lakes. Egg spots were first described in the literature by Wickler (1963) in *Haplochromis wingatii* (Boulenger, 1902 as cited in Lévêque et al.,

1991) as “egg-dummies”, which “trick” the female into pecking at them as if they were going to take the “egg” into their mouth for brooding. According to Wickler (1963), this mimicry positions the female to uptake the sperm being emitted by the male, which fertilizes the eggs in the female’s mouth.

A.

B.

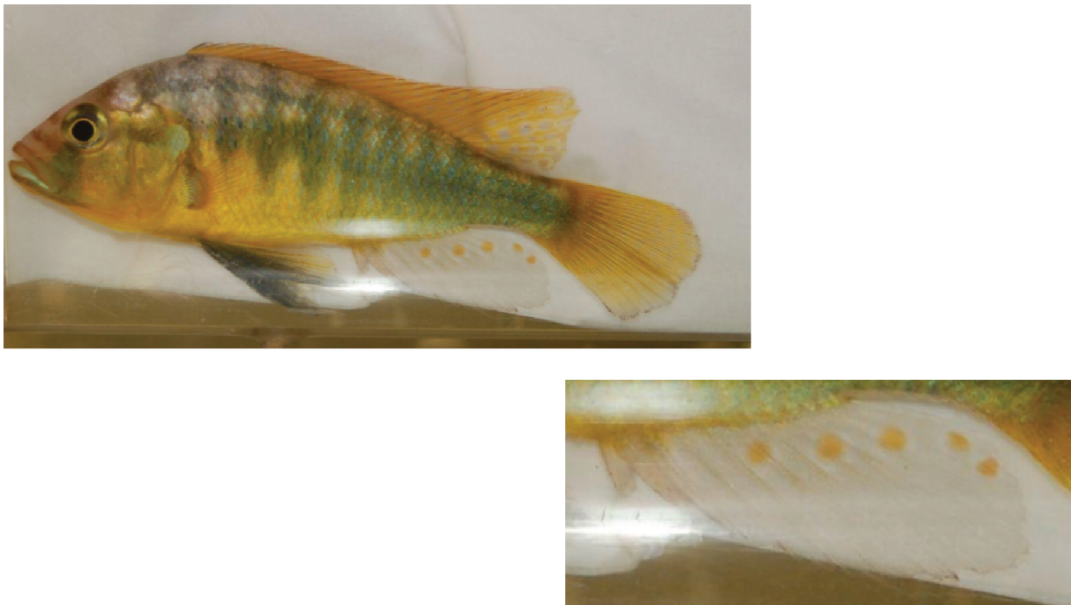


Figure 2.1: Photographs of male *M. benetos* & *A. burtoni* with anal fins splayed out. Each photograph is inset with a close-up of the anal fin and the egg spots of the fish against a white background.

Since Wickler's original hypothesis in 1963, it has become common in the literature to refer to the spots of carotenoid coloration on the fins of haplochromine cichlids as "egg dummies" or "egg spots." However, there has been disagreement over the veracity of the first tenet of Wickler's hypothesis: whether egg spots actually mimic eggs. Most notably, Jackson and Ribbink (1975) found that in many species, egg spots differ in size and color from eggs. However, Wickler's mimicry hypothesis seems to be supported by a "rough correspondence" between the color and size of wild-caught haplochromine cichlids and their eggs (Goldschmidt, 1991). It is important to note that neither of these studies have accounted for the differences between human and cichlid visual sensitivities while comparing eggs and egg spots. The ability to discriminate between different colors is inversely proportional to photoreceptor noise (see visual modeling below). Noise values for human long wavelength sensitive cones are approximately 0.02 (Wyszecki & Stiles, 1982), which is 5 to 8 times smaller than estimates of photoreceptor noise for fishes (Pignatelli et al., 2010; Cheney et al., 2019; Escobar-Camacho et al., 2019). Therefore, human color discrimination will be 5-8 times better than fish color discrimination. This suggests that although humans might be able to distinguish egg and egg spot colors, cichlids will be much less able to do so. This supports the importance of considering color discrimination for the animals' visual system, to prevent any human biases. The second component of Wickler's hypothesis, egg spot function, has also received study, which seems to vary in haplochromine species. For example, Metriaclima aurora (Burgess, 1976 as cited in Ciccotto et al., 2011) females lay more clutches with males that have egg spots over males without egg spots and prefer males with a

greater number of egg spots over those with a fewer number of egg spots (Hert, 1991). Conversely, female Astatotilapia burtoni (Günther, 1894 as cited in Daget et al., 1984) exhibit no preference between males with or without egg spots (Theis et al., 2012). In summary, Wickler's original hypothesis has led to a myriad of studies which have seemed to disagree on whether egg spots are egg mimics and have found differing egg spot function among species.

In this study, we test Wickler's egg mimicry hypothesis by quantifying egg and egg spot colors. In addition, we use visual models to determine whether two different species of haplochromine cichlids can distinguish between the color of the eggs and the color of the male egg spot, based on their individual visual system and the light present in their natural habitat.

2.3 Materials and Methods

2.3.1 Study Species

Two different species were selected for this study: *Metriaclima benetos* (Stauffer et al., 1997) and *Astatotilapia burtoni*. Both species are trichromats which use three different cone photoreceptors that process light at different wavelengths (Escobar-Camacho et al., 2017; Fernald & Liebman, 1980). *Metriaclima benetos* are lacustrine haplochromine cichlids endemic to Lake Malawi (Stauffer et al., 1997). Specifically, these animals are laboratory stock descended from a population found at Mazinzi Reef, a habitat with especially clear water where they dwell within 8 meters of the surface (Ribbink et al., 1983). *Metriaclima benetos* possess a trichromatic visual system with short, medium, and long wavelength sensitive photoreceptors (λmax =

379, 489, 522 nm; Hofmann et al., 2009, Jordan et al., 2006). *Astatotilapia burtoni* are riverine haplochromine cichlids found in the rivers and shore pools surrounding Lake Tanganyika, plus in the lake itself (Fernald, 1975; Fernald & Hirata, 1977). Like *M. benetos*, *A. burtoni* also have a trichomatic visual system but with shifted sensitivity towards longer wavelengths of light (λmax = 455, 523, and 562 nm; Fernald & Liebman, 1980). Compared to the clear waters of Mazinzi Reef, *A. burtoni* are found in more turbid environments. All of the fish utilized in this study are the result of multiple generations of breeding in the laboratory and were fed daily with commercial food. Each female whose eggs were measured came from a separate brood, while the males came from a mixed stock of several different broods.

2.3.2 Egg Spectra

Multiple breeding tanks were established for collecting eggs in the laboratory for both species using existing laboratory-raised populations. In each breeding tank, 4-5 females and 1 male were separated by a transparent barrier. The barriers were removed three times a week for 1 hour, during which time the male entered the female side of the tank and performed mating displays. Afterwards, the barriers were replaced and any mouthbrooding females were allowed to remain on the male side of the barrier for an additional hour. Eggs were then removed from mouthbrooding mothers (*M. benetos* n = 3 broods, *A. burtoni* n = 5 broods) and 5 eggs were randomly selected from the brood for measurement with spectrophotometer. The eggs were illuminated at 45° with a broad spectrum xenon Maglite (4D cell) that included UV wavelengths (350-700 nm). The reflected light was collected at 90° perpendicular to

the fish, and focused with a UV transparent Nikon camera lens (105 mm focal length) onto a 400 mm fiber optic cable and quantified by an Ocean Optics USB2000 spectrometer. Each egg's reflectance was measured 3 times and each measure is averaged from 50 scans made by the spectrophotometer. The relative reflectance of the eggs was determined by normalizing with the reflectance from a Spectralon diffuse reflectance standard (Labsphere, North Sutton, New Hampshire, USA). Each brood measured was taken from a different female.

2.3.3 Egg Spot and Anal Fin Spectra

Mature adult males of both species (*M. benetos* n = 7, *A. burtoni* n = 5) were randomly selected from breeding tanks for measurement of their egg spots. Each male was wrapped in a wet paper towel and his anal fin was flared manually by hand. The reflectance of both the egg spots and anal fin outside the spots of these fish were measured using a spectrophotometer on a white background using the same set up as for the eggs. Each egg spot and anal fin had its reflectance measured 3 times and each measure is averaged from 25 scans made by the spectrophotometer. *M. benetos* had an average of 2.7 egg spots and a range of 2-4 egg spots, whereas *A. burtoni* had an average of 4.0 egg spots and a range of 2-5 egg spots. Fewer scans were averaged for the live fish than the eggs in order to make measurements faster and avoid disruptions from the fish moving. Anal fin measures were taken halfway between the egg spot and the anal fin's juncture with the body.

2.3.4 Illuminance Fin Spectra

Irradiance, or the light (radiant energy) incident on a surface over time is an important factor when measuring and analyzing vision and color. In addition, side welling irradiance, or the background space light is what illuminates underwater objects. Side welling irradiance from previous measurements of the fluorescent lights in the laboratory (Philips fluorescent lamps F32T8/TL865 Plus Alto) were used for both species (Escobar-Camacho et al., 2019). Side welling irradiance from previous measurements in Lake Malawi was used for *M. benetos*, specifically the light measured at 3 meters below the surface at Thumbi West Island, a clear water site in Lake Malawi comparable to the fish's habitat at Mazinzi Reef (Sabbah et al., 2011). Side welling irradiance was measured in *Astatotilapia burtoni* habitats around southern Lake Tanganyika using an Ocean Optics spectrophotometer (USB2000; Dunedin, FL, USA), fitted with a 15 m long 400 μm diameter UV-transparent fiber. Measurements used a cosine corrector (CC-3, Ocean Optics) attached to the end of the optical fiber. Sidewelling irradiance measurements were taken at a depth of approximately 2 m on the bank of the Kalambo River (8.596232ºS, 31.183873ºE) and at a depth of approximately 2 m in Lake Tanganyika at Chipwa village (8.602067ºS, 31.186450ºE), where the Kalambo River enters Lake Tanganyika. The measurements were taken at 2 pm and 12 pm, respectively, on cloudless days in August of 2019 during the dry season, which typically experiences some fluctuations in turbidity (Langenberg, 2008). Each site is home to *A. burtoni* (W. Salzburger, personal communication).

2.3.5 Visual Model

The receptor noise limited (RNL) visual model was used to determine whether the fish could discriminate between the color of eggs and egg spots (Vorobyev & Osorio, 1998; Kelber et al., 2003). These quantitative models first calculate quantum catch (Q_i), the number of photons captured for each of the three photoreceptor types *i*. Quantum catch is calculated using the spectrum of the illuminant (*I*), the surface reflectance of an object (i.e. the target colors *S*), the lens transmission (*L*), and the receptor spectral sensitivities of the photoreceptors (R_i) (Kelber et al. 2003). The von Kries factor (K_i) is derived from the von Kries color constancy model, in which each receptor adapts independently to the background illumination, and acts as a color constancy correction. The photoreceptors were modeled for A_1 based visual pigments with peak wavelength sensitivities using the equations of Govordovskii et al. (2000). The visual model used in this study considers wavelengths of light from 350-700 nm.

$$Q_i = K_i \int_{350}^{700} R_i(\lambda)L(\lambda)S(\lambda)I(\lambda)d\lambda$$

$$K_i = \frac{1}{\int_{350}^{700} R_i(\lambda)L(\lambda)I(\lambda)d\lambda}$$

For each of the study species, we used the fishes' natural environment, visual system, and lens transmission. For *M. benetos*, we compared two illuminants: the fluorescent lights in the laboratory (Philips fluorescent lamps F32T8/TL865 Plus Alto) and spectra from Lake Malawi (Sabbah et al., 2011), both of which include UV light. For *M. benetos,* we used the previously measured lens transmission for the species (Escobar-Camacho et al., 2019) and the measured spectral sensitivities for the

species, λmax = 379, 489, 522 nm (Jordan et al., 2006). For *A. burtoni*, the illuminants used in the model also include the laboratory's fluorescent lights as well as two examples of *A. burtoni*'s natural habitats: Lake Tanganyika and Kalambo River. The lens transmission in *A. burtoni* has not yet been measured, so a longer wavelength cichlid lens transmission from *Melanochromis auratus* (Boulenger, 1897 as cited in Daget et al., 1984) is used instead. The two species have similar long-wavelength-sensitive opsin palettes which should utilize UV-blocking lenses (Hoffman et al., 2010). The spectral sensitivities of *A. burtoni* used in the visual model are λmax = 455, 523, 562 (Fernald & Liebman, 1980).

We compare the quantum catch values of the colors of two objects using the RNL model, taking receptor noise into account (Vorobyev & Osorio, 1998; Vorobyev et al., 2001; Koshitaka et al., 2008; Escobar-Camacho et al., 2019). This allows us to calculate ΔS, chromatic distance, whose units are just noticeable difference (JND). In order to calculate chromatic distance, we first use the quantum catch (Q_i) for each of the three photoreceptor types, i, to calculate the contrast between the pair of colors Δf_i (Siddiqi et al., 2004).

$$\Delta f_i = \ln\left[\frac{Q_i(\text{color 1})}{Q_i(\text{color 2})}\right]$$

The second component of the RNL model is calculating receptor noise, represented by the Weber fraction (ω). The Weber fraction for a single photoreceptor i is:

$$\omega_i = \frac{v_i}{\sqrt{n_i}}$$

where relative receptor noise is v and number of photoreceptors of photoreceptor type i is n_i (Vorobyev & Osorio, 1998; 2001). Additionally, we assigned a receptor noise

value for each cone class (Koshitaka et al., 2008), assuming the long (L) receptor to have a noise value of 0.16 (Escobar-Camacho et al., 2019). The value of 0.16 was quantified for *M. benetos*, however, that specific value is most relevant to the blue and red areas of the color space. Noise varies with color, so we also used two additional noise values quantified from the triggerfish *Rhinecanthus aculeatus* (Linnaeus, 1758 as cited in Myers, 1999): 0.1165 quantified for browns (more similar to egg spot colors) and 0.135 measured for blues (similar to *M. benetos* anal fins) (Cheney et al., 2019). Triggerfish are the only other fish (to our knowledge) where noise constants have been quantified. And although triggerfish are not haplochromine cichlids like *M. benetos*, they are trichromats with a complement of small, medium, and long cones (λmax = 413, 480, and 580 nm; Cheney et al., 2019) with visual sensitivities that are comparable to that of *M. benetos*. For each unique noise value used (0.16, 0.1165, and 0.135), the short (S) and medium (M) cone class noises values were calculated using their relative abundance in the retinal mosaic. In this calculation, *A. burtoni* has the same retinal mosaic (Fernald, 1981) as *M. benetos* (Dalton et al., 2014; Escobar-Camacho et al., 2017) with an S:M:L cone ratio of 1:2:2.

By bringing together the chromatic contrast Δf_i and Weber fraction ω_i we can calculate the chromatic distance ΔS (Vorobyev & Osorio, 1998). ΔS was calculated for a trichromatic visual system:

$$\Delta S = \sqrt{\frac{\omega_S{}^2(\Delta f_L - \Delta f_M)^2 + \omega_M{}^2(\Delta f_L - \Delta f_S)^2 + \omega_L{}^2(\Delta f_S - \Delta f_M)^2}{(\omega_S\omega_M)^2 + (\omega_S\omega_L)^2 + (\omega_M\omega_L)^2}}$$

ΔS values greater than 1 indicate that the fish can distinguish the two colors, while ΔS values less than 1 indicate that fish cannot distinguish the two colors (Siddiqi et al., 2004; Lind & Kelber, 2009; Olsson et al., 2018; Vorobyev & Osorio, 1998).

2.4 Results

2.4.1 Egg, egg spot, and fin reflectance data

We measured and compared the spectra of 40 eggs (15 *M. benetos* and 25 *A. burtoni*) from 8 mothers (3 *M. benetos* and 5 *A. burtoni*) within 2 hours of egg laying (Figure 2.2). The average *M. benetos* relative reflectance rises between 350-550 nm and falls between 550-675 nm (Figure 2.3). Interestingly, both species have eggs that reflect in the UV part of the spectrum as well as the blue-green and red regions of the spectrum, resulting in their characteristic "brown" color (to the human eye). Egg reflectance values were visualized by mother for *M. benetos* (Figure 2.2A) and *A. burtoni* (Figure 2.2B), and between species (Figure 2.2C).

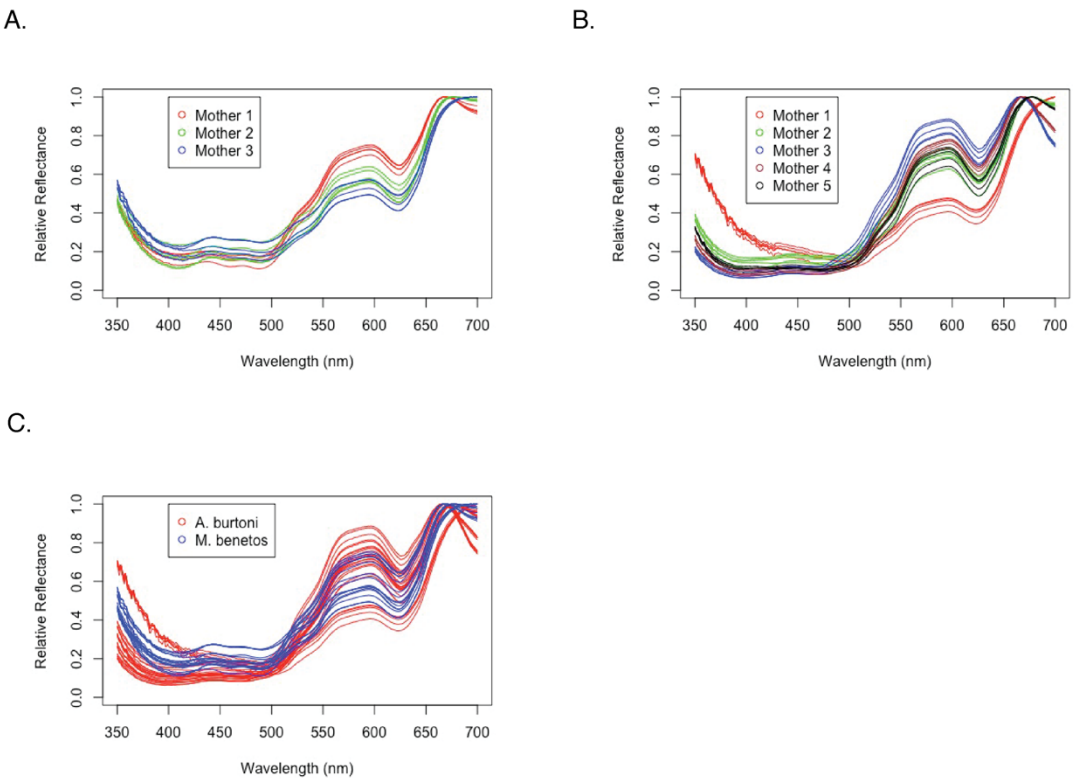
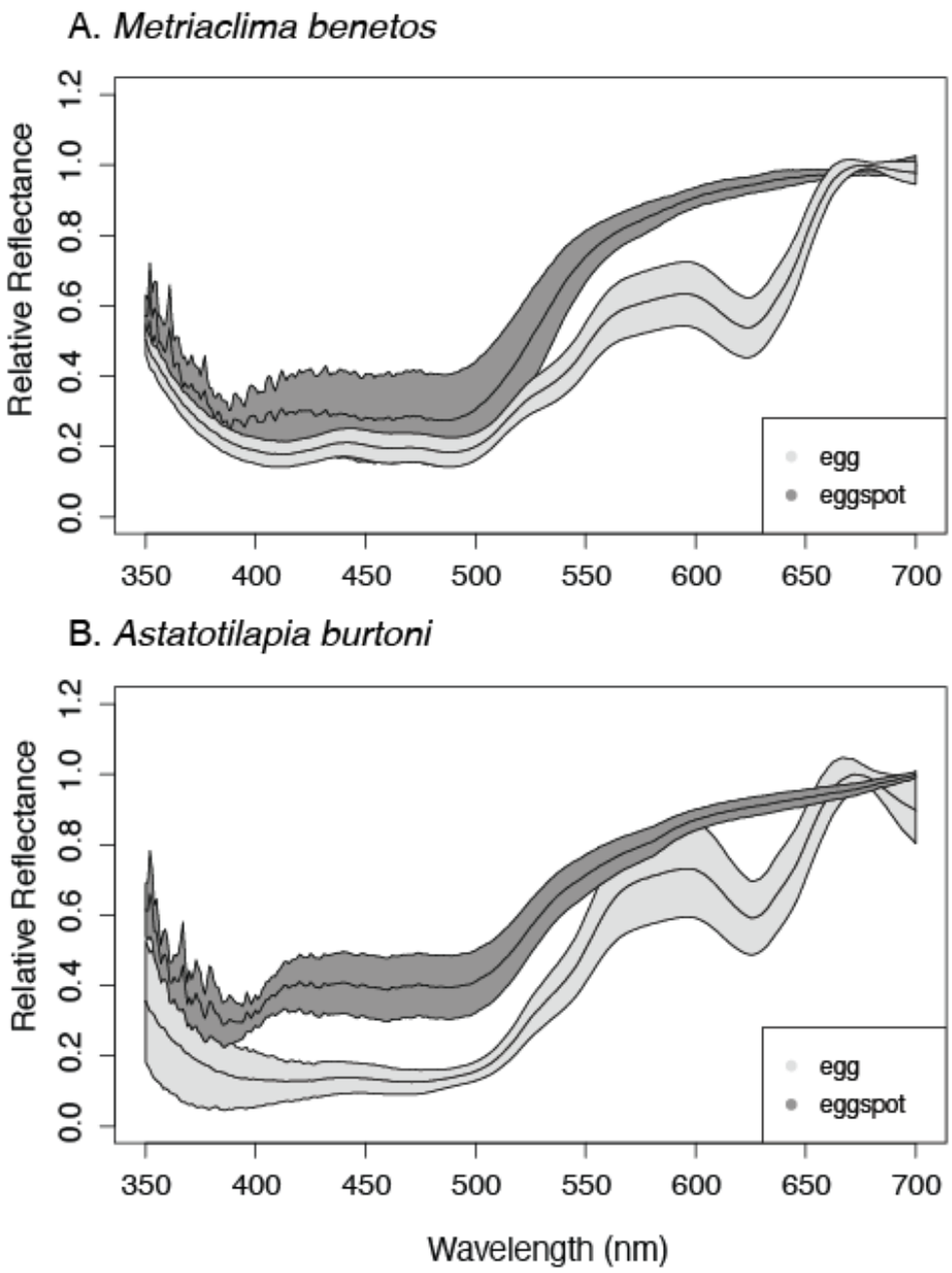


Figure 2.2: Relative reflectance of *M. benetos* and *A. burtoni* eggs. (A) Relative reflectance of each the *M. benetos* eggs by mother (5 eggs were taken randomly from each mother). (B) This figure shows the relative reflectance of each *A. burtoni* egg by mother (5 eggs were taken randomly from each mother). (C) Relative reflectance for all of the eggs measured in this study by species (15 *M. benetos* eggs and 25 *A. burtoni* eggs).

Figure 2.3: Relative reflectance of the average eggs and average egg spots for *M. benetos* and *A. burtoni.* (A) The relative reflectance of the average *M. benetos* egg spot as compared to the average *M. benetos* egg in solid lines, plus or minus one standard deviation in solid grey. Although the two stimuli have parallel reflectance profiles, there are differences in the relative magnitudes, particularly at wavelengths above 500 nm. (B). The relative reflectance of the average *A. burtoni* egg spot as compared to average *A. burtoni* egg in solid lines, plus or minus one standard deviation in solid grey. The stimuli differ between 350-550 nm as well as in the 600-700 nm range.


A. Metriaclima benetos
Relative Reflectance
0.0
0.2
0.4
0.6
0.8
1.0
1.2
350
400
450
500
550
600
650
700
egg
eggspot
B. Astatotilapia burtoni
Relative Reflectance
0.0
0.2
0.4
0.6
0.8
1.0
1.2
350
400
450
500
550
600
650
700
egg
eggspot
Wavelength (nm)

We measured and compared the spectra of 34 egg spots (19 for *M. benetos* and 15 for *A. burtoni*) from 12 fish (7 *M. benetos* males and 5 *A. burtoni* males; Figure 2.4). The average egg spot reflectance (Figure 2.3) demonstrates that both species have virtually identical average reflectance in 350-400 nm (the UV range) although the average M. benetos egg spot is more saturated with lower relative reflectance between 400-525 nm and higher relative reflectance between 525-700 nm. Egg spot reflectances were visualized by species (Figure 2.4). When plotted in their respective color spaces, the spectra of the individual eggs and egg spots stimulate the photoreceptors similarly (Figure 2.5).

Figure 2.4: Relative reflectance for all egg spots by species. This figure depicts the 19 *M. benetos* egg spots and 15 *A. burtoni* egg spots measured in this study by species.

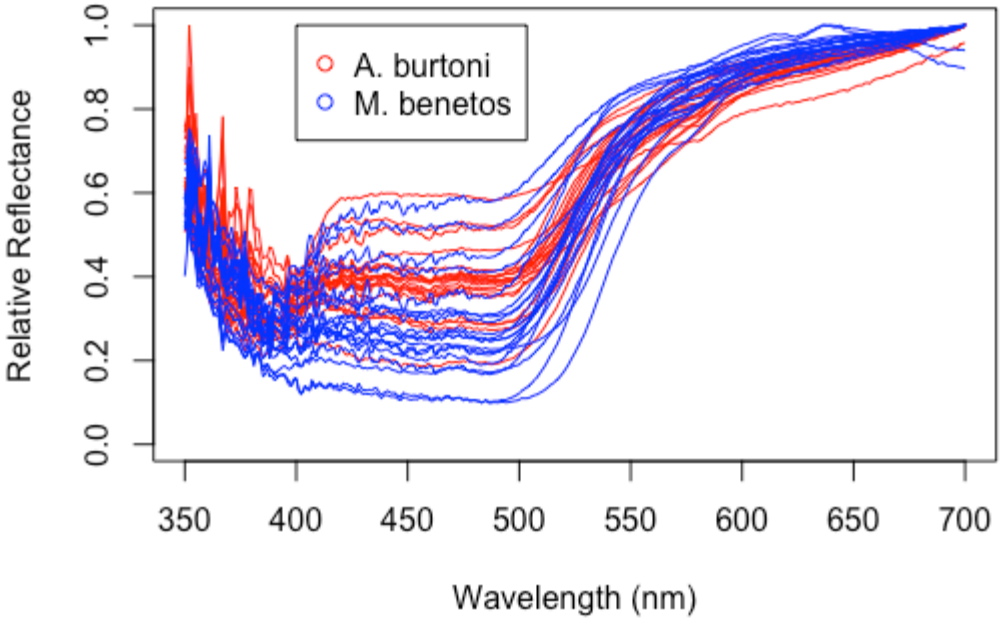
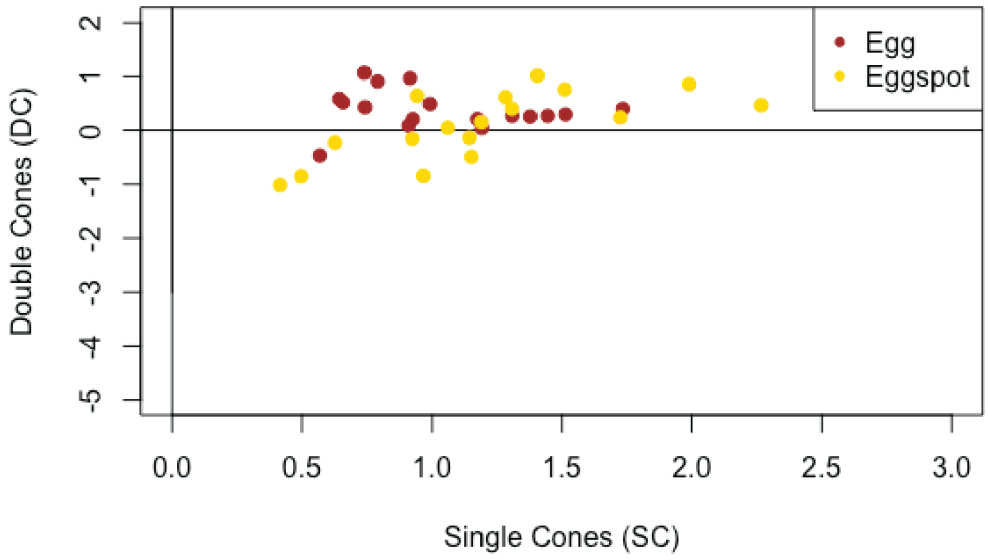
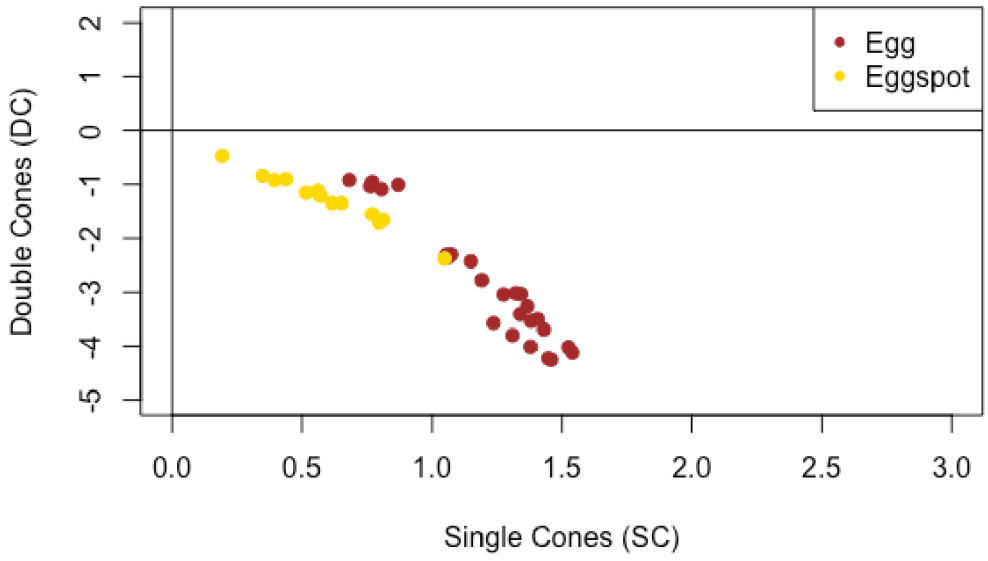

A. burtoni
M. benetos
Relative Reflectance
0.0
0.2
0.4
0.6
0.8
1.0
350
400
450
500
550
600
650
700
Wavelength (nm)

Figure 2.5: Egg and egg spot colors plotted in color space for *M. benetos* and *A. burtoni*. (A) Representation of how the colors of the *M. benetos* eggs and egg spots stimulate the photoreceptors under laboratory light, plotted in color space with the signals of the single cones on the x-axis and the summed signals in double cones on the y-axis. Pure opsin expression is assumed (no co-expression). Although there is more variation in egg spots than eggs, the two groups of stimuli show overlapping signals in the photoreceptors. (B) Representation of how the colors of the *A. burtoni* eggs and egg spots stimulate the photoreceptors under laboratory light, plotted in color space with the signals of the single cones on the x-axis and the summed signals in double cones on the y-axis. Pure opsin expression is assumed (no co-expression).

A.


Egg
Eggspot
Double Cones (DC)
2
1
0
-1
-2
-3
-4
-5
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Single Cones (SC)

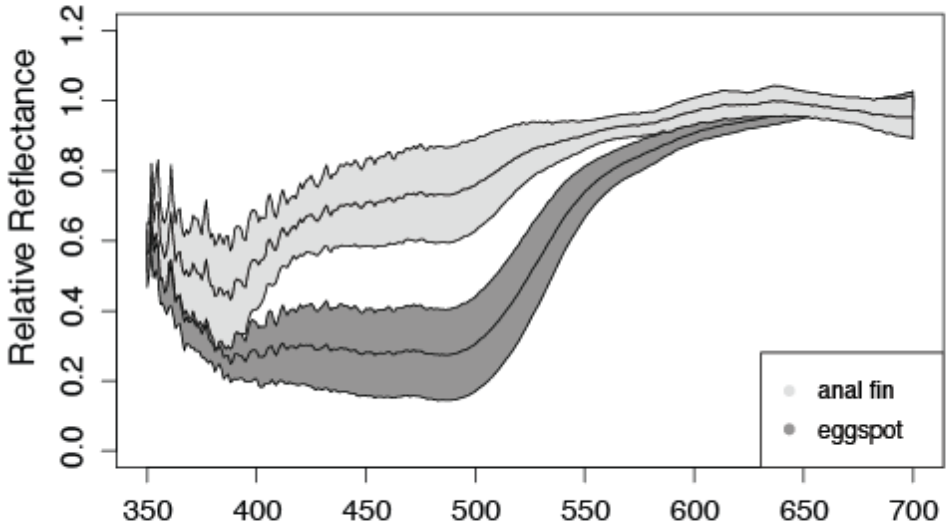
B.


Egg
Eggspot
Double Cones (DC)
2
1
0
-1
-2
-3
-4
-5
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Single Cones (SC)

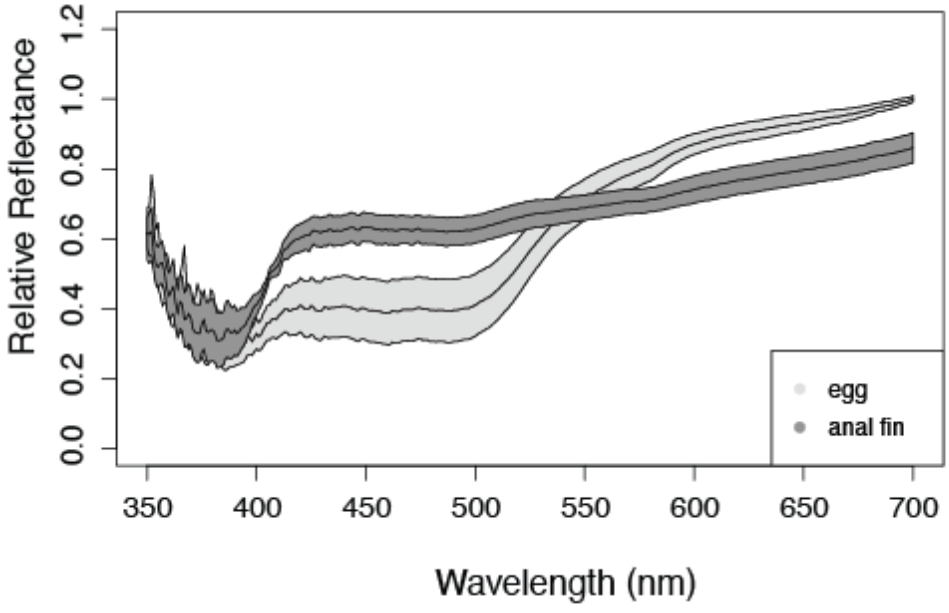
We measured and compared the spectra of 6 anal fin tissue measurements from 5 male *M. benetos*. A consistent pattern emerges for these reflectances: elevated reflectance around 350 nm declines to a minimum around 400 nm before steadily increasing across 500-700 nm (the visible light spectrum; Figure 2.6). We measured and compared the spectra of 5 anal fin tissue measurements from 5 male A. burtoni. Although the anal fins of *A. burtoni* appear somewhat transparent, the reflectance pattern of the anal fins is very similar to that of M. benetos (Figure 2.7). However, *A. burtoni* anal fin reflectance is less saturated than those of *M. benetos* (Figure 2.7). The relative reflectance spectra of both average and individual anal fins are noisy in the UV wavelengths of light (350-400 nm) due to low absolute flashlight illuminance.

Figure 2.6: Relative reflectance of the average eggs and average anal fins for *M. benetos* and *A. burtoni* (A) Relative reflectance of the average *M. benetos* egg and the average anal fin in solid lines, plus or minus one standard deviation in solid grey. The reflectances are similar at shorter wavelengths (350-400 nm) though differ particularly in middle wavelengths (400-550 nm). **(**B) Relative reflectance of the average *A. burtoni* egg spot and the average anal fin in solid lines, plus or minus one standard deviation in solid grey. Similar to *M. benetos*, the two stimuli differ in brightness between 400-550 nm.

A. *Metriaclima benetos*



B. *Astatotilapia burtoni*



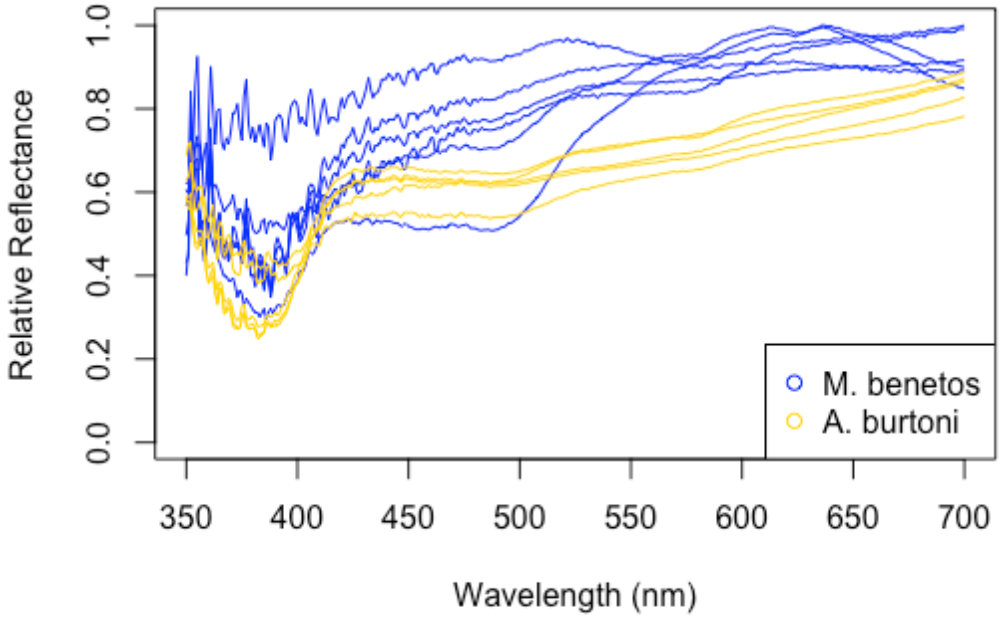


Figure 2.7: Relative reflectance for all anal fin spectra by species. Relative reflectance for all the anal fins measured in this study by species (6 *M. benetos* anal fins and 5 *A. burtoni* anal fins). Low absolute values make these measurements noisier than eggs or egg spots. Additionally, *M. benetos* have anal fins with higher brightness at wavelengths higher than 400 nm.

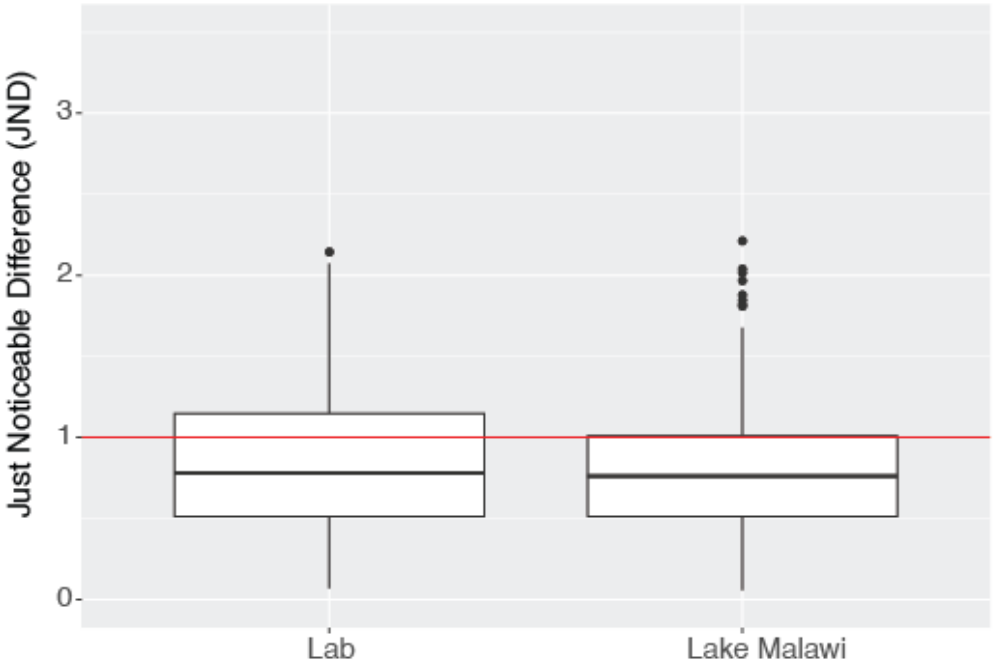
2.4.2 *Metriaclima benetos*

Though the egg and egg spot spectra differ (Figure 2.3), we wondered whether these differences are detectable by the fish. We measured color discrimination for all possible pairwise combinations of eggs (15 eggs from 3 female fish) and egg spots (19 egg spots from 7 male fish), or 285 combinations. These were calculated for each

of the illuminants to compare color discrimination in the laboratory versus in the wild. When calculating the chromatic distance using the noise constant calculated specifically from *M. benetos* visual behavior (Escobar-Camacho et al., 2019), the median chromatic distance is below 1 in both illuminant conditions (0.780 in the laboratory and 0.762 in Lake Malawi; Figure 2.8A; Table 2.1) indicating that *M. benetos* cannot distinguish between the colors of the two stimuli. The analysis with a noise value for the brown area of color space, 0.1165 (Cheney et al., 2019) gave similar results, though the median chromatic distance is just barely greater than 1 (1.07 under laboratory light and 1.05 under Lake Malawi light; Figure 2.8B; Table 2.1), indicating that the fish could just barely distinguish between the colors of egg spots and eggs at close distances such as a few centimeters. These calculations are a best-case scenario as they assume essentially zero viewing distances and do not include water turbidity.

Figure 2.8: The chromatic distance (ΔS) between eggs and egg spots for *M. benetos*. Chromatic distance was calculated with (A) a Weber fraction of 0.16 (Escobar-Camacho et al. 2019) specific to the cichlid visual system for red - blue color comparisons and (B) a Weber fraction of 0.1165 (Cheney et al. 2019) specific to the brown colors for the triggerfish visual system. Both calculations include two illuminants: laboratory fluorescent lights and at side welling irradiance in Lake Malawi, taken at 3 meters depth at Thumbi West Island (Sabbah *et al.* 2011), a clear water site at Lake Malawi comparable to the fish's habitat at Mazinzi Reef. (A) The median chromatic distance for each is less than 1, indicating that the fish cannot distinguish between the color of the two stimuli (eggs and egg spots) in the laboratory or in the wild, whereas in (B) the median chromatic distance is just above 1 in laboratory conditions and the wild. This indicates that the fish may be able to distinguish between the two colors at very close viewing distances.

A. *Metriaclima benetos*, weber fraction = 0.16



B. *Metriaclima benetos*, weber fraction = 0.1165

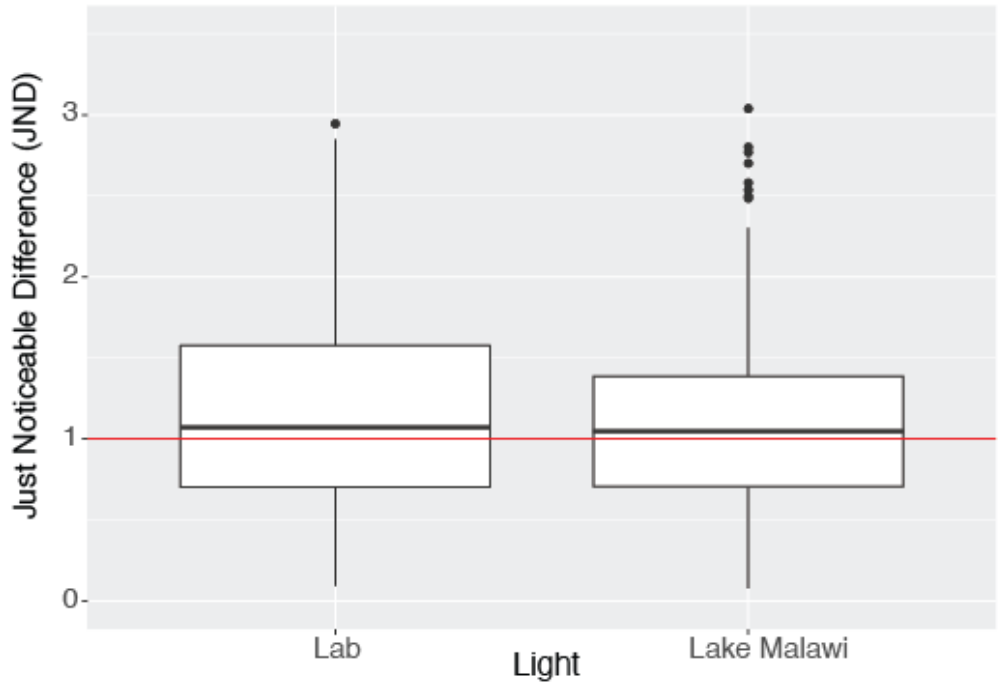


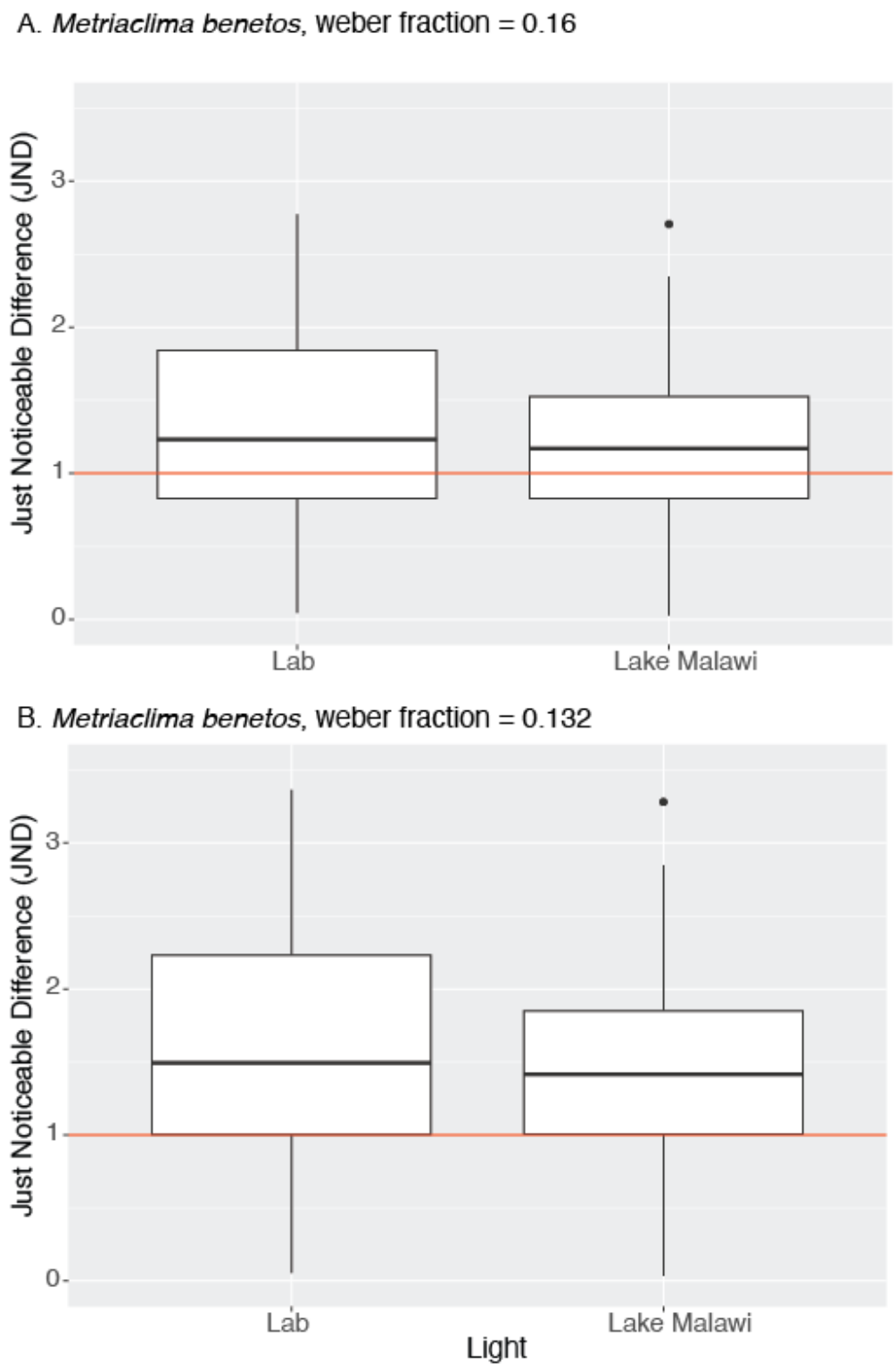
Table 2.1. Median chromatic distance for each instance of visual modeling conditions for *M. benetos*. The median chromatic distance between the stimulus colors of *M. benetos* under both illuminants using the relevant noise constants. NA not applicable.

		Eggs and Egg Spots		Anal Fins and Egg Spots	
		Illuminant		Illuminant	
		Laboratory	Lake Malawi	Laboratory	Lake Malawi
Noise Constant	0.16	0.780	0.762	1.23	1.17
	0.1165	1.07	1.05	NA	NA
	0.132	NA	NA	1.49	1.42

Chromatic distances for the anal fin versus egg spots were also examined. We measured color discrimination for all possible pairwise combinations of egg spots (19 egg spots from 7 male fish) and anal fins (6 anal fin measurements from 7 male fish). This yielded 114 combinations, which were calculated for each of the illuminants. When analyzed with the *M. benetos* noise constant of 0.16 (Escobar-Camacho et al., 2019), the median chromatic distance between the color of egg spots and the color of the male anal fin tissue (which appears translucent blue to the human eye) is above 1, with a median of 1.23 in the laboratory and 1.17 in Lake Malawi (Figure 2.9A; Table 2.1). When analyzed with the blue noise constant (0.132, Cheney et al., 2019), the median chromatic distance is above 1, with a median of 1.49 in the laboratory and 1.42 in Lake Malawi (Figure 2.9B; Table 2.1), indicating that M. benetos can distinguish between the two stimuli in both the laboratory and the clear waters of Lake Malawi at close distances.

Figure 2.9: The chromatic distance (ΔS) between anal fins and egg spots for *M. benetos*. Chromatic distance was calculated with (A) a Weber fraction of 0.16 (Escobar-Camacho et al. 2019) specific to the cichlid visual system for red - blue color comparisons and (B) a Weber fraction of 0.132 (Cheney et al. 2019) specific to the blue colors for the triggerfish visual system. Both calculations include two illuminants: the fluorescent light of the laboratory and at 3 meters below the surface at Thumbi West Island (Sabbah *et al.* 2011), a clear water site at Lake Malawi comparable to the fish's habitat at Mazinzi Reef. The median chromatic distance (regardless of the Weber fraction used) are higher than 1, indicating that the fish can

distinguish between the color of the two stimuli (anal fin and egg spots) under both laboratory conditions and in the wild.

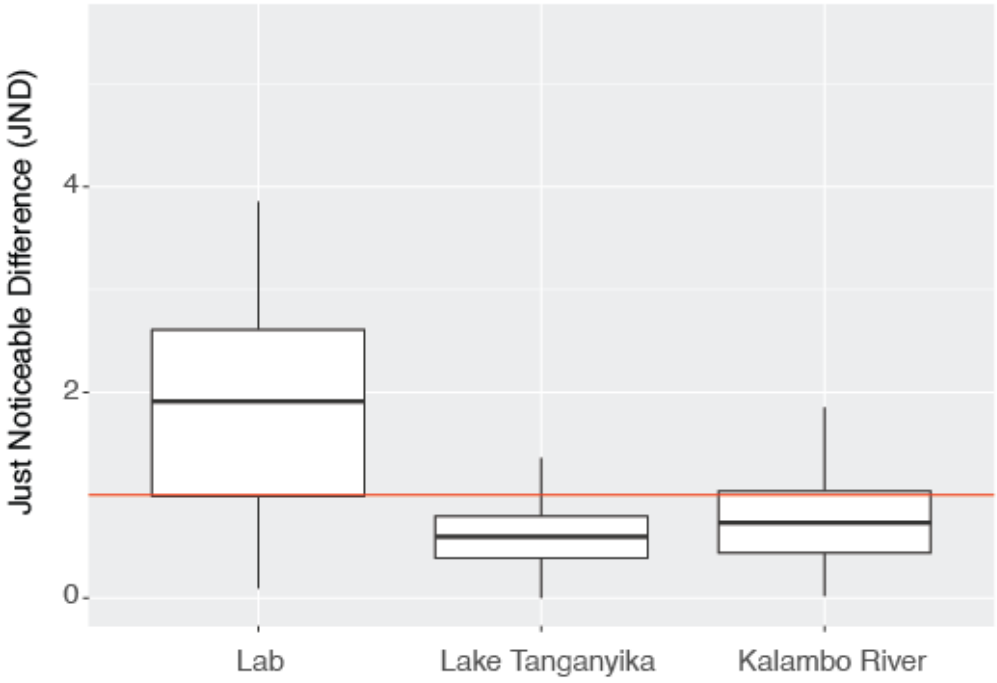


2.4.3 *Astatotilapia burtoni*

It was clear that there are differences in the spectra of *A. burtoni* eggs and egg spots (Figure 2.3), but we again measured whether or not these differences are discernable to the fish. We measured color discrimination for all possible pairwise combinations of eggs (25 eggs from 5 female fish) and egg spots (20 egg spots from 5 male fish). This yielded 500 combinations, which were calculated for each of the three illuminants: the laboratory, Lake Tanganyika, and Kalambo River. When calculating discrimination using the *Metriaclima benetos*-specific noise constant (0.16; Escobar-Camacho et al., 2019), the median chromatic distance is 1.92 under laboratory lighting (Figure 2.1A; Table 2.2). The chromatic distance in the natural habitats is much lower with medians of 0.599 (Lake Tanganyika; Table 2.2) and 0.732 (Kalambo River; Table 2.2). When calculated using the brown noise constant (0.1165) from Cheney et al. (2019), the median chromatic distance is at or below 1 in the native habitats with median chromatic distances of 0.822 (Lake Tanganyika; Table 2.2) and 1.01 (Kalambo River; Table 2.2). The median chromatic distance under laboratory lighting is 2.63 (Figure 2.10B; Table 2.2). Comparing the distribution of chromatic distances in natural habitat illuminants to those in laboratory lighting, it becomes clear that *A. burtoni* are more consistently able to distinguish eggs from egg spots in the fluorescent light of the laboratory than in the wild.

Figure 2.10: The chromatic distance (ΔS) between eggs and egg spots for *Astatotilapia burtoni*. Chromatic distance was calculated with (A) a Weber fraction of 0.16 (Escobar-Camacho et al. 2019) specific to the cichlid visual system for red - blue color comparisons and (B) a Weber fraction of 0.1165 (Cheney et al. 2019) specific to the brown colors for the triggerfish visual system. Both calculations include three illuminants: the fluorescent light of the laboratory and the sidewelling irradiance of Lake Tanganyika and Kalambo River. The median chromatic distance (regardless of the Weber fraction used) for natural habitat illuminants are below 1, indicating that the fish cannot distinguish between the color of eggs and egg spots in the wild, though they may be able to distinguish between the two stimuli at short viewing distances in the laboratory.

A. *Astatotilapia burtoni*, weber fraction = 0.16



B. *Astatotilapia burtoni*, weber fraction = 0.1165

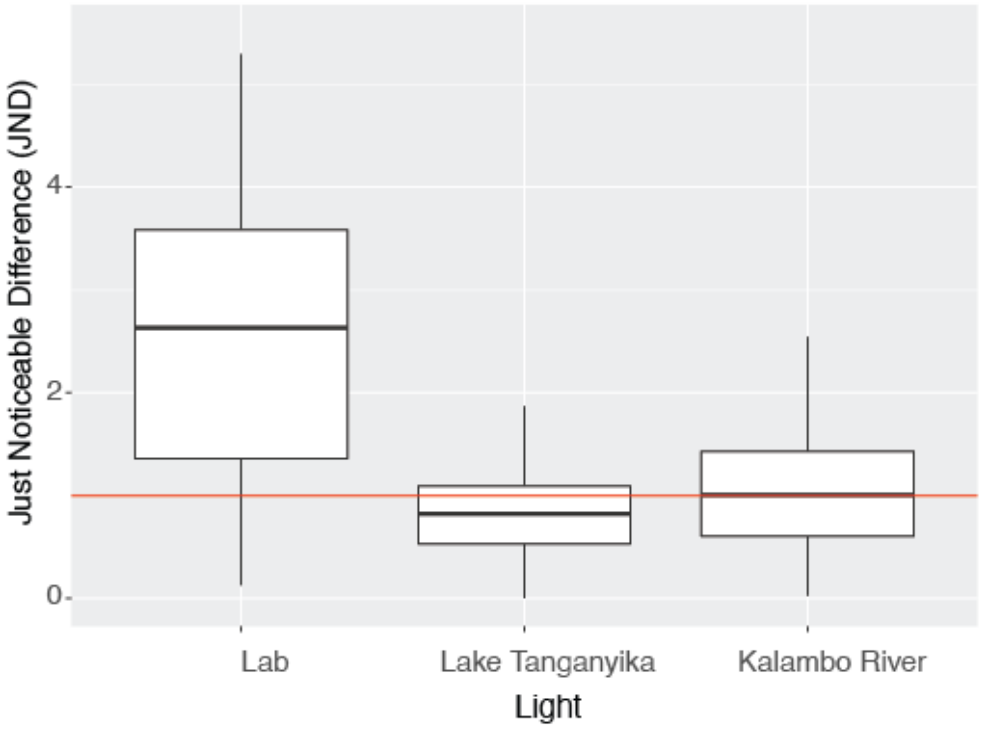


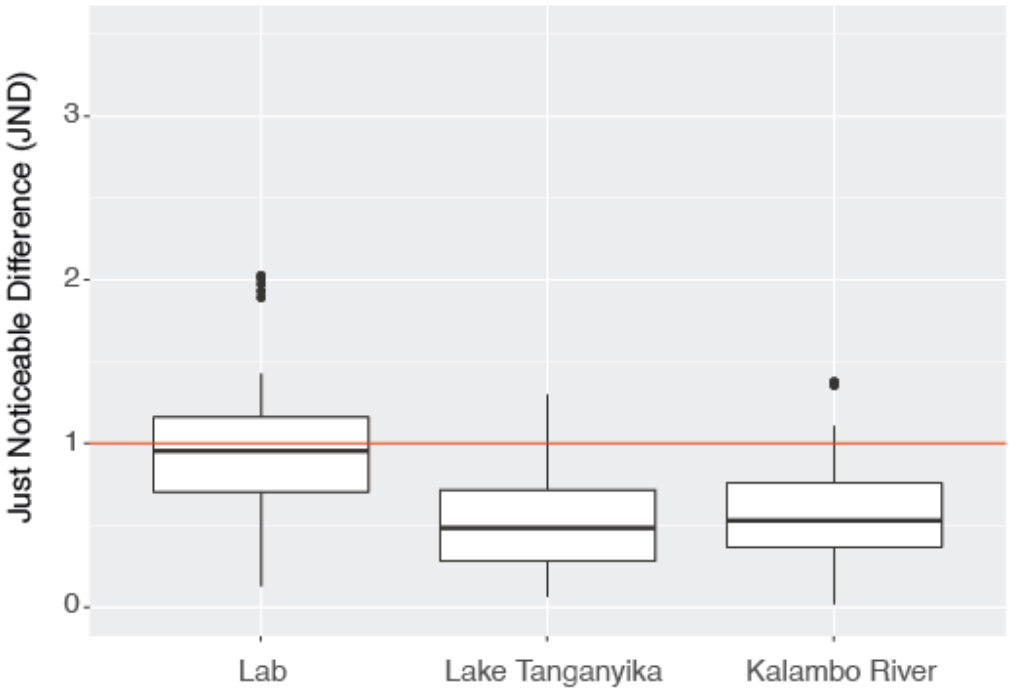
Table 2.2. Median chromatic distance for each instance of visual modeling conditions for *A. burtoni.* The median chromatic distance between the stimulus colors of *A. burtoni* under the three illuminants with the relevant noise constants.

		Egg and Egg Spot			Anal Fin and Egg Spot		
		Lab	Lake Tanganyika	Kalambo River	Lab	Lake Tanganyika	Kalambo River
Noise Constant	0.16	1.92	0.599	0.732	0.956	0.484	0.528
	0.1165	2.63	0.822	1.01	1.31	0.665	0.726

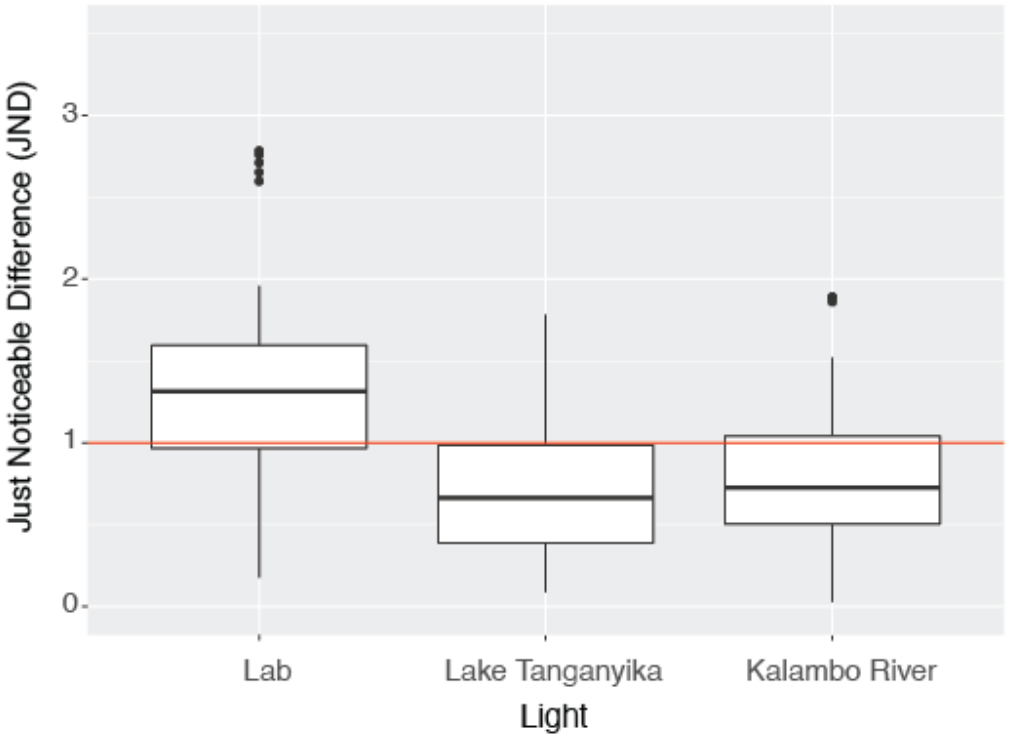
Chromatic distances for the *A. burtoni* anal fin versus egg spots were also examined. We measured color discrimination for all possible pairwise combinations of egg spots (20 egg spots from 5 male fish) and anal fins (5 anal fin measurements from 5 male fish). This yielded 100 combinations, which were calculated for each of the illuminants. When analyzed with the *M. benetos* noise constant of 0.16 (Escobar-Camacho et al., 2019), the median chromatic distance between the color of egg spots and the color of the male anal fin tissue is below 1, with a median of 0.956 in the laboratory, 0.484 at Lake Tanganyika, and 0.528 in Kalambo River (Figure 2.11A; Table 2.2). When analyzed with the brown noise constant (0.1165, Cheney et al., 2019), the median chromatic distance is slightly elevated, with a median of 1.31 in the laboratory, 0.665 at Lake Tanganyika, and 0.726 in Kalambo River (Figure 2.11B; Table 2.2), indicating that *A. burtoni* cannot distinguish between the two stimuli in their natural habitats but may be able to at close viewing distances in the laboratory.

Figure 2.11: The chromatic distance (ΔS) between anal fins and egg spots for *Astatotilapia burtoni*. Chromatic distance was calculated with (A) a Weber fraction of 0.16 (Escobar-Camacho et al. 2019) specific to the cichlid visual system for red - blue color comparisons and (B) a Weber fraction of 0.1165 (Cheney et al. 2019) specific to the brown colors for the triggerfish visual system. Both calculations include three illuminants: the fluorescent light of the laboratory and the sidewelling irradiances of Lake Tanganyika and Kalambo River. The median chromatic distance (regardless of the Weber fraction used) for natural habitat illuminants are below 1, indicating that the fish cannot distinguish between the color of the two stimuli (anal fins and egg spots) in the wild, though they may be able to distinguish between the two stimuli at very close viewing distances in the laboratory.

A. *Astatotilapia burtoni*, weber fraction = 0.16



B. *Astatotilapia burtoni*, weber fraction = 0.1165



2.5 Discussion

In this study we quantified the reflectance of the egg spots and eggs of laboratory-reared *M. benetos* and *A. burtoni* (Figure 2.3). The results of the visual model indicate that both *M. benetos* and *A. burtoni* under the lighting conditions of their natural habitat either cannot distinguish between the color of the egg spots and the eggs or can just barely discriminate them (Figures 2.8 and 2.10). Additionally, the reflectance of the *M. benetos* and *A. burtoni* anal fins were quantified (Figure 2.6). The visual model indicates that *M. benetos* can distinguish the color of the egg spot and the anal fin at close viewing distances both in the laboratory and the wild (Figure 2.9), whereas *A. burtoni* are unable to distinguish between the egg spots and anal fins in the wild, and only in the laboratory for the lower estimated noise value (Figure 2.11). While some of these results may be surprising, it agrees with previous behavioral studies of *A. burtoni* as we discuss below.

2.5.1 Color discrimination

The visual model used here is relatively simple and assumes that fish are observing eggs or egg spots at close range, i.e. zero viewing distance. For terrestrial organism that view objects through air, viewing distance has little impact. However, underwater viewing is complicated by the significant scattering of light (Lythgoe, 1988; McFarland et al., 1979). As viewing distance increases, the amount of background light scattering into view causes the light reflecting from two objects to become more and more similar. This degrades color discrimination quite rapidly (Wilkins et al., 2016). Egg and egg spot viewing distances can't be too large as cichlids have only

modest visual acuity (Dalton et al., 2017). A 3 mm diameter egg spot cannot be resolved at distances much beyond 1.5 m (Dalton et al., 2017). However, even modest viewing distances of 0.5 to 1 m, particularly in water with any turbidity, will rapidly degrade cichlid's color discrimination ability.

2.5.2 Wickler's Hypothesis

Wickler's egg mimicry hypothesis was the first to consider egg spots as an evolutionarily important phenotype and consists of two essential tenets. The first tenet asserts that egg spots are an accurate mimic of eggs. In fact, Wickler's hypothesis is the very reason we call the phenotype "egg spots." In this study, we accounted for the cichlid visual system while assessing the validity of this proposed mimicry. Our results are consistent with the idea that the color of the egg spot mimics the color of the egg under natural habitat illuminance for cichlid color vision. This is important because it lends support to a long-held assumption about an important phenotype in the widely-studied haplochromine cichlids. Some of these results show that the fish can distinguish between these colors at close viewing distances. In particular, the visual model showed that *A. burtoni* are able to distinguish between eggs and egg spots under fluorescent laboratory light. Astatotilapia burtoni are studied in many laboratories in the context of sexual selection and reproductive behavior. This study suggests that these fish can distinguish between the colors of the egg and the egg spot in the laboratory lighting, so perhaps behavioral studies which depend on visual behavior should attempt to replicate the illuminant of *A. burtoni*'s natural habitat. Although the statistical analysis of the chromatic distance reveals significant variation

within and between individuals of both species, it is important to remember that the JNDs produced by the visual model remain very small. In fact, recent studies suggest that a threshold of JND > 3 is a more conservative threshold for determining when an animal can distinguish two colors (Gómez et al., 2018; Maia & White, 2018), a metric which further solidifies the current interpretation of our results. Visual models are a valuable method because they quantify vision in a way that removes human bias; however, it is important to note that a model itself is a series of approximated calculations and cannot predict the behavior of a fish, which is affected by additional factors beyond those incorporated into the model. For example, although the model captures information about visual sensitivities, light, and color, it fails to consider a fish's individual differences in visual sensitivity from environmental plasticity, retinal wiring to specific color opponency, and the effect vision has on the fish's actual behavior. Another limitation of the model is that no light measurement can perfectly represent the variation that exists in underwater light conditions at these habitats. Additionally, it has been shown that the cichlid egg spot and nuptial coloration may exhibit environmental plasticity with regards to diet and light environment during development (Theis et al., 2017; Wright et al., 2018; Sefc et al., 2014). Although the development of this phenotype is not well-studied, it is worth noting that this study best represents well-fed fish who developed under the fluorescent lights of the laboratory. As such, there may be differences between the reflectance of these fish's egg spots and those of wild fish. With the limitations of this study in mind, these results (namely, the inability for the fish to distinguish between the color of the egg and the color of the egg spot) are likely applicable to other haplochromines with

similar visual systems and in similar habitats. However, in order to gain a more nuanced understanding of how this egg-egg spot mimicry may influence cichlid reproductive behavior, further studies comparing reproductive behavior or visual assays under both fluorescent lighting and simulated natural lighting with both laboratory and wild fish are necessary. Such studies could be especially valuable when designed not only to examine the egg spot and its role in the stages of reproductive behavior itself, but also to probe deeper into understanding the function of the egg spot in the broader context of the evolution of haplochromines.

The second proposition of Wickler's hypothesis speaks to the function of the egg spot. This second tenet posits that egg spots increase the male fertilization rate by exploiting female mouthbrooding behavior. In fact, the literature has consistently refuted this idea: fertilization rates do not differ between males with egg spots and males without egg spots (Hert, 1989; Hert, 1991; Theis et al., 2012). It is worth noting that two of these studies used *A. burtoni* and the closely related *Astatotilapia elegans* (Trewavas, 1933 as cited in Daget et al., 1984) for their laboratory experiments, which this study demonstrated may be able to distinguish egg and egg spot colors under laboratory lighting that they typically cannot in the wild.

The lack of a role for egg spots in enhancing fertilization leads us to any number of alternatives to Wickler's hypothesis. A potential alternative is that egg spots still perform an important role in haplochromine sexual selection, whether by interspecies differences in egg spot preferences or intraspecies preferences (in which body color and other phenotypes must play the important role of conspecific identification). Even if the egg spots have no particular effect on male fertilization, they may still have an

effect in male-male competition as an ornament or in female mate choice. Additionally, sensory exploitation may have shaped the evolution of the egg spot phenotype in the evolutionary past (Tobler, 2006; Egger et al., 2011). Of course, it is also possible that egg spots were an important phenotype while the haplochromines were undergoing explosive speciation, but have since lost their function (but not their form). If this were the case, the loss of function must be a recent enough change that the form of the now-vestigial trait has not yet degraded. Although females still peck at male egg spots, both in the laboratory and the wild, there has been no consistent evidence that indicates whether egg spots still play a functional role in sexual selection today. Indeed, none of these alternative hypotheses are mutually exclusive and in fact multiple interactions may play a role in egg spot function (if it still exists). Behavioral and molecular experiments to examine these hypotheses within the haplochromines and their related groups have the potential to shed light on such possibilities and examine the importance of this phenotype in the cichlid explosive radiation.

Although the function of egg spots in haplochromines still is not clear, this study does provide a basis for previous results. Notably, our data suggests that *A. burtoni* are unable to distinguish between the color of the anal fin and the color of the egg spot in the lighting of their natural habitat. This does not necessarily mean the fish cannot see the egg spot at all, simply that they cannot distinguish these two colors. The egg spots of adult haplochromines are surrounded by a ring of translucent tissue which may increase the spot's conspicuousness or create the illusion of an "egg" (the egg spots) against a similarly colored background (the anal fin). However, it is also worth noting

that *A. burtoni*'s inability to discriminate between these colors may explain why the phenotype does not appear to play a role in mate choice as demonstrated by Theis et al. (2015). *Astatotilapia burtoni* anal fins are more red-yellow in color while the Metriaclima species from Lake Malawi have a blue-white anal fin coloration. The function of egg spots in *M. benetos* has not yet been studied, though three related Lake Malawi Metriaclima species have been shown to have preferences for certain egg spot phenotypes. If these species can distinguish from the anal fins and egg spots of their species as their close relative *M. benetos* can, then it may support the hypothesis that egg spots are an important trait for mate choice within the Lake Malawi Metriaclima, as opposed to the egg spot's seeming unimportance in *A. burtoni*. This is especially interesting given the differences in water clarity between Lake Malawi, home to the Metriaclima, and the more turbid habitats of *A. burtoni*. Further study will show how sexually selected signals evolve differently in aquatic habitats with high and low visibility.

2.6 Conclusion

In this study, we have quantified the colors of the anal fin egg spots and eggs of two different laboratory reared haplochromine cichlids: *M. benetos* and *A. burtoni*. Further, we have used the RNL model in order to determine that these species are unable to distinguish the colors of their eggs and egg spots in the lighting of their natural habitat, though *A. burtoni* are likely able to distinguish between the colors under certain laboratory conditions. Though these two species have different visual sensitivities and environmental conditions, their similar results support long-standing

hypotheses of mimicry and sensory exploitation which led to these anal fin spots being known as “egg spots” in the first place.

Acknowledgements

We would like to thank Molly Westbrook and Kristen Behrens for their assistance with data collection and Daniel Escobar-Camacho for the use of his scripts and recommendations for the visual model. Underwater light spectra were collected with the help of Walter Salzburger, Tod Thiele, Venkatesh Subramanian, Nicholas Guilbeault, and Thomas Musisha. This work was supported by the National Eye Institute at the National Institutes of Health (R01EY024639B; KC and SJ) and the Human Frontiers in Science Program grant (RGY0079/2018; SJ).

Compliance with Ethical Standards

This work has been completed under the approval of the University of Maryland’s Institutional Animal Care and Use Committee (R-JUN-21-44).

Chapter 3: Cichlid species does not respond differentially to virtual stimuli in a behavioral assay

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3.1 Abstract

Cichlid fish have undergone explosive speciation resulting in a great deal of phenotypic variation that act as visual signals affecting inter- and intraspecific communication. However, phenotypes like egg spots and size are difficult to study because they are often confounded with one another and other variables such as age. Behavioral research has shown that using virtual stimuli is a robust methodology for such experiments. In this study, we used a receptor noise limited (RNL) visual model to develop visually salient virtual stimuli. Male *Metriaclima zebra* individuals were tested using virtual stimuli in a series of one- and two-way assays testing the effect of various phenotypes (egg spot presence, body color, movement, and size) on male aggression. We found that *M. zebra* responds to virtual stimuli with equal aggression and association as it does towards live fish. However, the test males respond with equal aggression and association towards virtual stimuli that differ in egg spot presence, body color, movement, and size. Therefore, *M. zebra* do not distinguish virtual stimuli making this approach uninformative. Although it is not yet clear why some fish are a better fit for this methodology than others, baseline aggression and territoriality are compelling variables to examine.

3.2 Introduction

Cichlid fishes of the African Rift Valley are a diverse group with wide variation in color patterns, ecology, and behavior (Ribbink et al., 1983; Seehausen, 1996; Barlow, 2008). This variation and their explosive radiation make them a useful model for studying evolution (Kocher, 2004), in particular the forces of natural selection and sexual selection driving speciation (Holzberg, 1978; Ribbink et al., 1983; Dominey, 1984; Albertson et al., 2005; Seehausen, 2006). Adult cichlids are often sexually dimorphic, wherein male cichlids are larger and more brightly colored than female cichlids (Ribbink et al., 1983; Seehausen et al., 1999). In the polyandrous species, the cryptically colored females swim above male territories and are courted by the colorful males who compete for territories and mating opportunities (Holzberg, 1978; Rogers & Barlow, 1991; Parker & Kornfield, 1996). Female cichlids are mouthbrooders who pick up their eggs in their mouths, which they carry until the fry can swim for themselves (Fryer & Iles, 1972) while the male cichlids simply provide the territories on which the pair mates and fertilize the eggs (Ribbinck et al., 1983). The difference in mating strategies between the sexes creates sexual conflict and results in sexual selection on dimorphic traits such as the bright colors, distinctive patterns, and body size of the males (Seehausen & van Alphen, 1998; Allender et al., 2003). These important visual signals have also been shown to maintain species coexistence (Seehausen & Schluter, 2004) and strongly influence male-male aggression (Dijstra et al., 2005). In fact, visual signals have been demonstrated repeatedly to be highly salient to cichlids when making social, aggressive, and

reproductive decisions (Maruska et al., 2012; Maruska & Sisneros, 2015; Chabrollese et al., 2017).

Specific visual signals such as size, behavioral vigor, and color have been demonstrated to be very important in male competition. Body size is a strong visual signal across numerous animal groups linked to dominance, reproductive success, and fighting acumen (Davies and Halliday, 1978; Wazlavek & Figler, 1989; Andersson, 1994; Arnott and Elwood, 2009) including African cichlids (Dijkstra et al., 2005). Even among other sympatric fish species of Lake Malawi, size is one of the most critical determiners a male uses to decide when to attack a potential rival (Pauers & Grudnowski, 2020). Further, in our study species *Metriaclima zebra* (Boulenger, 1899 as cited in Stauffer et al., 1997) body size is a critical determinant of fighting outcomes (Simões et al., 2008; Mellor et al., 2012). Behavioral vigor refers to the frequency with which a fish performs behaviors meant to indicate reproductive interest or aggression towards other animals. Although size and behavioral vigor are likely correlated, the exact relationship between them has not been documented. However, we do know that some cichlids who perform more aggressive behavior are more likely to win fights against size-matched contestants (Wazlavek & Figler, 1989). In several cichlid species, males attack conspecific colored fish more often than heterospecific colored fish (Dijkstra et al., 2006; 2007; Pauers et al., 2008). In fact, early male-male competition experiments on cichlids suggested that differently colored *M. zebra* exhibited assortative aggression towards conspecific-colored rivals over heterospecific-colored rivals (Holzberg, 1978; Wazlavek & Figler, 1989; Stauffer et al., 1997).

Although size, behavioral vigor, and color have been long indicated as major factors influencing male fight initiation and outcomes, other potential visual signals have demonstrated less consistent and conclusive results in the literature. One visual signal that is notable for its potential role in male-male competition is egg spots. It is well-documented that females prefer males with more contrasting color patterns (Baube et al., 1995; Brooks & Endler, 2001; Pauers et al., 2004) and males with conspecific patterns over heterospecific patterns (Couldridge & Alexander, 2002; Knight et al., 2004; Zoppoth et al., 2013). In fact, these trends indicate that pattern recognition may be an important element of conspecific recognition in these cichlids. However, there has been little research examining whether pattern plays an important role in male competition, as it is often difficult to separate the effects of color from pattern (Endler et al., 2018). Cichlid color and pattern can change in moments from any number of stimuli, including being handled, moved to a new environment, exposed to a novel stimulus, or winning/losing a fight with another male (Hurd, 1997; Maan et al., 2001; Maan & Sefc, 2013). This makes it a more difficult trait to examine in live fish. One such understudied element of cichlid color and pattern are the egg spots of the haplochromine cichlids.

Egg spots are an area of yellow, orange, or red coloration on the anal fin of a male fish which are typically surrounded by a translucent ring (Wickler, 1963, Hert, 1989; Hert, 1991). There is a great deal of natural variation in the number, size, and appearance of egg spots both within and between species (Goldschmidt, 1991; Hert, 1989; Theis et al., 2012). Since their first description in the literature by Wickler in 1963, these spots are referred to as “egg dummies” or “egg spots,” and for good

reason: cichlid fish cannot distinguish between the color of egg spots and the color of eggs in the light conditions of their natural habitat (Gonzalez et al., 2023). This unique phenotype has even been identified as a key innovation within the haplochromine cichlids that may have contributed to their rapid speciation (Salzburger et al., 2005). Despite indications that this phenotype is important, the question of whether or not egg spots play a role as a visual signal between males and females is difficult to determine. For example, *Metriaclima aurora* (Burgess, 1976 as cited in Hert 1992) prefer males with a greater number of egg spots over those with a fewer number of egg spots (Hert, 1991). Conversely, female *Astatotilapia burtoni* (Günther, 1894 as cited in Daget et al., 1984) exhibit no preference between males with or without egg spots (Theis et al., 2012). In summary, pattern has historically been a difficult trait to separate from color and there is disagreement in the literature on whether egg spots play a role as an important visual signal to male cichlids.

In using live fish to study any of these phenotypes (size, behavioral vigor, color, and egg spots), there are unavoidable limitations in an experimenter's ability to control for the other phenotypes. When testing with live fish, it is common for a researcher to size-match the test fish and stimulus fish as best they can to avoid size differences that can affect the outcome of a study. However, live animals are somewhat unpredictable and researchers cannot control every aspect of a test fish or a stimulus fish in an assay. For example, some fish may perform more vigorous reproductive displays, change their color or pattern, or simply not participate in an assay. This is a major limitation on our ability to disentangle how much or little each of these visual signals actually matter to the cichlids we are studying.

One methodology which allows scientists to control for these sorts of confounding variables, manipulate targeted individual variables, and limit pseudoreplication is the use of virtually animated animals in video playback experiments (Baldauf et al., 2008; 2009; 2011; Chouinard-Thuly et al., 2017). This method has become increasingly widespread in animal behavior research (Rosenthal, 1999; 2000; Baldauf et al., 2008; Baldauf et al., 2009; Chouinard-Thuly et al. 2017;) and has been successful in lizards (Clark et al., 1997), spiders (Clark & Uetz, 1990; Bednarsky et al., 2012), birds (reviewed in Evans & Marler, 1995), and multiple fish species (Rowland et al., 1995; Rosenthal, 1996; Nicoletto & Kodric-Brown, 1997; Butkowski et al., 2011), including several cichlid species (Balshine-Earn & Lotem, 1998; Baldauf et al., 2011; Fischer et al., 2014). The use of such stimuli ranges from the presentation of simple manipulated images or videos (Egger et al., 2011) to virtual fish which perform relevant behaviors, whether animated or recorded (Rosenthal & Evans, 1998; Wong & Rosenthal, 2006; Fisher et al., 2009).

In summary, technology provides an opportunity to robustly study phenotypes which are typically confounded by numerous other variables when using live fish. In this study, we first use visual modeling to create simplistic virtual stimuli which are cogent to *M. zebra*, then test how cichlids respond to the virtual stimuli. We then examine the role that four previously difficult-to-study phenotypes (egg spots, color, movement, and size) have on male association and aggression, using this assay for the first time in Lake Malawi cichlids.

3.3 Materials and Methods

3.3.1 Study Species

The Lake Malawi cichlid *Metriaclima zebra* was used in this study. Specifically, these animals are laboratory stock descended from a population found at Mazinzi Reef, a habitat with especially clear water where they can be found most commonly between 5 – 25 meters of the surface (Ribbink et al., 1983). All 55 of the males utilized in this study (referred to hereafter as "test males") are the result of multiple generations of breeding in the laboratory and were fed daily with commercial food. Each test male came from a mixed stock of several different broods and was individually housed with a refuge for two months prior to and during the experiment. During this time, the aquaria were cleaned weekly to ensure that the test males had visual contact with their neighbors. This ensured that the test males would both remain territorial by fighting across the glass with their reflections and neighbors (safely without physical contact) as well as prevent social isolation in the fish. Prior to being tested, all fish were measured (total length, TL, mean ± standard deviation = 95.58 mm ± 12.31 mm). After all experiments and preliminary data analysis were concluded, test males were anesthetized, sacrificed, and sexed by dissection.

3.3.2 Visual Modeling

It was essential to create virtual stimuli that the fish can discriminate when displayed on the monitor. This is especially important when testing whether there are behavioral differences towards a single phenotype such as egg spots. In order to test this, we used the receptor noise limited (RNL) visual model to determine whether the test

males could discriminate between the relevant visual signals being displayed on the monitor (Vorobyev & Osorio, 1998; Kelber et al., 2003), such as the color of the egg spot and the color of the anal fin or the body color of the gold stimulus and the blue stimulus. The illuminance of the gold body color and blue body color of the virtual stimuli were measured in five distinct body regions on the virtual stimulus (forehead, cheek, dorsal fin, midline midbody, and caudal peduncle), then averaged.

To ensure that these relevant colors are detectable and discernable by the fish when displayed on the monitor, we calculated color discrimination by first calculating quantum catch (Q_i), the number of photons captured for each of the three photoreceptor types *i*. Quantum catch is calculated using the spectrum of the illumination from the monitor (I_M), the lens transmission (L), and the receptor spectral sensitivities of the photoreceptors (R_i) (Kelber et al. 2003). The photoreceptor sensitivities (R_i) were modeled using vitamin A_1 based visual pigments with peak wavelength sensitivities using the equations of Govordovskii et al. (2000). The visual model used in this study considers wavelengths of light from 350-700 nm.

$$Q_i = K_i \int_{350}^{700} R_i(\lambda) L(\lambda) I_M(\lambda) d\lambda$$

Here K_i is the von Kries factor (K_i), which is derived from the von Kries color constancy model, in which each receptor adapts independently to the background illumination from the monitor (I_B), and acts as a color constancy correction.

$$K_i = \frac{1}{\int_{350}^{700} R_i(\lambda) L(\lambda) I_B(\lambda) d\lambda}$$

In the model, we used the illumination from the monitor and used the previously measured lens transmission (Hofmann et al., 2010) for *Metriaclima zebra* (Stauffer et

al., 1997) and the known maximum absorbance of each cone type in *M. zebra*, λmax = 368, 484, 528 nm (Parry et al., 2005).

We compare the quantum catch values of the colors of two objects being compared using the RNL model, taking receptor noise into account (Vorobyev & Osorio, 1998; Vorobyev et al., 2001; Koshitaka et al., 2008; Escobar-Camacho et al., 2019). This allows us to calculate ΔS, chromatic distance, whose units are just noticeable difference (JND), a measure of discriminability. In order to calculate chromatic distance, we first use the quantum catch (Q_i) for each of the three photoreceptor types, *i*, to calculate the contrast between the pair of colors Δf_i (Siddiqi et al., 2004).

$$\Delta f_i = \ln\left[\frac{Q_i(\text{color 1})}{Q_i(\text{color 2})}\right]$$

The second component of the RNL model is calculating receptor noise, represented by the Weber fraction (ω). The Weber fraction for a single photoreceptor *i* is:

$$\omega_i = \frac{v_i}{\sqrt{n_i}}$$

where relative receptor noise is *v* and the weber fraction is weighted by the number of photoreceptors of photoreceptor type *i* which is n_i (Vorobyev & Osorio, 1998; 2001; Champ et al., 2016). We assigned a receptor noise value for each cone class (Koshitaka et al., 2008), assuming the long (L) receptor to have a noise value of 0.16 (Escobar-Camacho et al., 2019). The short (S) and medium (M) cone class noise values were calculated using their relative abundance in the retinal mosaic, which has an S:M:L cone ratio of 1:2:2 (Dalton et al., 2014).

By bringing together the chromatic contrast Δf_i and Weber fraction ω_i we can calculate the chromatic distance ΔS (Vorobyev & Osorio, 1998). ΔS was calculated for a trichromatic visual system:

$$\Delta S = \sqrt{\frac{\omega_S{}^2(\Delta f_L - \Delta f_M)^2 + \omega_M{}^2(\Delta f_L - \Delta f_S)^2 + \omega_L{}^2(\Delta f_S - \Delta f_M)^2}{(\omega_S\omega_M)^2 + (\omega_S\omega_L)^2 + (\omega_M\omega_L)^2}}$$

ΔS values greater than 1 indicate that the fish can distinguish the two colors, while ΔS values less than 1 indicate that fish cannot distinguish the two colors (Vorobyev & Osorio, 1998; Siddiqi et al., 2004; Lind & Kelber, 2009; Olsson et al., 2018).

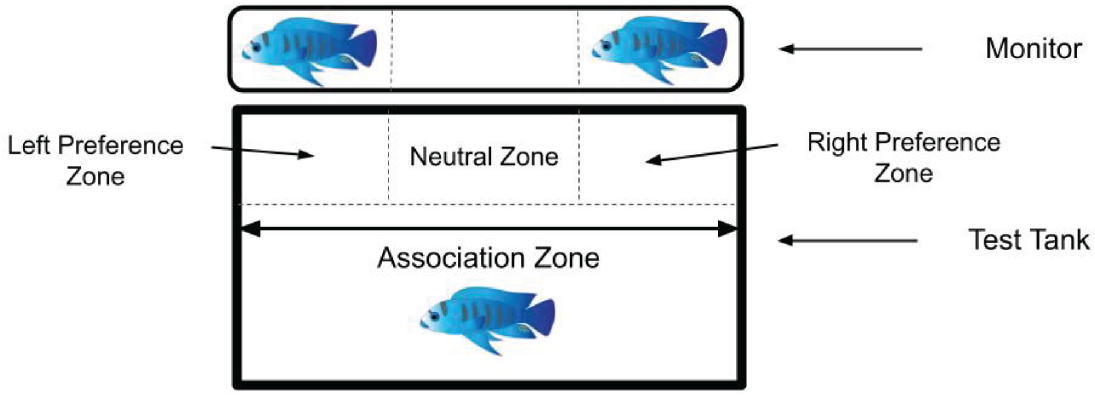
3.3.3 Control Experiment

Before investigating the effects of visual signals on male aggression, we first had to determine whether using virtual stimuli would be viable with *Metriaclima zebra*. In order to do so, we compared how a sample of 6 random test males responded behaviorally to a live stimulus fish and virtual stimulus fish. For this experiment and all but one of our subsequent experiments, we utilized a two-way male aggression assay to test behavior. The two-way assay consisted of a test tank (26.5 cm wide x 51 cm long x 30 cm deep) wherein the test males were placed. In the test tank, three of the four walls were made opaque with plastic and tape so that the test male could not see its reflection. Another aquarium of equal size (hereafter called the stimulus tank) was placed beside the uncovered wall of the test tank. This stimulus tank was divided into three compartments. The outer left and right compartments (16.5 cm long x 30 cm deep) were used for live stimulus and the central compartment was a neutral zone

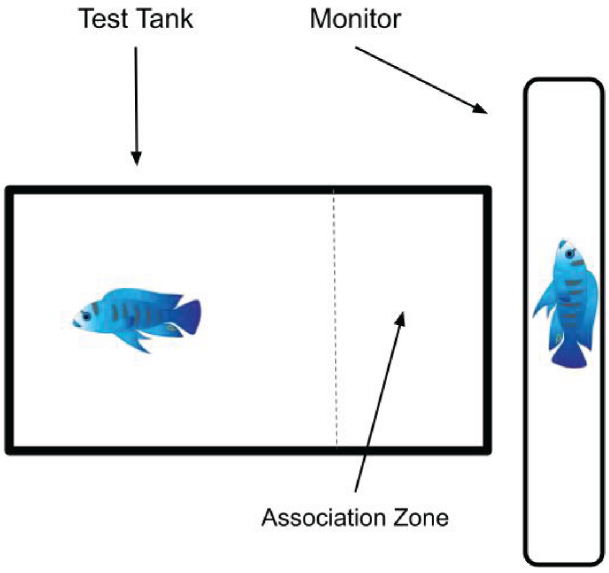
(18 cm long x 30 cm deep). The width of association zones inside the test tank was denoted as within one body length adjacent to each stimulus (Figure 3.1A). An opaque barrier was placed between the test tank and the stimulus tank prior to the start of experiments.

Figure 3.1: Behavioral assay diagrams. (A) Diagram depicting two-way male aggression assays using virtual stimuli displayed on a computer monitor, including annotations of the participation zone (area of the test tank one test male body length from the monitor) and the association zones (areas of the participation zone adjacent to the virtual stimuli). (B) Diagram depicting one-way male aggression assays using a virtual stimulus displayed on a computer monitor, including an annotation of the association zone (area of the test tank one test male body length from the monitor).

A. Two-Way Aggression Assay with Virtual Stimuli



B. One-Way Aggression Assay with Virtual Stimulus



At the start of a trial, a randomly selected test male was placed in the test tank. A live stimulus male was placed randomly in the left or the right compartment of the stimulus tank. The barrier between the test tank and the stimuli was removed and the behavior of the test male was recorded for 15 minutes (during initial control experiments) or 10 minutes (during all other experiments) using a Raspberry Pi camera mounted above the assay. At least six days later, the same test male was tested again with the live stimulus fish positions reversed (for example, if the stimulus fish was placed on the right side of the stimulus tank during the first trial, it was placed on the left side during the second trial). The total length (TL) of each test male was measured to the nearest 0.1 cm with a ruler. The live stimulus fish were size-matched to the test male within 0.5 cm of their TL. Test males were never shown either of their neighbors or one of their siblings during a trial to avoid effects of prior experience.

In addition to live stimulus fish, assays could also be performed using virtual stimulus fish. When testing with virtual stimuli, a computer monitor (21.5" 2009 iMac running Mac OS Yosemite version 10.10.5; Intel Core 2 Duo processor) was placed across from the uncovered wall of the test tank instead of the stimulus tank. A neutral grey background (R: 149, G: 149, B: 149) was displayed on the monitor, which had two outer windows (14 cm wide x 25 cm tall) that were used for virtual stimuli and a central window (18 cm wide x 25 cm tall) which was a neutral zone. The association zones were denoted as above (see Video 1). Virtual stimuli were created in Photoshop (version 25.1) and After Effects (version 24.0.3) by animating a photograph of a test male to move horizontally back-and-forth across the screen before briefly moving

rapidly up-and-down to mimic the cichlid quiver (see Video 1), a behavior used by haplochromine cichlids to both attract females and threaten males (Baerends & Baerends-Van Roon, 1950). Various color pattern and fish attributes were varied between two stimulus fish to assess their importance in stimulating male aggression (e.g. number of egg spots, color, movement). Prior to the trial, an opaque barrier was placed between the test tank and the monitor. Trials were conducted identically with virtual stimuli as with live stimuli and virtual stimulus fish were also size-matched to the test male within 0.5 cm of their TL, a small size difference that falls within the range suggested by previous work on male aggression in cichlids (Alcazar et al., 2014).

The videos recorded from these trials were analyzed using Bonsai (version 2.6.2) and Boris (version 7.13.9). All behaviors were measured once the test male visited both association zones. The three behaviors recorded were: time spent (sec), number of bites, and number of quivers. Time spent is defined as the number of seconds the test male spent in the association zone of each stimulus. A bite was defined as a snapping of the jaws combined with forward movement towards a target, and a quiver was defined as a display in which the test male erects its fins and shakes its body rapidly (e.g. Baerends & Baerends-Van Roon, 1950; Djikstra et al., 2006; Simões et al., 2008; Pauers & Grudnowski, 2020). Test fish that were physically identified as female after experimentation and non-participants (test males who spent less than one-third of the assay in the association zones) were removed from the dataset. All data was analyzed in R (version 2022.07.2+576) and behavior towards live stimuli and virtual stimuli were quantitatively compared using paired t-tests.

In analyzing the data, the participation of each test male was measured as the number of seconds the fish spent in the participation zone, the area of the tank within one body length of the stimuli (Figure 3.1). If the test male spent less than 200 seconds (one-third of the 600 second trial) in the participation zone, it was considered a non-participant during that trial. If a test male was a non-participant in both of the two trials, it was excluded from analysis. If a test male was a non-participant in one of the two trials, it was tested again in a "second chance" trial which mimicked the conditions of the trial in which it did not participate (i.e. with the same stimuli in the same positions as the trial in which it did not participate). At that point, the test male was then either marked as a participant (if it participated in the "second chance" trial) or a non-participant (if it did not participate in the "second chance" trial) and excluded from analysis.

3.3.4 Egg Spot Experiment

To examine the possible role that egg spots play in male aggression, we created virtual stimuli which differed only in the number of egg spots. These virtual stimuli moved as above, but the animated image was that of an *M. zebra* photographed in its natural habitat at Chilucha Reef (provided by Ad Konings) which was then digitally altered in Photoshop (version 25.1) to vary egg spot number. In one stimulus (hereafter called ES-), the virtual fish had its egg spots digitally removed. In the other stimulus (hereafter called ES+), the virtual fish was digitally altered to have 3 egg spots (the average number of egg spots possessed by the test males included in this

experiment; Figure 3.2A). These virtual stimuli were animated as described in the control experiment.

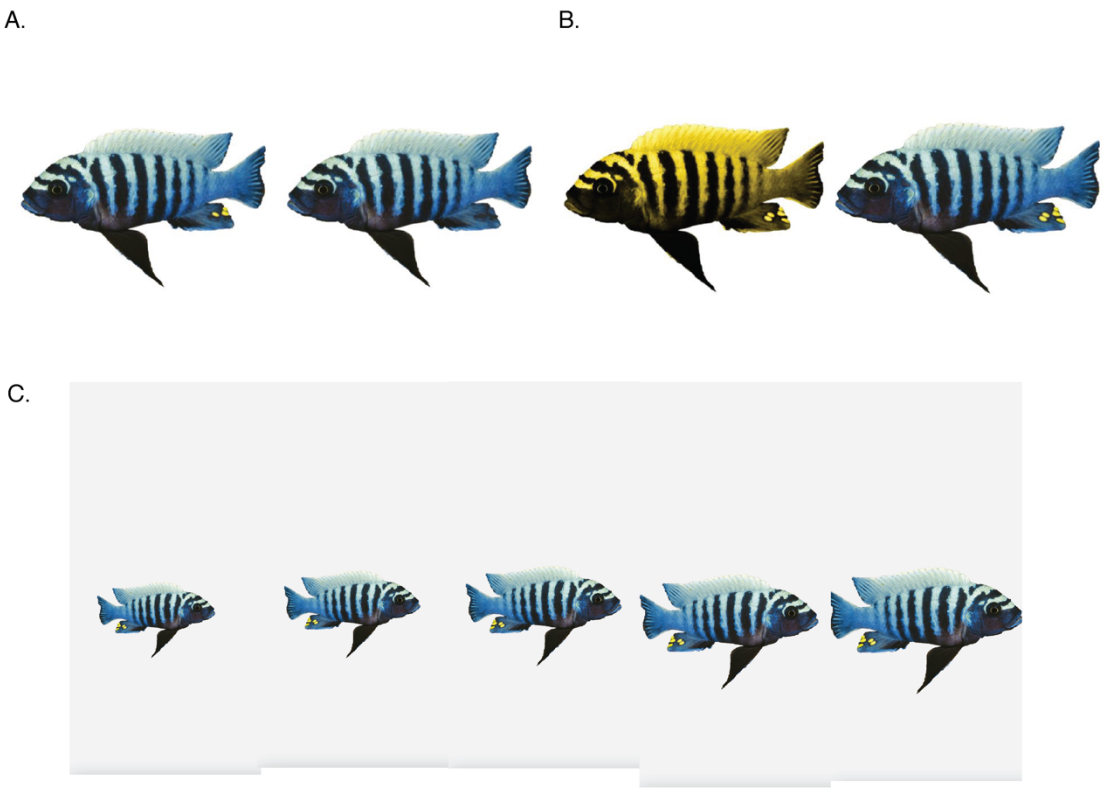


Figure 3.2: Stimuli for the egg spot, color, and size experiments. (A) A side-by-side depiction of the Photoshop-manipulated *M. zebra* images used in the ES+ and ES- stimuli. (B) A side-by-side depiction of the Photoshop-manipulated *M. zebra* images used in the gold and wild-type stimuli. (C). Cropped screenshots of each of the stimuli used in the size experiments to demonstrate the size difference between stimuli A through E.

At the start of a trial, a randomly selected test male was placed in the test tank. The virtual ES- stimulus was placed randomly on the left or the right side of the monitor and the ES+ stimulus on the other. The trials were conducted and the data was analyzed as described in the control experiment.

3.3.5 Color Experiment

To examine the role body coloration plays in male aggression, we created virtual stimuli which differed only in its color. These virtual stimuli were made from the same photo as the egg spot experiment and moved the same as all previous virtual stimuli. In one stimulus (hereafter called gold), the virtual fish had its body color digitally changed from blue to gold, but retaining its bar pattern. This was done by applying the hue and saturation (but not the brightness) of the photograph's gold egg spot to the rest of the virtual fish's body using the blending mode function in Photoshop. In the other stimulus (hereafter called wild-type), the virtual fish was not digitally altered (Figure 3.2B). These virtual stimuli were animated as described in the control experiment.

At the start of a trial, a randomly selected test male was placed in the test tank. The gold stimulus was placed randomly on the left or the right side of the monitor and the wild-type stimulus was placed on the other. The trials were conducted and the data was analyzed as in the control experiment.

3.3.6 Movement Experiment

To examine the role movement plays in male aggression, we created virtual stimuli which differed by whether or not they moved. These virtual stimuli were made from the same photo as the egg spot experiment. In one stimulus (hereafter called mobile), the virtual fish moved the same as all previous virtual stimuli (see Video 1). In the other stimulus (hereafter called immobile), the virtual fish was a static image which simply remained still in the center of its side of the monitor.

At the start of a trial, a randomly selected test male was placed in the test tank. The virtual mobile stimulus was placed randomly on the left or the right compartment of the monitor and the immobile stimulus on the other. The trials were conducted and the data was analyzed as in the previous experiments.

3.3.7 Size Experiment

In order to examine the effects of size on male competition in *M. zebra*, we utilized a multiple-comparisons one-way male aggression assay. The one-way assay consisted of a test tank (26.5 cm wide x 51 cm long x 30 cm deep) wherein the test male was placed. In the test tank, all the walls except one were made opaque with plastic and tape so that the test male could not see its reflection. We placed the computer monitor adjacent to the uncovered wall. A single virtual stimulus was displayed on the monitor (Figure 3.1B). An opaque barrier was placed between the test tank and the monitor and removed at the start of the test.

Virtual stimuli were made in Photoshop (version 25.1) and After Effects (version 24.0.3) using the same photograph as the egg spot, color, pattern, and movement

experiments and moved in the same pattern as the previous virtual stimuli. However, for this experiment, five stimuli were made which differed only in size. Each of the five stimuli, labeled A through E, were virtually manipulated as follows: Stimulus A is a total length of 7.1 cm, Stimulus B is a total length of 8.3 cm, Stimulus C is 9.5 cm, Stimulus D is 10.7 cm, and Stimulus E is 12.0 cm. The sizes of these stimuli were determined by the average and standard deviation in total length of the test fish so as to best represent the variation in size that exists in the laboratory (Figure 3.2C). It is worth noting that an increase in the total length of the stimulus fish also caused a proportional increase in the size of the stimulus fish's egg spots. In addition to the five test stimuli, a sixth control stimulus was also used which had no fish but simply the background color.

At the start of a trial, a randomly selected test male was placed in the test tank. A randomly determined virtual stimulus was displayed on the monitor. Then the barrier between the test tank and the stimuli was removed and the behavior of the test male was recorded for 10 minutes using a Raspberry Pi camera mounted above the assay. After a 10 minute rest period, the same test male was tested again with another randomly determined virtual stimulus. This process was repeated until the test male had been shown each of the five different-sized stimuli and the blank control stimulus. The videos recorded from these trials were analyzed as previously described.

3.4 Results

3.4.1 Visual Modeling

Before running behavior experiments, we used visual modeling to ensure the test males could discriminate between visual stimuli displayed on the monitor. We measured the spectra of the virtual egg spots, virtual anal fin, virtual gold body, and virtual blue body (Figure 3.3). We then calculated chromatic distance for the color of the virtual egg spots and the color of the virtual anal fin, to ensure that the test males can discriminate between the virtual stimuli. We measured color discrimination for all possible pairwise combinations of egg spot colors (3 egg spots from the photograph) and anal fin colors (2 measures from the photograph), or 6 combinations. The median chromatic distances of all the egg spot colors compared to each anal fin color are greater than 1 (4.95 and 5.49; Figure 3.4), indicating that the fish could distinguish between the colors of egg spots and the anal fin on the monitor. This demonstrates that the test males could discriminate between the ES+ and ES- stimuli used in the egg spot experiment.

Next, we calculated chromatic distance for the body color of the virtual gold stimulus and the body color of the virtual blue (or wild-type) stimulus. We measured color discrimination for the average gold body color and average blue body color (Figure 3.3B). The chromatic distance between the gold and the blue color is greater than 1 (2.10), indicating that the test males can distinguish between the body colors of the gold and blue virtual stimuli. This demonstrates that the test males could discriminate between the gold and blue stimuli used in the color experiment.

A. B.

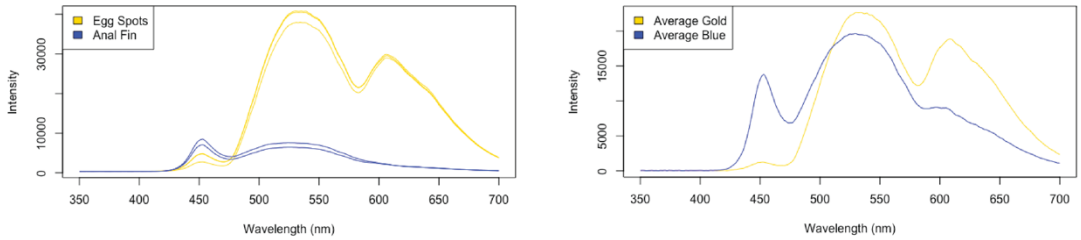


Figure 3.3: Spectra of virtual stimuli. (A) Virtual Egg Spots and Virtual Anal Fin. The spectra of the virtual egg spots and the spectra of the virtual anal fin from the egg spot experiment. (B) Virtual Gold and Blue Spectra. The spectra of the average gold and average blue body colors on the virtual stimuli from the color experiment.

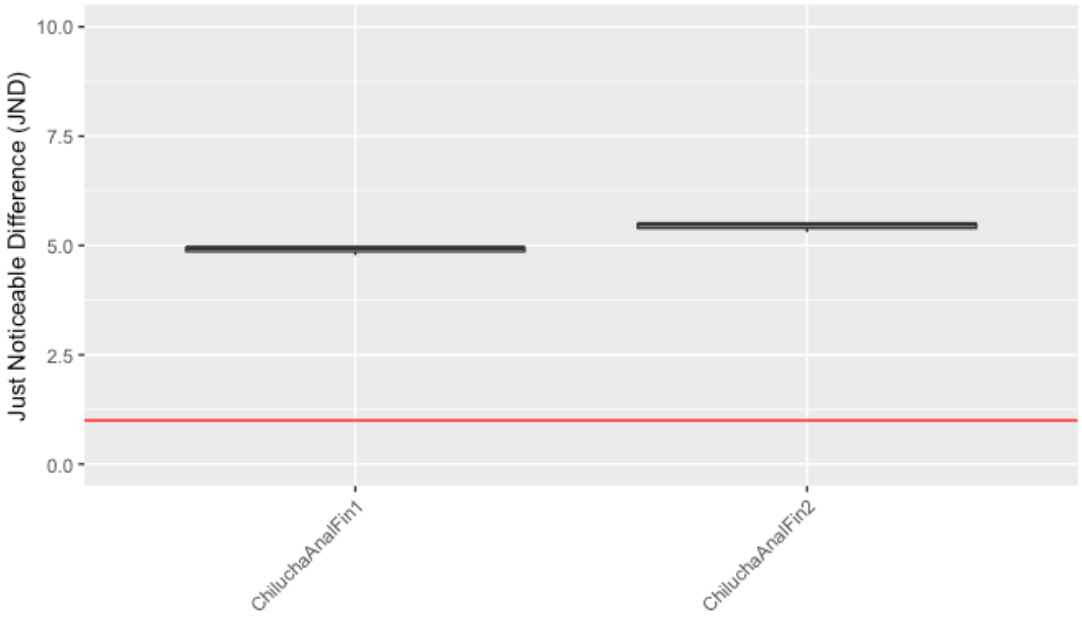


Figure 3.4: Chromatic distance of egg spots from anal fins. The chromatic distance (ΔS) between the colors of the egg spots and each anal fin color. Chromatic distance was calculated with a Weber fraction of 0.16 (Escobar-Camacho et al. 2019) specific to the cichlid visual system for red - blue color comparisons. The calculations use the illumination from the computer monitor as the background adapting light for von Kries normalization. The median chromatic distance for each is greater than 1, indicating that the fish can distinguish between the the egg spots and the anal fin of the virtual.

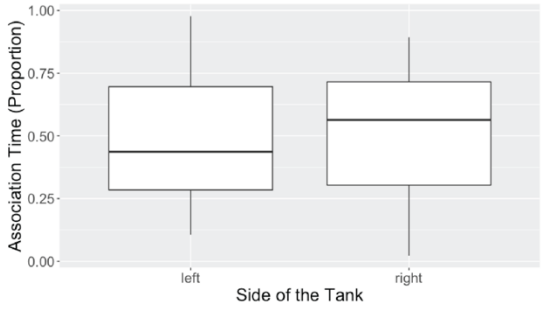
3.4.2 Control Experiment

We first tested several controls to ensure that the assay was working. The controls measured whether fish had any tank side biases, whether test males responded differentially to stimuli versus no stimuli (an empty compartment), and whether there were response differences to live stimuli or virtual stimuli. A total of 12 test males

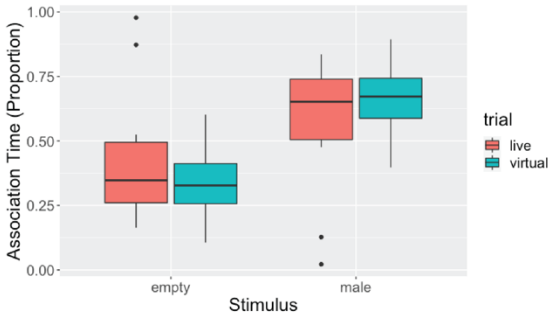
were used for this assay, 2 of which were non-participants and 2 of which were females which were removed from the data. In assessing whether fish had a side preference, we found that test males did not associate differently with the left side (median = 0.44) versus the right side (median = 0.56) of the test tank (regardless of stimuli positions; n = 8, t = -0.54398, df = 15, p-value = 0.5945; Figure 3.5A). We subsequently found with an ANOVA that test males spent significantly more time with a conspecific male (median = 0.67) than with an empty compartment (median = 0.33, n = 8, F value = 4.178, p value = 0.05). This was true for both the live stimulus and the virtual stimulus, though test males spent time with live stimulus fish (median = 0.50) and a virtual stimulus fish (median = 0.50) equally (n = 8, F value = 0, p value = 1; Figure 3.5B). With regards to biting, test males bite at stimulus males (average = 7.13 bites) significantly more than the empty compartment (average = 1.98 bites, n = 8, F value = 15.770, p = 0.0004326), but there is no difference between the number of bites directed at virtual (average = 3.75 bites) or live stimuli (average = 4.82 bites, n = 8, F value = 0.509, p = 0.4814097; Figure 3.5C). With regards to quivering, test males quiver at stimulus males (average = 0.44 quivers) and empty compartments (average = 0.03 quivers) equally (n = 8, F value = 2.180, p = 0.151) and test males quiver at live stimuli (average = 0.16 quivers) and virtual stimuli (average = 0.50 quivers) equally (n = 8, F value = 1.171, p = 0.288; Figure 3.5D).

Figure 3.5: Control experiment results. (A) The proportion of time spent by test males on each side of the test tank, regardless of the positioning of the live and virtual stimuli. There is no difference in test male associations with the left and right association zones, indicating that the test males did not have an intrinsic bias for either side of the test tank ($n = 8$, $t = -0.54398$, $df = 15$, p-value = 0.5945). (B) The proportion of time spent by test males in the association zone associating with either an empty compartment or a male stimulus, either a live male or a virtual male. Test males have a significant association with a male over an empty compartment (median = 0.33, $n = 8$, F value = 4.178, p value = 0.05), but there was no significant difference in the association for a live fish stimulus or a virtual fish stimulus ($n = 8$, F value = 0, p value = 1). (C) Test males exhibited a higher number of bites for a male stimulus over an empty compartment, but there was no significant difference in the number of bites for a live fish stimulus or a virtual fish stimulus ($n = 8$, F value = 0.509, $p = 0.4814097$). (D) Test males exhibited a very low number of quivers overall. There was no significant difference in the number of quivers directed at an empty compartment or a male stimulus ($n = 8$, F value = 2.180, $p = 0.151$), nor between a live male stimulus and a virtual fish stimulus ($n = 8$, F value = 1.171, $p = 0.288$).

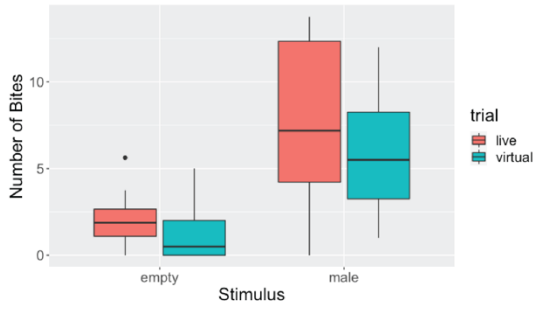
A.


Association Time (Proportion)
1.00
0.75
0.50
0.25
0.00
left
right
Side of the Tank

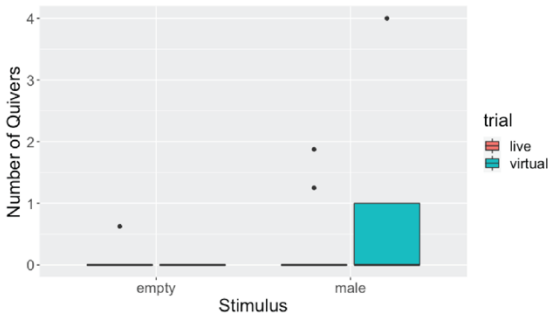
B.


Association Time (Proportion)
1.00
0.75
0.50
0.25
0.00
empty
male
Stimulus
trial
live
virtual

C.


Number of Bites
10
5
0
empty
male
Stimulus
trial
live
virtual

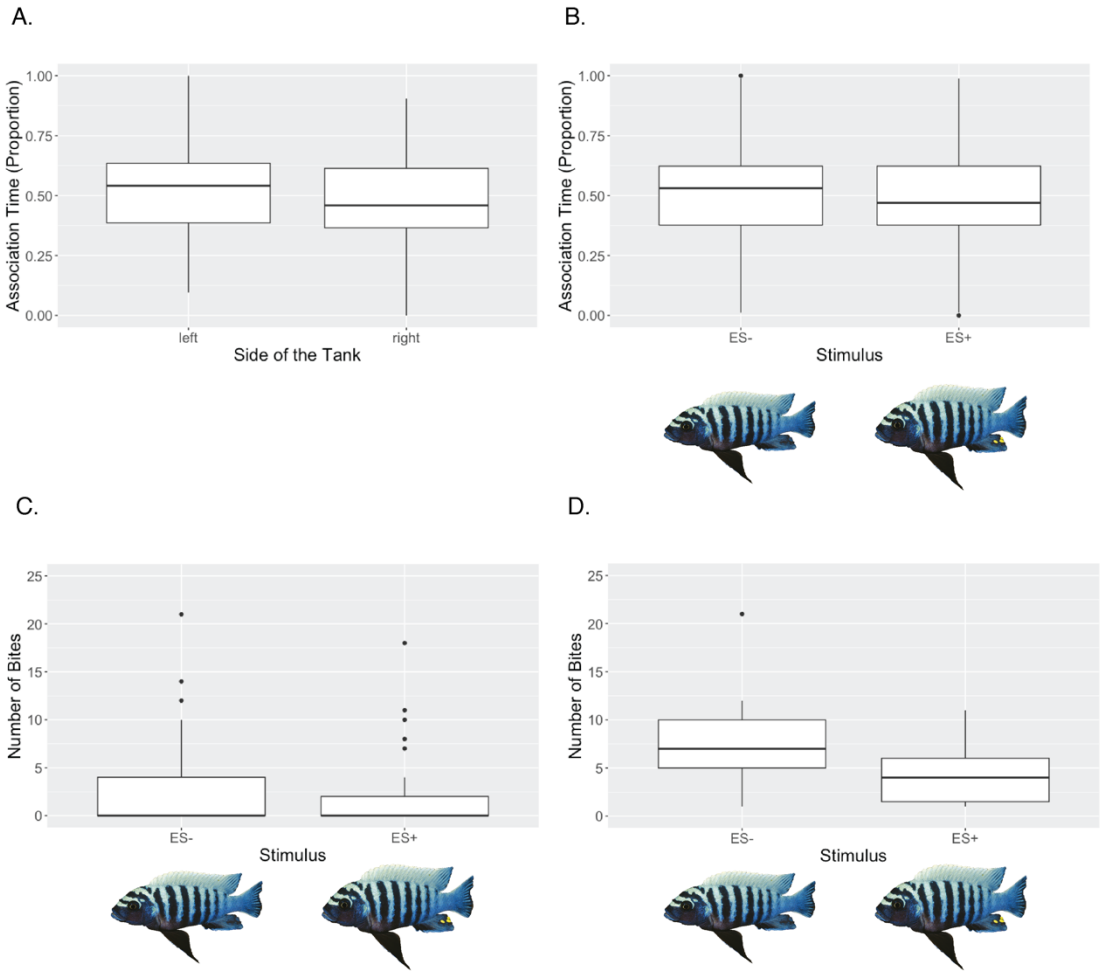
D.


Number of Quivers
4
3
2
1
0
empty
male
Stimulus
trial
live
virtual

3.4.3 Egg Spot Experiment

We then tested whether test males responded differentially to virtual stimuli with egg spots or without egg spots. A total of 39 test males were used for this assay, 5 of which were non-participants and 4 of which were females which were removed from the data. We found that test males did not associate differently with the left side (median = 0.54) or the right side (median = 0.46) of the test tank (regardless of stimuli positions; n = 33, t = 1.3391, df = 59, p-value = 0.1857; Figure 3.6A). We subsequently found that test males did not associate differently with ES+ stimuli (median = 0.47) stimuli over ES- stimuli (median = 0.53, n = 33, t = 0.45617, df = 59, p-value = 0.6499; Figure 3.6B). With regards to biting, test males bit towards ES+ stimulus fish (average = 1.60 bites) and ES- stimulus fish (average = 2.52 bites) equally (n = 33, t = 1.9597, df = 59, p-value = 0.05477; Figure 3.6C). When considering only the test males who exhibited at least 1 bite in each trial, this does not change: test males bite towards ES+ stimulus fish (average = 4.43 bites) and ES- stimulus fish (average = 8.43 bites) equally (n = 6, t = 1.4491, df = 6, p-value = 0.1975; Figure 3.6D). With regards to quivering, test males did not exhibit any quivers in this experiment.

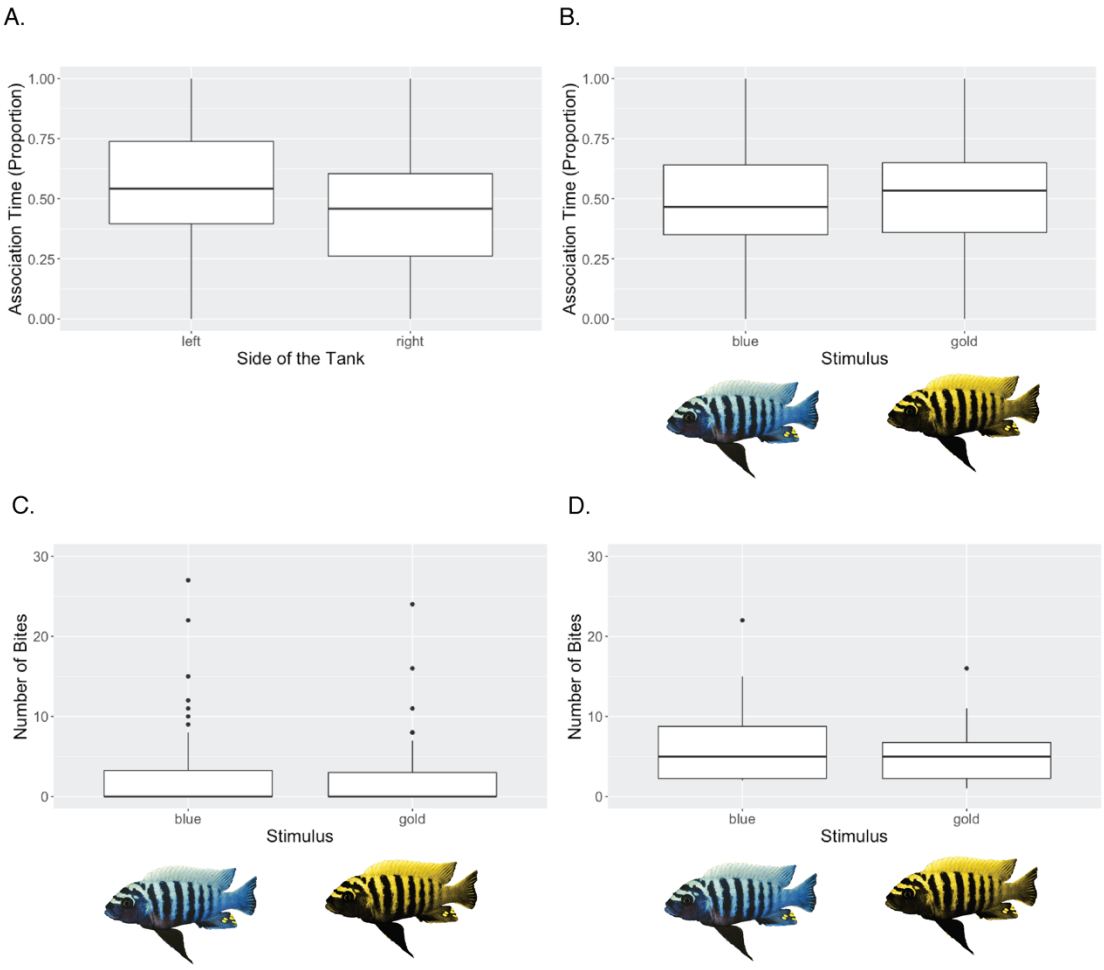
Figure 3.6: Egg spot experiment result summary. (A) The time spent by test males on each side of the test tank, regardless of the positioning of the live and virtual stimuli. There is no difference in test male associations with the left and right association zones, indicating that the test males did not have an intrinsic bias for either side of the test tank ($n = 33$, $t = 1.3391$, $df = 59$, p-value = 0.1857). (B) The proportion of time spent by test males in the association zone associating with either virtual stimuli with egg spots or virtual stimuli without egg spots. Test males did not have differential association with either stimulus ($n = 33$, $t = 0.45617$, $df = 59$, p-value = 0.6499). (C) Test males exhibited no difference in the number of bites directed at ES+ or ES- stimuli ($n = 33$, $t = 1.9597$, $df = 59$, p-value = 0.05477). (D) When considering only test males who exhibited aggressive behavior in both trials, there is no difference in the number of bites directed at ES+ or ES- stimuli ($n = 6$, $t = 1.4491$, $df = 6$, p-value = 0.1975).


A.
B.
C.
D.
Association Time (Proportion)
1.00
0.75
0.50
0.25
0.00
left
right
Side of the Tank
ES-
ES+
Stimulus
Number of Bites
25
20
15
10
5
0

3.4.4 Color Experiment

We then tested whether test males responded differentially to virtual stimuli with gold or wild-type (blue) body color. A total of 48 test males were used for this assay, 6 of which were non-participants and 4 of which were females which were removed from the data. We found that test males associated slightly more often with the left side (median = 0.54) over the right side (median = 0.46) of the tank (regardless of stimuli positions; n = 38, t = 2.2608, p-value = 0.02667; Figure 3.7A). We subsequently found that test males associate with gold stimuli (median = 0.53) and wild-type stimuli (median = 0.47) equally (n = 38, t = 0.45617, p-value = 0.6499; Figure 3.7B). With regards to biting, test males bit towards gold (average = 2.08 bites) and wild-type (average = 2.70 bites) stimulus fish equally (n = 38, t = 1.7853, p-value = 0.07825; Figure 3.7C). When considering only the test males who exhibited at least 1 bite in each trial, this does not change: test males bit towards gold stimulus fish (average = 5.39 bites) and wild-type stimulus fish (average = 6.72 bites) equally (n = 18, t = 1.0851, p-value = 0.293; Figure 3.7D). With regards to quivering, test males did not exhibit any quivers in this experiment.

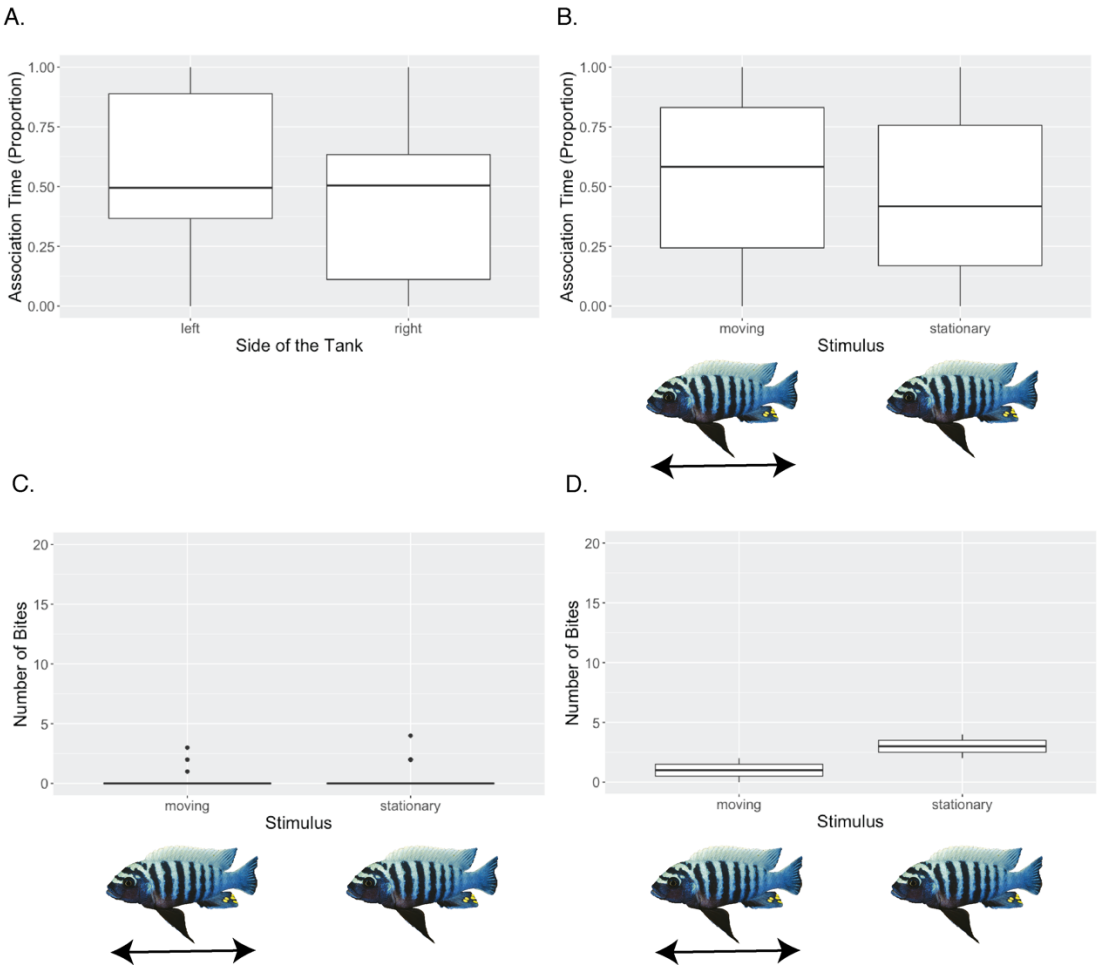
Figure 3.7: Color experiment result summary. (A) The time spent by test males on each side of the test tank, regardless of the positioning of the live and virtual stimuli. Test males exhibited a slight preference for associating with the left side of the test tank ($n = 38$, $t = 2.2608$, p-value = 0.02667). (B) The proportion of time spent by test males in the association zone associating with either virtual stimuli with wild-type body coloration or virtual stimuli with gold body coloration. Test males did not have differential association with either stimulus ($n = 38$, $t = 0.45617$, p-value = 0.6499). (C) Test males exhibited no difference in the number of bites directed at wild-type or gold stimuli ($n = 38$, $t = 1.7853$, p-value = 0.07825). (D) When considering only test males who exhibited aggressive behavior in both trials, there is no difference in the number of bites directed at wild-type and gold stimuli ($n = 18$, $t = 1.0851$, p-value = 0.293).


A.
B.
C.
D.
Association Time (Proportion)
1.00
0.75
0.50
0.25
0.00
left
right
Side of the Tank
blue
gold
Stimulus
Number of Bites
30
20
10
0

3.4.5 Movement Experiment

We then tested whether test males responded differentially to mobile or immobile virtual stimuli. A total of 48 test males were used for this assay, 4 of which were non-participants and 1 of which was a female. These test fish were removed from the data. We found that test males associated with the left side of the tank (median = 0.50) and the right side of the tank (median = 0.50) equally (regardless of stimuli positions; n = 10, t = 0.66479, p-value = 0.5146; Figure 3.8A). We subsequently found that test males associate with mobile stimuli (median = 0.58) and immobile (median = 0.42) stimuli equally (n = 10, t = 0.25758, p-value = 0.7997; Figure 3.8B). With regards to biting, test males bit towards mobile stimuli (average = 0.32 bites) and immobile stimuli (average = 0.53 bites) equally (n = 10, t = -0.67612, p-value = 0.5076; Figure 3.8C). When considering only the test males who exhibited at least 1 bite in each trial, this does not change: test males bit towards mobile stimuli (average = 1 bite) and immobile stimuli (average = 3 bites) equally (n = 1, t = -1, p-value = 0.5; Figure 3.8D). With regards to quivering, test males did not exhibit any quivers in this experiment.

Figure 3.8: Movement experiment result summary. (A) The proportion of time spent by test males on each side of the test tank, regardless of the positioning of the live and virtual stimuli. Test males associated equally with the left and the right sides of the test tank ($n = 10$, $t = 0.66479$, p-value = 0.5146). (B) The proportion of time spent by test males in the association zone associating with either mobile virtual stimuli or immobile virtual stimuli. Test males did not have differential association with either stimulus ($n = 10$, $t = 0.25758$, p-value = 0.7997). (C) Test males exhibited no difference in the number of bites directed at mobile or immobile stimuli ($n = 10$, $t = -0.67612$, p-value = 0.5076). (D) When considering only test males who exhibited aggressive behavior in both trials, there is no difference in the number of bites directed at mobile and immobile stimuli ($n = 1$, $t = -1$, p-value = 0.5).


A.
B.
C.
D.
Association Time (Proportion)
1.00
0.75
0.50
0.25
0.00
left
right
Side of the Tank
moving
stationary
Stimulus
Number of Bites
20
15
10
5
0

3.4.6 Size Experiment

Finally, we tested whether test males responded differentially to virtual stimuli of various sizes. A total of 6 test males were used for this assay, 1 of which was a non-participant which was removed from the data (there were no females among the test fish for this experiment). According to the linear model, test males associate equally with the blank control stimulus (0.87) and with the differently-sized fish stimuli (0.75), indicating that the test fish spend the same amount of time in the association zone, regardless of whether a moving stimulus or a blank screen being displayed ($n = 5$, $t = -1.382$, $p = 0.178$; Figure 3.9A). With regards to biting, test males bit more towards stimulus fish (average = 5.96 bites) than towards the blank control (average = 1.20 bites, $n = 5$, $t = 2.310$, $p = 0.0288$), and the order of stimulus presentation was not a significant factor in bite distribution towards fish stimuli or blank control ($n = 5$, $t = 1.261$, $p = 0.2180$; Figure 3.9B). When considering only data from the stimulus trials, test fish bit equally towards stimuli of different sizes: Size A received an average of 3.4 bites, Size B 6.0 bites, Size C 6.4 bites, Size D 7.4 bites, and Size E 6.6 bites ($n = 5$, $t = 1.059$, p value = 0.301) and the order of stimulus presentation was not a significant factor in bite distribution towards fish stimuli of different sizes ($n =5$, $t = -1.267$, $p = 0.218$; Figure 3.9C). With regards to quivering, test males did not exhibit any quivers in this experiment. Finally, the difference in total length (TL) between the test male and the stimulus fish did not play a significant role in the number of bites directed at any given stimulus (quadratic linear model, $n = 5$, $t = 1.578$, p-value = 0.128, Figure 3.9D).

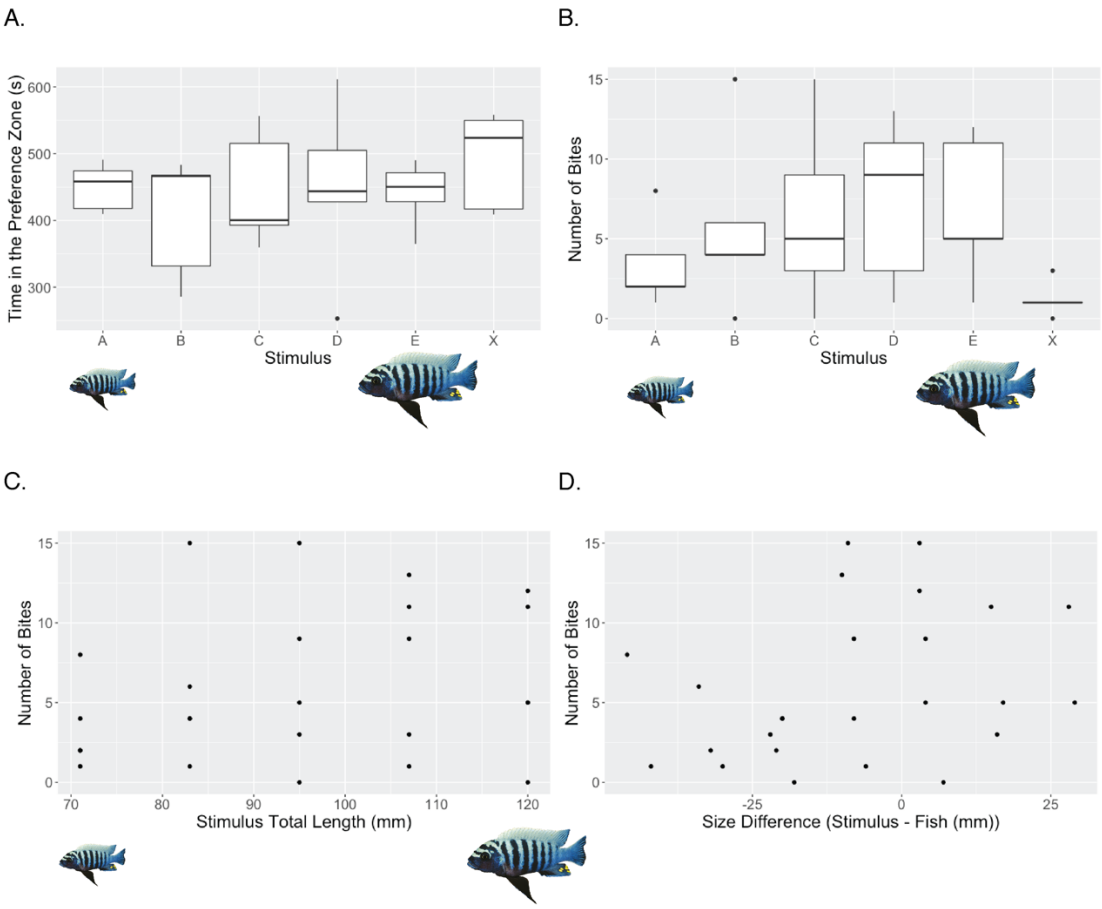


Figure 3.9: Size experiment results summary. (A) The proportion of time spent by test males with each stimulus. Test males associated equally with the blank control stimulus (X) and with the differently-sized fish stimuli (A-E), indicating that the test fish spends the same amount of time in the association zone regardless of whether a moving stimulus or a blank screen is being displayed on the monitor (n = 5, t = -1.382, p = 0.178). (B) Test males bit more towards virtual stimulus fish (A – E) than towards the blank control stimulus (X; n = 5, t = 2.310, p = 0.0288). (C) Test males bit equally towards virtual fish stimuli, regardless of the stimuli's total length (TL; n = 5, t = 1.059, p value = 0.301). (D) Test males bit equally towards virtual fish stimuli, regardless of the difference in total length (TL) between the virtual stimulus fish and the test male (n = 5, t = 1.578, p-value = 0.128).

3.5 Discussion

In this study, we tested how *M. zebra* males respond to simplistic virtual stimuli, determining firstly that *M. zebra* males spent more time and behaved more aggressively towards stimuli (whether live or virtual) than towards an empty compartment. This suggests that males can, at minimum, recognize a novel stimulus across the glass of the aquarium and respond to it. Further, males behave aggressively towards virtual stimuli in a manner equivalent to live fish stimuli (Figure 3.5), suggesting that the fish indeed recognize the virtual stimuli as intruder fish. The results of further experimentation indicate that egg spot presence, body color, movement, and size do not provoke differential aggressive behavior from *M. zebra* (Figures 3.5 – 3.9). Although the egg spot and pattern results are novel to this study, the results found with regards to body color, movement, and size are both surprising and seemingly contrary to the well-established and consistent findings in the literature. However, we believe that these unexpected findings reflect the essential differences from classic male aggression assays required by using virtual stimuli.

To consider whether the assay is working, we consider several major assumptions of this assay. The first assumption is that the fish can discriminate differences in virtual stimuli being displayed on the monitor. In order to validate this, we used the receptor noise limited (RNL) visual model to demonstrate that the test males can discriminate the differences between the ES+ and ES- stimuli used in the egg spot experiment and the gold and blue stimuli used in the color experiment. By using visual modeling, based on data from behavioral experiments, we quantitatively

validated that the test males' visual system should have been able to discriminate between these stimuli (Figure 3.4).

The second assumption to address is the question of whether the test males are truly exhibiting aggressive behavior towards the stimuli on the other side of aquarium glass or if they are simply fighting their own reflections, as many aggressive fish species including cichlids are known to do (Tinbergen, 1951; Verbeek et al., 2007; Desjardins & Fernald, 2010). To address this concern, two main factors are worth considering. Firstly, if the fish were simply biting its reflection, we would expect to see an equivalent number of bites directed at the blank control stimulus as were directed at the various-sized fish stimuli in the size experiment. However, the test males exhibited significantly fewer bites towards the blank control than towards fish stimuli in the size experiment (Figure 3.9). Secondly, across all of the test males in all of the experiments conducted, only a single test male performed just one bite while in the neutral zone between the two stimuli. If test males were simply biting their reflections, we would expect to see a much higher number of bites in the neutral zone across our two-way male aggression tests.

The third assumption to briefly assess is that the test fish do not have an inherent bias for one side of the tank over the other. In one of our experiments, this assumption was violated. During the color experiment, the test males demonstrated a bias towards the left side of the test tank. It is unclear why this preference arose, but worth noting that this circumstance is controlled for by our methodology. By performing each assay twice with each test male and reversing the positions of the stimuli, we can remain confident that our overall assessment of the test male's

behavior is as precise as possible despite the unpredictable side bias exhibited by the test males in these two experiments. Having validated three of the major assumptions of our experiments, let us now consider an alternative explanation for these seemingly unusual results.

In classic male aggression assays, such as the resident-intruder assay, test males are often separated from the stimulus intruder males by glass so that the fish in the assay cannot physically harm one another (Huntingford, 1980; Bakker & Sevenster, 1983; Dingemanse et al., 2007; Arnott & Elwood, 2009; Verbeek et al., 2007). This remains the same when displaying virtual fish from a monitor across glass: the test male never comes into physical contact with its potential rival. However, while live fish respond behaviorally as a trial proceeds, virtual fish continue the exact same loop of behavior for the entire trial. As such, the mutual assessment of a potential rival that occurs in trials with a live stimulus male does not occur with a virtual stimulus male. Considering the results of these experiments, it seems possible that this mutual assessment period between rival males may be essential when a test male is distributing bites towards potential rivals. Another factor to consider is the "dear enemy" hypothesis, which has been demonstrated to occur in other, unrelated fish species (Getty, 1981; 1987; Pelabon et al., 1999) and cichlids (Leiser & Itzkowitz, 1989). Under this hypothesis, when population density is high and/or male territories are close together, one of the most important factors that males use to decide what potential rivals to attack is familiarity. Males in territory-dense regions focus their attacks on unknown males over known males. In this assay, males are bombarded by consistent visual intrusions from only unknown males. It may be

that when conditioned as they are prior to these experiments, test males become primed to simply react aggressively towards any unknown rival and therefore not attribute any difference to potential rivals in accordance with their color, body patterns, or size. Ultimately, it could be any one of these potential factors or a combination of them that explain the results.

The overall neutral results of these experiments demonstrate that *M. zebra* do not respond differentially to virtual stimuli. This lack of response is similar to the failure of other cichlids to respond to virtual stimuli including Nile tilapia (personal communication, Rui Oliveira) and Mozambique tilapia (Wackermannova et al., 2017). This indicates that this methodology may not work for certain fish species. In contrast, experiments using virtual stimuli have shown great promise (and differential results) in other fish such as three-spined stickleback, (Künzler & Bakker, 1998; 2001; Bakker et al., 1999; Mazzi et al., 2003), Siamese fighting fish (Clotfelter et al. 2006), Swordtail fish (Wong & Rosenthal 2006), and other cichlids (Baldauf et al., 2009; 2011; Fischer et al., 2014; Egger et al., 2011). This suggests that some species may be more amenable to virtual experiments than others. It is not clear exactly what makes some fish good candidates for this method, but one possibility is that the ideal species have high levels of baseline aggression and are bolder in their exploration of the test tank. Future work investigating this methodology will need to untangle the relationships between these variables in order to determine for which species virtual stimuli will prove enlightening.

3.6 Conclusion

In this study, we demonstrated that *M. zebra* males respond to simplistic virtual stimuli, performing aggressive bite behaviors in a manner equivalent to bites directed at live fish. Further experimentation showed that egg spot presence, body color, movement, and size do not provoke differential aggressive behavior from *M. zebra*. Although these results seem to conflict with the known male aggression literature regarding live cichlids, it is consistent with other behavioral studies using virtual stimuli. This suggests that cichlid species differ in whether virtual stimuli can be used. This methodology may test fundamentally different questions than its classical counterparts when it comes to behavior experiments with fish and other aquatic animals.

Acknowledgements

We would like to thank Kristen Behrens for her assistance with animal care, Karen Gu for her instruction on how to sex test animals, and Ad Konings for providing the high-quality photographs of *Metriaclima zebra* used to create the virtual stimuli. This work was supported by the National Eye Institute at the National Institutes of Health (R01EY024639B; KC).

Compliance with Ethical Standards

This work has been completed under the approval of the University of Maryland's Institutional Animal Care and Use Committee (R-JUN-21-44).

Chapter 4: The Fish Eye View: Visual modeling provides insight on the viewing distances at which visual tasks occur in a Lake Malawi cichlid

4.1 Abstract

Cichlids have a multimodal sensory system but vision is notably important to them, particularly for cichlids that are native to shallow, clear water habitats like the rocky shore haplochromines of Lake Malawi. Male cichlids use a distinct set of visual signals in order to secure matings, compete with other males, and defend territories. However, little is understood about how these visual signals are received by conspecifics in the underwater environment where the scattering of light can deteriorate visual signals over relatively short distances. In this study, I calculated chromatic distance as a function of viewing distance for *Metriaclima zebra* body colors against the space light, the rocky habitat of Lake Malawi, themselves, and colors of sympatric heterospecifics. The visual model showed that *M. zebra* body colors are discriminable from the space light at distances, but from the rocks at shorter distance (but comparable to the spacing of male territories). Additionally, the visual model shows that *M. zebra* is highly discriminable from yellow *M. aurora* but not so from blue heterospecifics *M. callainos* and *C. afra*. This study indicates the importance of evaluating the salience of visual signals in the context of an animal's visual system, habitat and viewing distances.

4.2 Introduction

Vision is a particularly salient sensory modality for many animal species, both terrestrial and aquatic. Among the aquatic species, color vision has been the focus of much study in cichlids. Cichlids are one of the largest known vertebrate lineages (Turner et al., 2001) whose explosive radiation makes them an ideal study system for studying evolution (Kocher et al., 2004). Cichlid fish have a multimodal sensory system (reviewed in Escobar-Camacho & Carleton, 2015) but vision is notably important for its daily role in everyday behaviors such as courting females (Maan et al., 2006; Seehausen et al., 2008; Selz et al., 2014; Field et al., 2018) and assessing rival males (Holzberg, 1978; Schröder, 1980; Dijkstra et al., 2006; Pauers & Grudnowski, 2020). Some cichlid behaviors include visual signals that are used to both court females and intimidate rival males, such as the "quiver" behavior (Baerends & Baerends-Van Roon, 1950). When quivering, a male fish turns perpendicular to the cichlid receiving the signal before flaring their dorsal and anal fin and shaking their body rapidly. This movement is thought to show off the various color patterns of the fish and demonstrate its vigor (Wazlavek & Figler, 1989). The role of cichlid coloration in behavioral signals like quivering has also been demonstrated to play an important role in the speciation of this massive phylogenetic family (Terai et al., 2002; 2006; Carleton et al., 2005; Spady et al., 2005). Due to the clarity of the water, vision is especially important for the cichlids that live in the shallow waters of Lake Malawi.

In Lake Malawi, the shallow, rocky waters are the habitat for an estimate of 700-800 cichlid fish species (Konings, 1995; Snoeks, 2001; Turner et al., 2001). One such

species is *Metriaclima zebra*, whose males are identified by their blue body and black bar pattern (Ribbinck et al. 1983). These fish live in especially clear water where they can be found most between 5 – 25 meters of the surface amidst other blue-bodied fish such as *Cynotipalia afra* or *Metriaclima callainos* and yellow-bodied fish such as *Metriaclima aurora* (Ribbink et al., 1983). Males of different species form a patchwork of breeding territories in the rocky habitat. Living among other cichlids, being able to differentiate between conspecifics and heterospecifics is another important visual task that these fish need to perform to successfully court females and defend their territories. Evidence has shown that color and pattern matter in Lake Malawi; female cichlids have been shown to mate preferentially with conspecifics (Couldridge & Alexander, 2002). Similarly, male cichlids have been shown to preferentially fight males with similar color and similar pattern (Dijkstra et al., 2006; Pauers et al. 2008). In fact, differently colored *M. zebra* exhibit assortative aggression towards conspecific-colored rivals over heterospecific-colored rivals (Holzberg, 1978; Wazlavek & Figler, 1989; Stauffer et al., 1997). This demonstrates that the body color of potential rivals is an important signal of their body pattern being received by the male cichlids of Lake Malawi.

Another potentially important visual signal from the body pattern of Lake Malawi signals is the egg spot. Egg spots are an area of yellow coloration on the anal fin of a male fish (Wickler, 1963). In some cichlids, this color has been shown to mimic the color of cichlid eggs (Gonzalez et al., 2023). There is a great deal of natural variation in the number, size, and appearance of egg spots both within and between species (Goldschmidt, 1991; Hert, 1989; Theis et al., 2012), but this unique phenotype has

even been identified as a key innovation that may have contributed to the explosive speciation of cichlids (Salzburger et al., 2005). Despite indication that this phenotype is important, the question of whether egg spots play a role as a visual signal between males and females in Lake Malawi is difficult to determine. While *M. zebra* and *M. aurora* prefer males with a greater number of egg spots over those with a fewer number of egg spots (Couldridge & Alexander, 2002; Hert, 1991), the closesly-related Malawi cichlid *Metriaclima lombardoi* prefer males with larger egg spots over males with smaller ones (Couldridge 2002), and female *Astatotilapia calliptera* exhibit no preference between males with or without egg spots (Theis et al., 2015). Although the exact role of egg spots in sexual selection remains unclear, the egg spot is clearly a relevant visual signal to some of the fish in Lake Malawi. Both body color and egg spots are visual signals being received by the cichlids of Lake Malawi and being able to distinguish these patterns is therefore a visual task that these fish must be able to do to receive information about the potential mates and rivals around them. Although there have been numerous studies affecting how pattern affects interactions between males and females, those are often under controlled laboratory settings in close quarters. We don't know how the visual signals conveyed by cichlid color pattern or egg spots operate over distance as opposed to in close quarters. For terrestrial animals that view objects through air, viewing distance has only small effects on perception. However, viewing objects through water is complicated by the significant scattering of light (Lythgoe, 1968; McFarland et al., 1979). As viewing distance increases, the amount of background light scattering into view causes the light reflecting from two objects to become more and more similar. This degrades

color discrimination over distance more rapidly than viewing through air (Wilkins et al. 2016) and makes distance a much more important factor to consider when considering fish vision. In addition, males display against different backgrounds in male competition and female choice contexts. When displaying to another male, males remain on their rocky territory, displaying against the background of rocks (Ribbinck et al., 1983). This reinforces ownership of territory, but is also a darker background. When displaying to a female, males leave their territory and rise to meet females approximately 1 m above the lek (Kocher, personal communication). In these instances, the males are displaying against the background of the space light before attempting to lead females back to their territory (Ribbink et al., 1983). This means that the visual tasks of courting females and assessing rival males occur against very different backgrounds, which provide different contrasts against the color patterns of the fish.

In this study, I use visual modeling to provide clarity and context on the everyday visual tasks performed by male *M. zebra*. First I calculate the distances at which *M. zebra* colors are discriminable from two biologically relevant backgrounds: the space light of Lake Malawi and the rocks on which *M. zebra* form territories. Then I use visual modeling to calculate the distances at which the discrimination can be made between *M. zebra* blue and conspecific black bar, conspecific yellow egg spots, yellow heterospecific *M. aurora*, blue heterospecific *M. callainos*, and blue heterospecific *C. afra*.

4.3 Materials and Methods

4.3.1 Study Species and Color Spectra

The Lake Malawi cichlid *Metriaclima zebra* was modeled for this study because its visual system is well-documented (Dalton et al., 2014) and the reflectance spectra of wild fish were available to use in the visual model. The species possess a trichromatic visual system with short, medium, and long wavelength sensitive photoreceptors (λmax = 368, 484, 528 nm; Parry et al., 2005).

Visual modeling was broken into three categories based on the biological context the model was meant to represent. First, I modeled male *M. zebra* courting females by calculating the discriminability over distance of *M. zebra* body colors (blue body, black bar, and yellow egg spot) against space light. Then, I modeled male *M. zebra* assessing conspecific rivals against the rocks of Lake Malawi by calculating the discriminability over distance of *M. zebra* body colors (body blue, body black, and egg spot yellow) against Lake Malawi rocks. Finally, I modeled scenarios of male *M. zebra* identifying con- and heterospecifics by calculating the discriminability over distance of *M. zebra*'s blue body against *M. zebra* black bar, *M. zebra* yellow egg spot, *M. aurora*'s yellow body, *M. callainos*'s blue body, and *C. afra*'s blue body. All spectra were taken from wild fish that live in Lake Malawi. Wherever multiple spectra for the fish were available, the most saturated spectra were selected so as to best represent the nuptial coloration of each species. The spectra used are previous measurements taken by Dalton et al. (2010) with light spectra taken from Sabbah et al. (2011, Table 4.1).

Table 4.1: Summary of previously measured spectra used in the visual model. All the relevant spectra used in the visual model are shown with detail about the type of spectra they represent and the original source in which each was measured and published.

Visual Modeling Spectra	Reference
M. zebra blue body (reflectance)	Dalton et al., 2010
M. zebra black body (reflectance)	Dalton et al., 2010
M. zebra yellow egg spot (reflectance)	Dalton et al., 2010
Lake Malawi (Otter Point) Space Light (5 m depth, radiance)	Sabbah et al., 2011
Lake Malawi (Otter Point) Irradiance (5 m depth)	Sabbah et al., 2011
Lake Malawi rocks (reflectance)	Dalton et al., 2010
M. aurora yellow body (reflectance)	Dalton et al., 2010
M. callainos blue body (reflectance)	Dalton et al., 2010
C. afra blue body (reflectance)	Dalton et al., 2010

4.3.2 Visual Model

I used the receptor noise limited (RNL) visual model to calculate the chromatic distance between each pair of the space light or measured colors (Vorobyev & Osorio, 1998; Kelber et al., 2003), specifically *M. zebra* blue body, *M. zebra* black bar, *M. zebra* egg spot, *M. aurora* yellow body, *M. callainos* blue body, and *C. afra* blue body. To calculate color discrimination, I first calculated quantum catch (Q_i), the number of photons captured for each of the three photoreceptor types *i*. Quantum catch is calculated slightly differently depending on whether one is calculating the photons captured from the space light (sidewelling radiance I_{RAD}) or the reflectance of an object (target colors *S*).

When calculating the quantum catch of the space light (I_{RAD}), I used the lens transmission (L), and the receptor spectral sensitivities of the photoreceptors (R_i) (Kelber et al. 2003). The photoreceptor sensitivities (R_i) were modeled using vitamin A_1 based visual pigments with peak wavelength sensitivities using the equations of Govordovskii et al. (2000). The visual model used in this study considers wavelengths of light from 350-700 nm.

$$Q_i = K_i \int_{350}^{700} R_i(\lambda) L(\lambda) I_{RAD}(\lambda) d\lambda$$

Here K_i is the von Kries factor (K_i), which is derived from the von Kries color constancy model, in which each receptor adapts independently to the sidewelling irradiance of Lake Malawi (I_{IRR}), and acts as a color constancy correction. In the model, we used the previously measured lens transmission (Hofmann et al., 2010) for *Metriaclima zebra* and the known maximum absorbance of each cone type in *M. zebra*.

$$K_i = \frac{1}{\int_{350}^{700} R_i(\lambda)L(\lambda)I_{IRR}(\lambda)d\lambda}$$

When calculating the quantum catch for the reflectance (such as the Lake Malawi rocks or the body colors of the relevant cichlids), the illuminant is the sidewelling irradiance of Lake Malawi (I_{IRR}). This is multiplied by the reflectance of an object (i.e. the target colors *S*). To account for the effects of viewing distance (*r*), two factors must be incorporated. First, the observed beam of reflected light from a fish color is exponentially attenuated with distance based on the water's attenuation coefficient (α). Second, the sidewelling radiance (I_{RAD}) is scattered into the reflected beam and exponentially increases with distance (Lythgoe 1968). The quantum catch as a function of viewing distances is therefore given by the following, which includes the lens transmission (L) and the photoreceptor sensitivities (R_i).

$$Q_i = K_i \int_{350}^{700} R_i(\lambda)L(\lambda)[I_{IRR}(\lambda)S(\lambda)e^{-\alpha r} + I_{RAD}(\lambda)(1 - e^{-\alpha r})]d\lambda$$

We then compare the quantum catch values of the two sources being compared using the RNL model, taking receptor noise into account (Vorobyev & Osorio, 1998; Vorobyev et al., 2001; Koshitaka et al., 2008; Escobar-Camacho et al., 2019). The quantum catches for any two sources (e.g. space light or for object reflectance) are comparable measures that are neurally compared and assessed as a ratio. This allows us to calculate ΔS, chromatic distance, whose units are just noticeable difference (JND), a measure of discriminability. To calculate chromatic distance, we first use the quantum catch (Q_i) for each of the three photoreceptor types, *i*, to calculate the contrast between the pair of colors Δf_i (Siddiqi et al., 2004).

$$\Delta f_i = \ln\left[\frac{Q_i(\text{color 1})}{Q_i(\text{color 2})}\right]$$

The second component of the RNL model is the receptor noise, represented by the Weber fraction (ω). The Weber fraction for a single photoreceptor *i* is:

$$\omega_i = \frac{v_i}{\sqrt{n_i}}$$

where relative receptor noise is *v* and the weber fraction is weighted by the number of photoreceptors of photoreceptor type *i* which is n_i (Vorobyev & Osorio, 1998; 2001; Champ et al., 2016). Additionally, we assigned a receptor noise value for each cone class (Koshitaka et al., 2008), assuming the long (L) receptor to have a noise value of 0.1 (Escobar-Camacho et al., in prep). The short (S) and medium (M) cone class noise values were calculated using their relative abundance in the retinal mosaic, which has an S:M:L cone ratio of 1:2:2 (Dalton et al., 2014).

By bringing together the chromatic contrast Δf_i and Weber fraction ω_i we can calculate the chromatic distance ΔS (Vorobyev & Osorio, 1998). ΔS was calculated for a trichromatic visual system:

$$\Delta S = \sqrt{\frac{\omega_S{}^2(\Delta f_L - \Delta f_M)^2 + \omega_M{}^2(\Delta f_L - \Delta f_S)^2 + \omega_L{}^2(\Delta f_S - \Delta f_M)^2}{(\omega_S\omega_M)^2 + (\omega_S\omega_L)^2 + (\omega_M\omega_L)^2}}$$

ΔS values greater than 1 indicate that the fish can distinguish the two colors, while ΔS values less than 1 indicate that fish cannot distinguish the two colors (Vorobyev & Osorio, 1998; Siddiqi et al., 2004; Lind & Kelber, 2009; Olsson et al., 2018).

4.4 Results

4.4.1 Courting Females

Chromatic distance between the space light of Lake Malawi and the body colors of *M. zebra* is considered to model how discriminable male *M. zebra* are from the background when leaving their territory to court females. We measured color discrimination for space light against *M. zebra* blue body, space light against *M. zebra* black bar, and space light against *M. zebra* yellow egg spot as a function of viewing distance. The chromatic distance between the space light and *M. zebra* blue body remains above 1 (and therefore discriminable) from a viewing distance of 0 m (3.61 JND) to a viewing distance to 5 m (0.93 JND) at which point the two stimuli become indiscriminable to the fish (Figure 4.1A). The chromatic distance between the space light and *M. zebra* black bar remains above 1 (and therefore discriminable) from a viewing distance of 0 m (3.83 JND) to a viewing distance to 6 m (0.97 JND) at which point the two stimuli become indiscriminable to the fish (Figure 4.1B). The chromatic distance between the space light and *M. zebra* yellow egg spot remains above 1 (and therefore discriminable) from a viewing distance of 0 m (3.08 JND) to a viewing distance to 3 m (0.76 JND) at which point the two stimuli become indiscriminable to the fish (Figure 4.1C). Chromatic distance values for each distance can be found in Table 4.2.

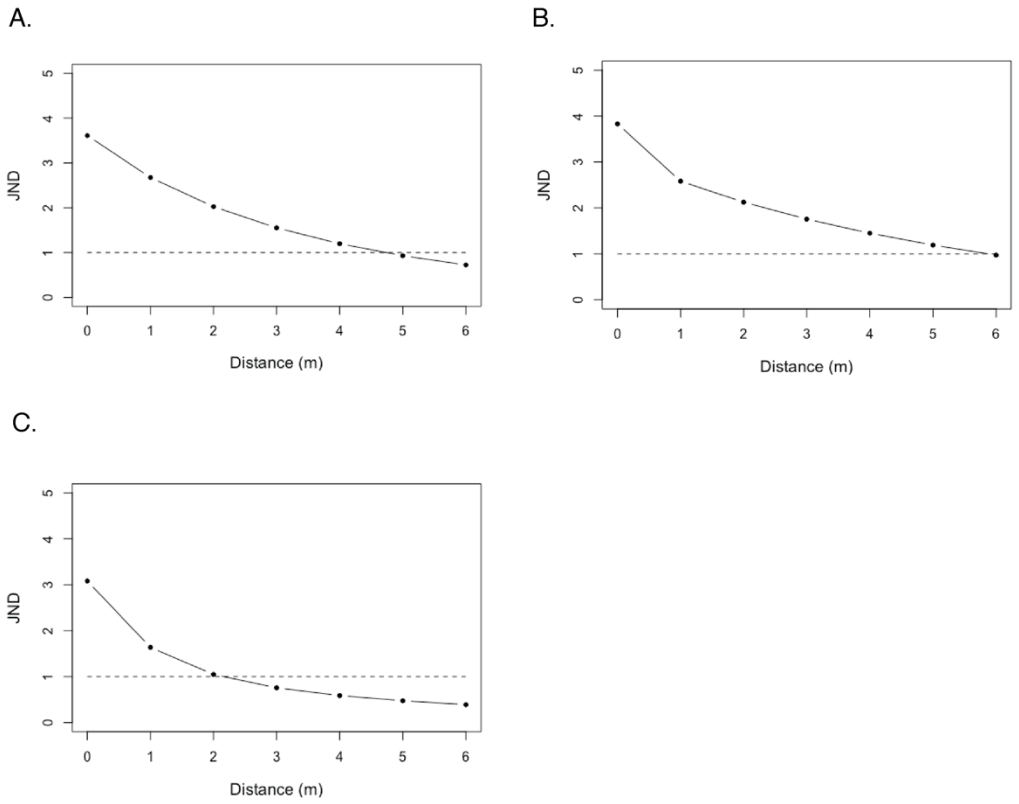


Figure 4.1: Discrimination of *M. zebra* body colors against space light in Lake Malawi. (A) The chromatic distance between the space light and *M. zebra* blue body remains above 1 to a viewing distance to approximately 4 m (1.20 JND). (B) The chromatic distance between the space light and *M. zebra* black bar remains above 1 to a viewing distance to approximately 5 m (1.19 JND). (C) The chromatic distance between the space light and *M. zebra* yellow egg spot remains above 1 (and therefore discriminable) to a viewing distance to approximately 2 m (1.05 JND).

Table 4.2: Chromatic distance values for each stimulus pair across viewing distance (*r*). The resulting chromatic distance (ΔS) values for viewing distance (r) for each stimulus pair calculated using the visual model. Each value is rounded to the hundredth place. Values greater than 1 indicate that the fish can distinguish the two stimuli at viewing distance (r) and are bolded.

Stimulus Pair	Viewing Distance (*r*) in meters						
	0	1	2	3	4	5	6
M. zebra blue body & space light	**3.61**	**2.68**	**2.03**	**1.55**	**1.20**	0.93	0.72
M. zebra black bar & space light	**3.83**	**2.58**	**2.12**	**1.76**	**1.45**	**1.19**	0.97
M. zebra yellow egg spot & space light	**3.08**	**1.64**	**1.05**	0.76	0.59	0.47	0.39
M. zebra blue body & rocks	**2.97**	**1.38**	0.97	0.75	0.60	0.49	0.40
M. zebra black bar & rocks	**2.01**	0.28	0.13	0.08	0.06	0.04	0.03
M. zebra yellow egg spot & rocks	**5.70**	**3.49**	**2.43**	**1.78**	**1.34**	**1.02**	0.79
M. zebra blue body *M. zebra* black bar	**1.14**	**1.12**	0.86	0.69	0.56	0.48	0.40
M. zebra blue body & *M. zebra* yellow egg spot	**6.62**	**4.10**	**2.74**	**1.91**	**1.37**	**1.00**	0.74
M. zebra blue body & *M. aurora* yellow body	**7.40**	**4.32**	**2.82**	**1.94**	**1.38**	**1.01**	0.75
M. zebra blue body & *M. callainos* blue body	0.96	0.63	0.41	0.27	0.17	0.11	0.08
M. zebra blue body & *C. afra* blue body	**2.69**	**1.81**	**1.26**	0.90	0.65	0.47	0.35

4.4.2 Assessing Males

Chromatic distance between the rocks of Lake Malawi and the body colors of *M. zebra* was considered to model how discriminable male *M. zebra* are from the background when assessing potential rivals intruding on their territory. We measured color discrimination for the rocks against *M. zebra* blue body, the rocks against *M. zebra* black bar, and the rocks against *M. zebra* yellow egg spot. The chromatic distance between the rocks and *M. zebra* blue body remains above 1 (and therefore discriminable) from a viewing distance of 0 m (2.97 JND) to a viewing distance of 2 m (0.97 JND) at which point the two colors become indiscriminable to the fish (Figure 4.2A). The chromatic distance between the rocks and *M. zebra* black bar remains above 1 (and therefore discriminable) from a viewing distance of 0 m (2.01 JND) to a viewing distance of 1 m (0.278 JND) at which point the two colors become indiscriminable to the fish (Figure 4.2B). The chromatic distance between the rocks and *M. zebra* yellow egg spot remains above 1 (and therefore discriminable) from a viewing distance of 0 m (5.70 JND) to a viewing distance to 6 m (0.79 JND) at which point the two colors become indiscriminable to the fish (Figure 4.2C). Chromatic distance values for each distance can be found in Table 4.2.

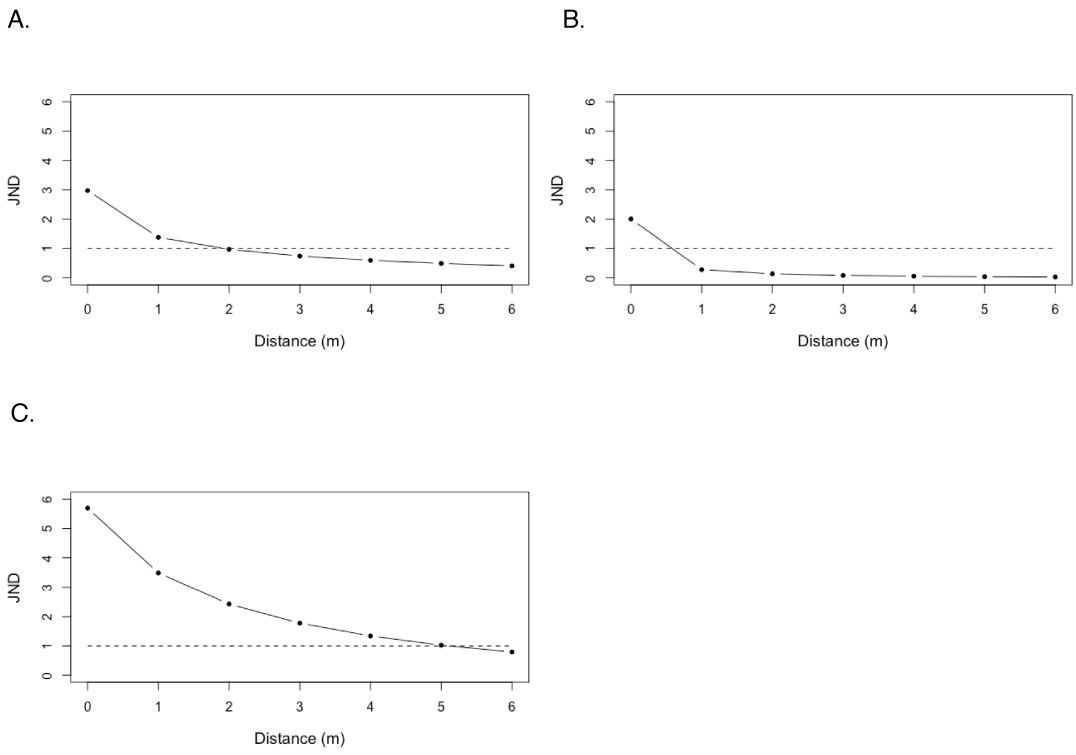


Figure 4.2: Discrimination of *M. zebra* body colors against the rocks in Lake Malawi. (A) The chromatic distance between the rocks and M. zebra blue body remains above 1 (and therefore discriminable) from a viewing distance of 0 m (2.97 JND) to a viewing distance of approximately 1 m (1.38 JND. (B) The chromatic distance between the rocks and M. zebra black bar remains above 1 (and therefore discriminable) from a viewing distance of 0 m (2.01 JND) before becoming indiscriminable. (C) The chromatic distance between the rocks and M. zebra yellow egg spot remains above 1 (and therefore discriminable) from a viewing distance of 0 m (5.70 JND) to a viewing distance to approximately 5 m (1.02 JND).

4.4.3 Identifying Con- and Heterospecifics

Chromatic distance between the blue body of *M. zebra* and the other body colors of conspecifics was considered to model if and at what viewing distances *M. zebra* can identify conspecifics solely by the visual signals of their body colors. We measured color discrimination for *M. zebra* blue body and *M. zebra* black bar, *M. zebra* blue body against *M. zebra* yellow egg spot. The chromatic distance between *M. zebra* blue body and *M. zebra* black bar remains above 1 (and therefore discriminable) from a viewing distance of 0 m (1.14 JND) to a viewing distance of 3 m (0.86 JND) at which point the two colors become indiscriminable to the fish (Figure 4.4A). The chromatic between *M. zebra* blue body and *M. zebra* yellow egg spot remains above 1 (and therefore discriminable) from a viewing distance of 0 m (6.62 JND) to a viewing distance of 6 m (0.74 JND) at which point the two colors become indiscriminable to the fish (Figure 4.4B). Chromatic distance values for each distance can be found in Table 4.2.

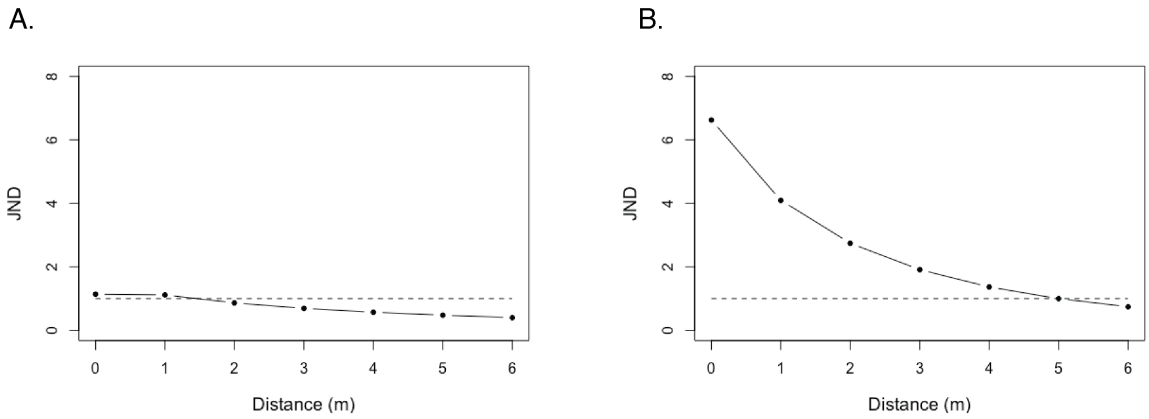


Figure 4.3: Discrimination of *M. zebra* blue body against the body colors of conspecifics in Lake Malawi. (A) The chromatic distance between *M. zebra* blue body and *M. zebra* black bar remains above 1 (and therefore discriminable) from a viewing distance of 0 m (1.14 JND) to a viewing distance of approximately 1 m (1.12 JND. (B) The chromatic between *M. zebra* blue body and *M. zebra* yellow egg spot remains above 1 (and therefore discriminable) from a viewing distance of 0 m (6.62 JND) to a viewing distance of approximately 5 m (1.00 JND).

Chromatic distance between the blue body of *M. zebra* and the body colors of heterospecifics was considered to model if and at what viewing distances *M. zebra* can identify con- and heterospecific fish solely by the visual signals of their body colors. We measured color discrimination for *M. zebra* blue body and *M. aurora* yellow body, the *M. zebra* blue body against *M. callainos* blue body, and *M. zebra* blue body against *C. afra* blue body. The chromatic distance between *M. zebra* blue body and *M. aurora* yellow body remains above 1 (and therefore discriminable) from a viewing distance of 0 m (7.40 JND) to a viewing distance of 6 m (0.75 JND) at which point the two stimuli become indiscriminable to the fish (Figure 4.4A). The

chromatic between *M. zebra* blue body and *C. afra* blue body is below 1 (and therefore not discriminable) from a viewing distance of 0 m (0.96 JND, Figure 4.4B). The chromatic distance between *M. zebra* blue body and *C. afra* blue body remains above 1 (and therefore discriminable) from a viewing distance of 0 m (2.69 JND) to a viewing distance of 3 m (0.90 JND) at which point the two stimuli become indiscriminable to the fish (Figure 4.4C). Chromatic distance values for each distance can be found in Table 4.2.

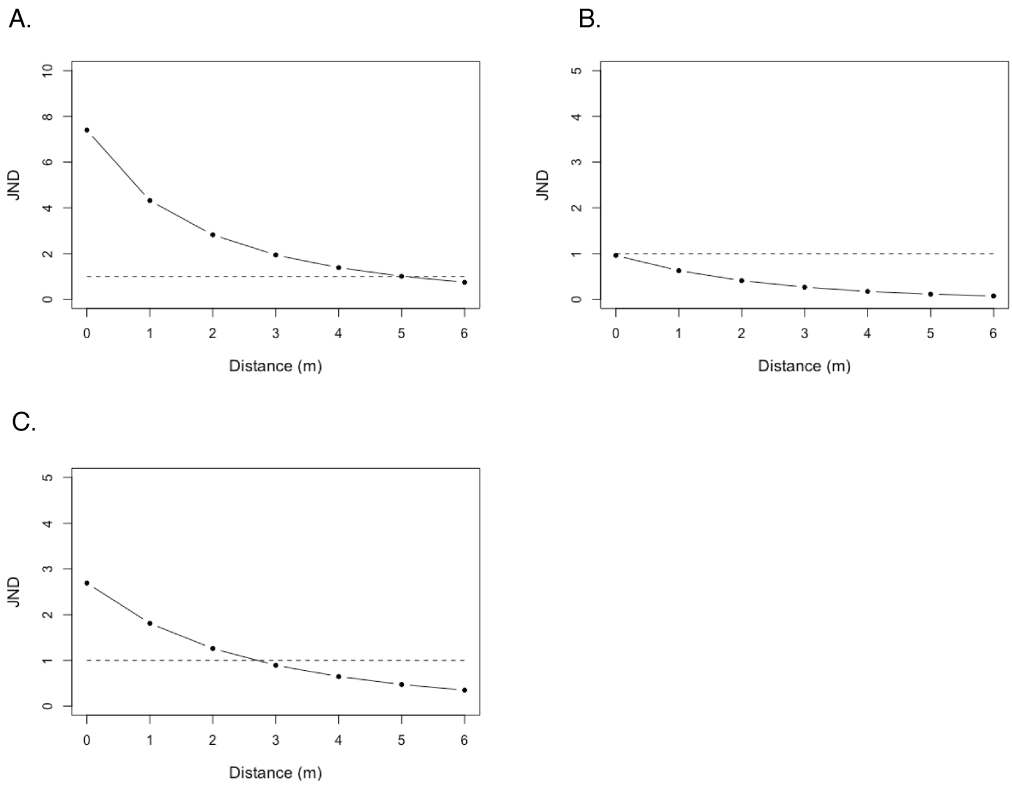


Figure 4.4: Discrimination of *M. zebra* blue body against the body colors of heterospecifics in Lake Malawi. (A) The chromatic distance between *M. zebra* blue body and *M. aurora* yellow body remains above 1 (and therefore discriminable) from a viewing distance of 0 m (7.40 JND) to a viewing distance of approximately 5 m (1.01 JND. (B) The chromatic between *M. zebra* blue body and *C. afra* blue body is below 1 (and therefore not discriminable) from a viewing distance of 0 m (0.96 JND). (C) The chromatic distance between *M. zebra* blue body and *C. afra* blue body remains above 1 (and therefore discriminable) from a viewing distance of 0 m (2.69 JND) to a viewing distance of approximately 2 m (1.26 JND).

4.5 Discussion

In this study, I calculated chromatic distance over multiple viewing distances for *M. zebra*, and determined that the blue body and black bars of *M. zebra* are discriminable against the space light of their habitat to a viewing distance of 5 meters, while *M. zebra* egg spots are more difficult to distinguish from the space light from further than 2 meters away. Conversely, I found that the blue body and black bars of *M. zebra* cannot be discriminated from the dark rocks of their habitat further than 1 or 2 meters, while *M. zebra* egg spots stand out better against the rocks to a viewing distance of 5 meters. Further, while the blue body of *M. zebra* is discriminable from the yellow body of heterospecific *M. aurora* out to 5 meters, my results show that it is indiscriminable from the blue body of heterospecific *M. callainos*. Surprisingly, the blue body of *M. zebra* can be discriminated from the blue body of heterospecific *C. afra* to a viewing distance of 2 meters. Integrating viewing distance into cichlid visual modeling is novel to this study and these results highlight that male *M. zebra* may be less conspicuous against their territories on the rocks than previously estimated. However, before interrogating the implications of the visual modeling, it is important to reflect on the limitations and knowledge gaps inherent to the visual model itself.

4.5.1 Photoreceptor Noise

The visual model used here improves upon my previous work by integrating viewing distance and light attenuation, something that significantly complicates underwater vision. The scattering of background light into and out of view affects the light reflected from two objects and makes them indiscriminable at smaller viewing

distances than when viewing the same two objects on land (Lythgoe, 1968). By accounting for this process in the visual model, we can more confidently evaluate how discriminable two objects are at increasing viewing distances underwater. However, an aspect of the visual model that has remained a limitation is our estimation of photoreceptor noise.

Vision in animals relies on photoreceptors, which have noise that puts a fundamental limit on their sensitivity. Noise in rods consists of photon-like events from spontaneous activation of photopigment rhodopsin and continuous activity of other components of the phototransduction cascade (Baylor et al., 1980; Baylor et al., 1984; Rieke & Baylor, 1986). To the best of our understanding (based on research in monkeys), quantal fluctuations are responsible for the noise in rod photoreceptors (Schneeweis & Schnapf, 2000). Therefore, detection sensitivity of rod responses scales with square root of the background light level (Schneeweis & Schnapf, 2000). However, color vision relies primarily on cone photoreceptors and noise is much less understood in cones. The rate of spontaneous activation is higher in cones than in rods (Rieke & Baylor, 2000), but research in primates indicates that noise originates downstream of the photopigment, mostly from gating transitions in cGMP-gated channels (Angueyra & Rieke, 2013). The relationship between signal to noise in cones is still not well understood, though research in trichromatic fish like *M. zebra* indicate that noise may vary across color space (Cheney et al., 2019; Escobar-Camacho et al., 2019; Green et al., 2022). In this study, I follow Weber's Law, assuming that receptor noise is consistent between receptor types and is proportional to the percentage of those receptor types present in the retina (Vorobyev & Osorio,

1998; 2001; Champ et al., 2016). The noise values in this model were calculated from behavioral experiments that found noise in the blue area of the color space to be 0.1 in the long wavelength-sensitive cones (Escobar-Camacho et al., in prep). This value was used because it is a more precise measure than our previous best estimate, which was a noise constant of 0.16 estimated to be a general cichlid noise constant (Escobar-Camacho et al., 2019). As such, the noise values used in this model represent our current best estimate of how noise affects the cones of these fish. But it is important to note that there is evidence in the Picasso triggerfish (*Rhinecanthus aculeatus*), another trichromatic fish, that discrimination thresholds estimated by this model may be higher than predicted for (1) colors that differ in saturation and (2) highly saturated colors distant from the achromatic point (Green et al., 2023). Although there is some evidence that color saturation becomes less impactful in underwater vision as viewing distance increases (Lythgoe, 1968), it is worth noting that some of the cichlid colors compared in this model are much more saturated than the colors they are being compared to. For example, the rocks of Lake Malawi and the black bars of *M. zebra* reflect light at levels of very low saturation and as such, they may be even less discriminable from the other colors they are compared to than estimated in this study. With this limitation of the visual model in mind, there are a number of notable takeaways from this visual model that may have impacts on our understanding of how female courtship, male assessment, and identifying heterospecifics in the rocky habitats of Lake Malawi.

4.5.2 Courting Females

Body color has been shown to be the dominant factor in the cichlid mate choice hierarchy (Seehausen & van Alpha, 1998) and plays a significant role in female choice (Maan et al., 2006; Seehausen et al. 2008; Selz et al., 2014). Female cichlids prefer males with more saturated body colors and more contrasting body colors (Pauers et al., 2004). The results of this study align with the literature, showing that male *M. zebra* court females against the background on which their body colors are most contrasting (Figure 4.1). After initial courtship, males attempt to lead females back to their territories (as described in Juntti et al., 2016) as part of their stereotyped reproductive behaviors. Once on their territories, *M. zebra* males are much less conspicuous against the rock background (Figure 4.3) except for their egg spots, which contrast against the rocks much more than they do against the space light (Figures 4.1, 4.3). This sheds interesting new light on Wickler's hypothesis, which states that cichlid egg spots are egg mimics that "trick" females into pecking at the male anal fin, thereby positioning the mouthbrooding females to increase fertilization (Wickler, 1963). In the time since this hypothesis, scientists have struggled to find consistent evidence as to the role of egg spots in female choice (reviewed in Tobler, 2006 and Table 3 of Gonzalez et al., 2023). Those results seem to indicate that egg spot number or size may be an important visual signal for female choice in Lake Malawi cichlids (Hert, 1991; Couldridge, 2002; Couldridge & Alexander, 2002). This study shows that egg spots are most conspicuous during circling and egg laying rather than during female assessment of male courtship efforts. Therefore, it may be, regardless of whether egg spots increase fertilization now or in evolutionary history,

that egg spots are unlikely to play a role in female choice for species wherein the males display to females in clear water habitats, the space light of which acts as a low-contrasting background for egg spots. In fact, this seems to provide further support for Wickler's hypothesis, indicating that the time when females are most likely to confuse eggs with egg spots is during circling and egg laying on the rocks. Further, this does leave open the possibility that egg spots could still play a role in male assessment and competition for such cichlid species.

4.5.3 Assessing Males

The results of this model indicate that when viewing conspecifics against the dark brown rocks of Lake Malawi, *M. zebra* cannot discriminate the blue body and black bar from the background from very far away and no further than 2 or 1 meters respectively. However, it is important to keep in mind the fishes' ability to perceive not just the individual colors, but also the pattern that the two colors form. Cichlids have a modest ability to discriminate two stimuli separated in space relative to the background, based on their visual acuity (Dalton et al., 2017). Because of their photoreceptor spacing, a 4 mm wide black bar cannot be resolved beyond a viewing distance of 2 meters by these fish (Dalton et al., 2017). The agreement of the fish's ability to discriminate black from blue with their visual acuity in discriminating the black-and-blue pattern may indicate that this visual signal is most useful at close distances of 2 meters or less. Interestingly, this coincides with the 2 meter radius of the average male territory (Genner, 1999). The overlapping of visual acuity, chromatic discrimination, and territory size may indicate that there is a not-yet-understood relationship between the cichlid vision system and male territoriality.

4.5.4 Identifying Con- and Heterospecifics

In assessing *M. zebra*'s ability to discriminate conspecifics from heterospecifics, we first evaluated how well they can discriminate the within-pattern colors of their own body from one another. Comparing *M. zebra*'s blue body to *M. zebra*'s black bar, the results indicate that the fish can discriminate between the blue and the black and therefore see the pattern on conspecifics in the habitat to a distance of 1 meter. This suggests that although the fish's visual acuity allows them to resolve the bars at a distance of 2 meters, they cannot resolve the bar pattern on other fish until they get even closer. Further, this suggests that males may only need to identify conspecifics at extremely short distances, such as when a potential rival is intruding on the territory. This finding aligns with our previous results that show visual tasks against the background of the Lake Malawi rocks may be happening at short viewing distances and the suggestion that male cichlids may not need to react to many stimuli outside of their territory. In contrast to these results, I found that *M. zebra* are able to discriminate *M. zebra* blue from *M. zebra* egg spots. The visual model shows that these two colors are conspicuous and discriminable across viewing distances out to 5 meters, demonstrating that it is a visual signal with high within-body contrast, and suggesting that egg spots are a salient signal which could play a role in cichlid social interactions such as male aggression.

The visual model indicates that *M. zebra* are able to discriminate between yellow fish and blue fish, given the high chromatic distance values across viewing distance produced by comparing *M. zebra*'s blue body and *M. aurora*'s yellow body. The fish can discriminate between conspecifics and *M. aurora* out to a viewing distance of

nearly 5 meters. However, *M. zebra* are unable to discriminate between conspecifics and blue heterospecifics such as *M. callainos*, which is indistinguishable from *M. zebra* even at a viewing distance of 0 meters, and *C. afra*, which is discriminable from *M. zebra* slightly beyond a viewing distance of 2 meters. This finding provides some support for the negative frequency-dependent hypothesis, demonstrating that fish with different body colors can be discriminated from conspecifics that may drive the assortative aggression some cichlids have demonstrated towards similarly-colored males (Dijkstra et al., 2006). To the best of my knowledge, evidence for this hypothesis has not yet been found in Lake Malawi and could represent a viable course of further research. In addition, these findings demonstrate that signals from other sensory pathways may play an important role in male competition and the identification of con- and heterospecifics. It also highlights the importance that pattern plays in allowing males to distinguish themselves from other, similarly-colored, heterospceifics. Research on this species and other haplochromine cichlids has demonstrated that acoustic signals (Simões et al., 2008; Bertucci et al., 2012; Amorim et al., 2019) and olfactory signals (Field et al., 2018) are sufficient to change a male's response to a potential rival. Of the less-studied sensory modalities, olfaction has also been demonstrated to be necessary to see female preference for conspecifics in Lake Malawi cichlids (Plenderleith et al. 2005). Continuing to explore the role of non-visual sensory modalities in cichlid sexual selection could prove fruitful and impact our understanding of the explosive radiation of east African cichlids.

4.6 Conclusion

In this study, I present a method for calculating chromatic distance across viewing distance by using light attenuation data. By using previously measured light and reflectance spectra taken at Lake Malawi, I calculated the chromatic distance as a function of viewing distance for stimulus pairs representing three everyday visual tasks performed by *M. zebra*: courting females, assessing males, and identifying con- and heterospecifics. These results suggest that *M. zebra* are conspicuous and discriminable against the space light of Lake Malawi out to a viewing distance of approximately 5 meters and *M. zebra* are discriminable against the rocks of Lake Malawi at shorter viewing distances to approximately 2 meters. I also found that although *M. zebra* egg spots are conspicuous and discriminable against *M. zebra*'s blue body, *M. zebra* blue and black are only discriminable at short viewing distances. In addition, while *M. zebra* can be distinguished from yellow *M. aurora* to viewing distances of 5 meters, from *C. afra* to viewing distances of 2 meters, but they are indistinguishable from blue *M. callainos*. Together, these findings provide support for avenues of future research on male competition and both con- and heterospecific identification.

Acknowledgements

I would like to thank Daniel Escobar-Camacho for the noise constant used in this study and his thoughts on the ecology of Lake Malawi. This work was supported by the National Eye Institute at the National Institutes of Health (R01EY024639B; KC) and the National Science Foundation (DEB-1830753; Thomas Kocher).

Compliance with Ethical Standards

This work has been completed under the approval of the University of Maryland's Institutional Animal Care and Use Committee (R-JUN-21-44).

Chapter 5: Synthesis

5.1 Introduction

The study of evolution has become one of the major branches in the field of biology since the time of Darwin and his contemporaries. Cichlids have long been identified as a powerful system within which to study evolution due to their recent explosive radiation in the lakes of the African rift valley and the surrounding rivers (Kocher et al. 2004). The explosive speciation of cichlids is often attributed to a number of factors ranging from habitat adaptation to sexual selection on cichlid body color and patterns (Holzberg 1978; Ribbink et al. 1983; Hert 1991; Kornfield & Smith 2000; Kocher et al. 2004). However, efforts to study these body colors and patterns, such as egg spots, have conflicted and produced an unclear picture of the role these visual signals play in cichlid reproduction and therefore cichlid speciation (reviewed in Tobler 2006). Given the great deal of research that has been conducted on the cichlid visual system, we are now able to use the receptor noise limited (RNL) visual model to provide clarity on the salience of egg spots and other relevant phenotypes to cichlid fish. The work presented in this thesis has focused on using a visual modeling approach to better understand cichlid ecology, behavior, and reproduction, the main results of which are summarized as follows.

5.2 Main Inferences

5.2.1 Cichlids cannot discriminate between the color of egg spots and eggs

In 1963, egg spots were described on *Haplochromis wingatii* and hypothesized to be egg mimics which played a role in fertilization for mouthbrooding cichlids (Wickler).

However, studies could not agree on whether either component of this hypothesis were supported by animal behavior (Jackson & Ribbink, 1975; Goldschmidt, 1991; Hert, 1989; Hert, 1991; Theis et al., 2012). Further, some studies found that egg spots played a role in female choice (Hert 1989; Hert 1991; Couldridge 2002; Couldridge & Alexander 2002) while others found that female choice was not acting on egg spots (Henning & Meyer, 2012; Theis et al., 2012; Theis et al., 2015). There was no emergent hypothesis or pattern that is able to satisfactorily answer whether the haplochromine egg spot played a role in reproduction. However, given that the RNL visual model provided an opportunity to examine the primary tenet of Wickler's hypothesis, it stood to reason that calculating the chromatic distance between egg spots and eggs would provide some clarity. By quantifying the reflectance of egg spots and eggs of laboratory-reared *M. benetos* and *A. burtoni*, I was able to determine that neither haplochromine species can discriminate between the color of the egg spots and the color of the egg. Although there is known to be some variance in the color of egg spots across cichlid species (Wickler, 1963, Lehtonen & Meyer, 2011), it stands to reason that these findings hold true for other lacustrine and riverine species related to these two species and living in similar visual environments.

5.2.2 *M. zebra* do not behave differentially towards differing virtual stimuli

The cichlid fish of east Africa have undergone an explosive speciation resulting in a great deal of phenotypic variation, producing visual signals which affect inter- and intraspecific communication (Holzberg, 1978; Ribbink et al., 1983; Dominey, 1984; Kocher, 2004; Albertson et al., 2005; Seehausen, 2006). However, some of these phenotypes, such as egg spots and size, have proven difficult to study because these

phenotypes are often correlated with other variables such as age, and hard to examine in isolation from other phenotypes (Lehtonin & Meyer, 2011). Virtual stimuli have been shown to allow researchers to robustly examine such questions due to a scientist's ability to create stimuli that differ only in the phenotype of focus (Baldauf et al., 2008; 2009; 2011; Chouinard-Thuly et al., 2017). In order to produce such virtual stimuli, I used the RNL visual model to create pairs of visually salient virtual stimuli that differed from one another only in singular phenotypes (egg spot presence, body color, movement, and size). The results showed that although *M. zebra* respond to virtual stimuli and live stimuli with equal aggression, these cichlids also respond with equal aggression towards the virtual stimuli that differ in egg spot presence, body color, movement, and size. Therefore, *M. zebra* do not distinguish between differential virtual stimuli, making this approach uninformative in this species.

5.2.3 *M. zebra* are more conspicuous and discriminable against space light than the rocks of Lake Malawi

Scientists have long studied how body color and pattern affects interactions between males and females, although often under controlled laboratory settings that necessitate close quarters. It is not well understood how such signals operate over distance, especially in underwater environments where the scattering of light limits the effectiveness of the signal (Lythgoe, 1968; McFarland et al., 1979; Wilkins et al., 2016). In addition, male cichlids display against different backgrounds in different contexts. When displaying to another male, males remain on the brown to red rocks of their territory (Ribbinck et al., 1983). In contrast, males leave their territory when courting females, displaying their color and patterns against the blue-green

background of the space light before attempting to lead females back to their territory (Ribbink et al., 1983). Since displaying against different backgrounds changes how visual signals are perceived, I calculated the chromatic distance as a function of viewing distance for *M.* zebra body colors against each background. These results indicate that *M. zebra* males are more conspicuous and discriminable against the space light of Lake Malawi than the rocks of Lake Malawi.

5.3 Future Directions

This thesis produced several key insights into the role of egg spots and other visual signals in cichlid sexual selection through careful and quantitative consideration of the cichlid visual system. By demonstrating the way in which important visual signals are conspicuous and discriminable in both the wild and the lab, I have shown that there is still much to learn both in how we quantify animal vision and how animal vision impacts behavior. Further, these studies have highlighted areas of study that require deeper examination to continue to unravel the importance of both egg spots and color vision in cichlid evolution.

5.3.1 The role of egg spots in sexual selection

Body color is a key feature used in mate choice in cichlids (Seehausen & van Alpha, 1998) and plays a significant role in female preference (Maan et al., 2006; Seehausen et al. 2008; Selz et al., 2014). Female cichlids prefer males with more saturated body colors and more contrasting body colors (Pauers et al., 2004). The results of Chapter 4 show that male *M. zebra* court females against the space light, where their body colors are most contrasting. This study demonstrates that once males have led females

back to their territories on the rocks, their blue-and-black bars are much less conspicuous against the rock background. However, while the fish are circling the egg spots come into view much more clearly due to the fish's visual acuity. In addition to seeing the egg spots more clearly, the yellow egg spots are discriminable and conspicuous against the blue and black colors of the male. Wickler's hypothesis raises the possibility that cichlid egg spots are egg mimics that "trick" females into pecking at the male anal fin, thereby positioning the mouthbrooding females to increase fertilization (Wickler, 1963). Scientists have struggled to find consistent evidence as to the role of egg spots in female choice (reviewed in Tobler, 2006 and Table 3 of Gonzalez et al., 2023). Those results seem to indicate that egg spot number or size may be an important visual signal for female choice in Lake Malawi cichlids (Hert, 1991; Couldridge, 2002; Couldridge & Alexander, 2002). We know from these studies that egg spots can only be seen when the male is (A) flaring and displaying the anal fin in courtship displays and reproduction and (B) at close enough viewing distances that the animal's visual acuity allows the egg spots to be perceived. This provides further support for Wickler's hypothesis, indicating that the time when females are most likely to confuse eggs with egg spots is during circling and egg laying on the rocks. Therefore, if egg spots play a role in female choice, it is more likely to be at the fertilization stage of mating than at the mate assessment stage, an important distinction that is not always separated in classic behavior assays that measure female choice by association and not actual fertilization. Further, this does leave open the possibility that egg spots could still play a role in male assessment and competition for such cichlid species. In addition, it suggests that studying the role of

egg spots in male-male competition, especially for cichlids who inhabit more turbid environments, is an opportunity to shed light on the function of this elusive phenotype.

5.3.2 The source and nature of photoreceptor noise in cichlids

Vision in animals relies on two kinds of photoreceptors, which have inherent noise that affect their perception of signals. In the first kind of photoreceptor, rods, noise has been well-documented and relatively understood (Baylor et al., 1980; Baylor et al., 1984; Rieke & Baylor, 1986; Schneeweis & Schnapf, 2000). However, noise is less documented and understood in the second type of photoreceptor: cones. Although cGMP-gated channels seem to be the source of noise in cones (Angueyra & Rieke, 2013), we have seen that noise in cones varies across the color space (Cheney et al., 2019; Escober-Camacho et al., 2019; Green et al., 2022; Escobar-Camacho et al., in prep). In these studies, I assume that receptor noise is consistent between receptor types and is proportional to the percentage of those receptor types in the retinal mosaic (Vorobyev & Osorio, 1998; 2001; Champ et al., 2016). However, as my understanding of noise evolves over time and new noise values become available, I attempt to use the most relevant and precise noise values, whether they come from behavior experiments in cichlids or triggerfish (Escobar-Camacho et al., 2019; Cheney et al., 2019; Escobar-Camacho et al., in prep). However, it remains that the field still does not have a strong grasp of where cone noise comes from. As such, the noise values used in these models represent our current best estimate of how noise affects the cones of these fish at the time these models were calculated. However, further research that combines quantitative and behavioral methodologies to

illuminate why noise changes with regards to the color space is an avenue for further research that could prove tremendously valuable to our understanding of color vision.

5.3.3 Multimodal sensation in conspecific and heterospecific identification

The results of Chapter 4 indicate that the *M. zebra* eye is worse at discriminating blue conspecifics and heterospecifics than the human eye. Although their visual acuity indicates that the fish should be able to resolve the bars of conspecific blue-and-black patterns at a viewing distance of 2 meters, the visual model demonstrates that their ability to discriminate between blue and black is constrained to viewing distances of 1 meter or less. This suggests that males may only be able to visually distinguish conspecific blue males from heterospecific blue fish at extremely short distances. This fits with trends that have been noted in Lake Victoria and the negative frequency-dependent hypothesis, indicating that male fish assortatively aggress towards same-colored males (Dijkstra et al., 2006). Further, research on haplochromine cichlids has shown both acoustic signals (Simões et al., 2008; Bertucci et al., 2012) and olfactory signals (Plenderleith et al. 2005; Field et al., 2018) impact male-male interactions and can increase or decrease aggression. The use of classical behavioral assays to examine the role of multimodal signals in modulating the aggression of Lake Malawi males towards both blue and yellow-bodied rivals could prove a fruitful exploration that enhances our understanding of the negative frequency-dependent hypothesis and the explosive radiation of east African cichlids.

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