

RESEARCH ARTICLE

Color discrimination thresholds in a cichlid fish: *Metriaclima benetos*

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ABSTRACT

Color vision is essential for animals as it allows them to detect, recognize and discriminate between colored objects. Studies analyzing color vision require an integrative approach, combining behavioral experiments, physiological models and quantitative analyses of photoreceptor stimulation. Here, we demonstrate, for the first time, the limits of chromatic discrimination in *Metriaclima benetos*, a rock-dwelling cichlid from Lake Malawi, using behavioral experiments and visual modeling. Fish were trained to discriminate between colored stimuli. Color discrimination thresholds were quantified by testing fish chromatic discrimination between the rewarded stimulus and distracter stimuli that varied in chromatic distance (ΔS). This was done under fluorescent lights alone and with additional violet lights. Our results provide two main outcomes. First, cichlid color discrimination thresholds correspond with predictions from the receptor noise limited (RNL) model but only if we assume a Weber fraction higher than the typical value of 5%. Second, cichlids may exhibit limited color constancy under certain lighting conditions as most individuals failed to discriminate colors when violet light was added. We further used the color discrimination thresholds obtained from these experiments to model color discrimination of actual fish colors and backgrounds under natural lighting for Lake Malawi. We found that, for *M. benetos*, blue is most chromatically contrasting against yellows and space-light, which might be important for discriminating male nuptial colorations and detecting males against the background. This study highlights the importance of lab-based behavioral experiments in understanding color vision and in parameterizing the assumptions of the RNL vision model for different species.

KEY WORDS: Behavior, Cichlids, Color constancy, Color discrimination threshold, Fish vision

INTRODUCTION

Behavioral investigations studying the relevance of color patterns in nature are essential for understanding the evolution of animal coloration patterns. One way to understand the adaptive significance of animal coloration is through color perception. Color vision is the capacity of discriminating color regardless of brightness. It is essential for many living organisms because it facilitates the

detection, discrimination and recognition of colored objects. Several behaviors can be mediated by color vision, such as predator or prey detection, finding mates, and other interspecific and intraspecific social interactions.

An integrative approach is necessary to comprehensively investigate color vision in animals, which combines physiological models, behavioral experiments and data on photoreceptor spectral sensitivities. Most vertebrates exhibit multiple spectral cone types, as has been shown through physiological (microspectrophotometry, MSP) (Bowmaker, 1984) and molecular experiments (opsin gene analysis) (Davies et al., 2012). However, behavioral experiments are necessary to confirm color vision because photoreceptors can be used for a variety of visual tasks including achromatic (luminance) vision, motion detection, polarized vision or phototaxis (Kelber and Osorio, 2010; Douglas and Hawryshyn, 1990). In addition, theoretical visual models have been developed to predict the visual capabilities of animals in studies of visual ecology; thus, behaviorally testing for color vision enables us to determine whether assumptions of theoretical visual models are met.

Color discrimination thresholds can be estimated using color vision models such as the receptor noise limited (RNL) model (Vorobyev and Osorio, 1998). This model, based on physiological principles, has two main assumptions: (1) chromatic contrast is achieved through color opponency, where signals from at least two spectrally different photoreceptors are compared, and (2) the limits of color discrimination are set by receptor noise, originating in the proximal visual pathway. The estimation of receptor noise is critical for the correct prediction of color discrimination thresholds (Bitton et al., 2017; Lind and Kelber, 2009). Noise in the receptor channel is related to the Weber fraction (ω). This is a constant that describes the signal-to-noise ratio that sets discrimination thresholds to the smallest difference in chromatic contrast that can be detected, a just noticeable difference (JND) (Lind et al., 2014; Olsson et al., 2018; Vorobyev et al., 1998). Thus, values below 1 JND are indicative of color pairs that are indistinguishable, while values above 1 JND are indicative of color pairs that can be distinguished (Siddiqi et al., 2004).

Receptor noise in single cells can be measured through electrophysiology experiments. It can also be estimated based on the relative number of photoreceptor cell types and knowing or assuming the noise for one photoreceptor type (Vorobyev et al., 2001). Noise can also be inferred by adjusting the noise parameter of the model such that an estimated threshold fits the behaviorally determined color discrimination threshold (Olsson et al., 2018). This is based on the assumption that if noise can be used to estimate the limits of color discrimination, then color-discrimination thresholds can also be used to estimate receptor noise (Olsson et al., 2018).

When viewing colored objects, environmental light may also influence color discrimination. Because the light spectrum hitting an object can change, the capacity of the visual system to perceive colors consistently regardless of changes in illumination may aid

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object recognition (Hurlbert, 2007). Without color constancy, color vision would be unreliable because colors would change under different illumination. Unsurprisingly, a wide range of animals exhibit color constancy (Olsson et al., 2016), including fish (Dörr and Neumeyer, 2000; Ingle, 1985; Neumeyer et al., 2002). One of the ways color constancy is achieved is through photoreceptor adaptation, where photoreceptors adapt to stimulus intensity by changing their sensitivity (Barbur and Spang, 2008; Kamermans et al., 1998). To account for color constancy, color vision models use the von Kries color constancy model where photoreceptors adapt independently to the background illumination (Dörr and Neumeyer, 1996; Neumeyer, 1981). One way to quantify color constancy is to perform vision-mediated behavioral experiments under different illuminations.

Fish are an ideal system to study color vision because of their diverse set of visual pigments and the highly variable light environments they inhabit. Behavioral methods are useful for quantifying the limits of color. These studies often use a classical conditioning approach where fish are trained to a reward stimulus to test for visual capabilities (Champ et al., 2016; Cheney et al., 2013; Escobar-Camacho et al., 2017; Mitchell et al., 2017; Neumeyer, 1992; Pignatelli et al., 2010; Risner et al., 2006; Siebeck et al., 2008, 2014). Characterizing the detection thresholds for chromatic signals is important because it will contribute to an understanding of color vision and the perception of coloration patterns in nature.

Among teleosts, cichlids are a great model for vision-mediated behavioral experiments in laboratory conditions (Escobar-Camacho et al., 2017; Satoh et al., 2016; Schluessel et al., 2012, 2014, 2015, 2018). Cichlids are one of the most diverse vertebrate clades, with approximately 2000 species widely distributed across the globe (Friedman et al., 2013; Turner et al., 2001; www.fishbase.org). Like many taxa, cichlids use color vision to detect, identify and discriminate different foods, offspring (if they are mouth-brooders) and sexual mates (Escobar-Camacho and Carleton, 2015; Price et al., 2008; Seehausen and van Alphen, 1998; Seehausen et al., 1997), as some can only be distinguished based on nuptial coloration patterns (Stauffer et al., 1997). Species of the genus *Metriaclima* are ideal for chromatic discrimination experiments because their visual system has been characterized through MSP, opsin gene expression (Carleton, 2009; Carleton et al., 2008; Jordan et al., 2006) and analysis of retinal anatomy (Dalton et al., 2017; Escobar-Camacho et al., 2017). They are also bold as they are highly territorial, which makes them excellent candidates for performing visual tasks. In this study, we used *Metriaclima benetos* (Stauffer et al., 1997), a rock-dwelling cichlid endemic to Mazinzi Reef in the southeastern arm of Lake Malawi. Like many other haplochromines, *M. benetos* exhibits sexual dimorphism where males are pale blue while females are brown with green highlights. Breeding males have a nuptial coloration of bright light blue (Stauffer et al., 1997). Females use this coloration to select a breeding partner from a large lek, which includes multiple conspecifics and multiple species. Color discrimination is therefore important for both species recognition and mate selection.

In a previous study, we showed that cichlids could be trained to discriminate between different colors regardless of brightness (Escobar-Camacho et al., 2017). Cichlids successfully discriminated a rewarded blue stimulus from several other colors (grays, yellows, red, green, brown, orange, pink). However, they made errors when discriminating blue from purple (Escobar-Camacho et al., 2017). To better understand the limits of cichlid color discrimination, we wanted to quantify color discrimination thresholds following previous studies (Champ et al., 2016; Cheney et al., 2019). Our goals were to

answer the following questions. (1) What are cichlid color discrimination thresholds and how do they compare with those predicted by the RNL model? (2) Does color discrimination change under different illumination? (3) How do these lab-based behavioral results help us understand and interpret cichlid visual tasks in the wild? In this study, we performed a series of color discrimination experiments and psychometric analyses, and measured color discrimination thresholds between a range of blue and purple stimuli. We also report the potential limitations of color constancy in cichlids and further discuss the color discrimination thresholds obtained in this research and their implications for color perception in the wild based on field data from Lake Malawi.

MATERIALS AND METHODS

Behavioral approach, fish training and testing

Fish were trained and tested in the Tropical Aquaculture facility at the University of Maryland, USA, under the guidelines of the University of Maryland Institutional Animal Care and Use Committee protocol (#R15-54). *Metriaclima benetos* were held in individual tanks of 26×50×30 cm (Fig. S1A) with continuous water flow from the same recirculating system, and were fed daily during training and testing periods. White paper was used to block the sides of the tank so that the fish could not see the experimenter or other fish.

We used seven adult lab-raised *M. benetos* individuals for our experiments. To measure discrimination thresholds, we used a similar approach to the experiments of von Frisch (1914), in which bees were trained to associate a reward with a specific colored stimulus chosen from among a number of distracter stimuli that varied in brightness. We trained fish to recognize blue as the rewarded stimulus through classical conditioning, and then tested their capacity to discriminate blue from other colors that varied in chromatic hue and brightness. We chose blue as our rewarded stimulus because blue is the primary body coloration of male *M. benetos* (Stauffer et al., 1997).

To train the fish, a feeding apparatus was created by attaching a feeding tube to a syringe filled with a mix of fish flakes and water (Fig. S1B). Initially, food was delivered in the front of the tank with the feeding apparatus. Once the fish learned to bite/tap the tube in order to obtain food, a flat-laminated, blue-colored circle was attached to the end above the tube using Velcro. This allowed the fish to begin to associate the color stimulus with food. After they learned to tap the blue stimulus attached to the feeder tube, a laminated card with a blue circle in the center was presented to the fish (Fig. S1C). Initially, the laminated card had the feeding tube attached just above the blue circle with Velcro. Finally, once the fish had become comfortable tapping the stimuli in the presence of the tube attached to the laminated card, they were shown a laminated card without the feeding tube. As soon as the fish tapped the card independently of food being present in the tank, they were given several preliminary assays (~30) in a binary choice test (see experiment 1) to confirm that they could discriminate blue from other colors (green, yellow and orange). This lasted for several months and when they succeeded 75% of the time, testing started. Training began in March and was completed by July 2017. All fish had different learning performance, with some learning faster than others, but after approximately 2–4 months, all fish were ready for testing.

During testing, in order to make sure fish could see all stimuli before choosing, they were lured towards the back of the tank with the feeding apparatus while the color cards were placed in the front

section of the tank. When the feeder was subsequently removed, the fish turned to make a choice (as per Escobar-Camacho et al., 2017); the experimenter could see which color stimulus the fish tapped with a mirror placed above the tank. Finally, in order to reinforce memory, the rewarded blue stimulus alone was presented to the fish before every testing session.

Fish were tested either once or twice a day (morning and afternoon) with five tests in each testing session for a total of up to 10 tests per day. However, fish did not always respond to a particular stimulus on a given day. Typically, fish were tested at least 3 times per week, though there were weeks when no testing was performed. Experiments were run between July 2017 and August 2018.

Visual modeling

For testing color discrimination thresholds, we analyzed how a series of colors differentially stimulates photoreceptors. This was done by calculating quantum catch (Q), which is the number of photons absorbed by a given photoreceptor. Calculation of quantum catch includes (1) the spectral cone sensitivities of *M. benetos*, (2) the reflectance spectrum of an object (e.g. colored circle), (3) the lens transmission and (4) the spectrum of environmental light (Fig. 1).

Metriaclima benetos exhibits three visual pigments with a peak absorbance (λ_{\max}) of 379, 489 and 522 nm (Fig. 1A) (Jordan et al., 2006). These are classified as short, medium and long (denoted by subscripts S, M and L, respectively) wavelength-sensitive cones. Quantum catch was calculated for each of these cone types using Eqn 1 (Dalton et al., 2010) where R_i is the sensitivity of receptor i , L is the lens transmission, S is the color reflectance, I is the

illuminant and K is the von Kries factor for receptor i :

$$Q = K_i \int_{300}^{750} R_i(\lambda)L(\lambda)S(\lambda)I(\lambda)d\lambda. \quad (1)$$

The spectral sensitivity $R_i(\lambda)$ represents the fraction of photons of wavelength absorbed by photoreceptor i (F_{abs}) (Johnsen, 2012):

$$R_i(\lambda) = F_{\text{abs}}(\lambda) = (1 - e^{-kA(\lambda)l}), \quad (2)$$

where k is the absorption coefficient of the photoreceptor at the peak absorption wavelength, $A(\lambda)$ is the wavelength-dependent absorbance of the photoreceptor normalized to a peak of 1 (Govardovskii et al., 2000) and l is the length of the photoreceptor outer segment in μm . Values of l and k were obtained from MSP measurements (Carleton et al., 2000; Dalton et al., 2014).

In addition, the von Kries factor (Eqn 3) is derived from the von Kries color constancy model in which each receptor adapts independently to the background illumination:

$$K_i = \frac{1}{\int R_i(\lambda)L(\lambda)I(\lambda)d\lambda}. \quad (3)$$

In order to obtain the luminance input from double cones, we also modeled combined quantum catches of double cones (Eqn 4), which are thought to mediate luminance vision in teleosts (Pignatelli et al., 2010; Siebeck et al., 2014):

$$Q_{\text{DC}} = \frac{Q_{\text{M}} + Q_{\text{L}}}{2}. \quad (4)$$

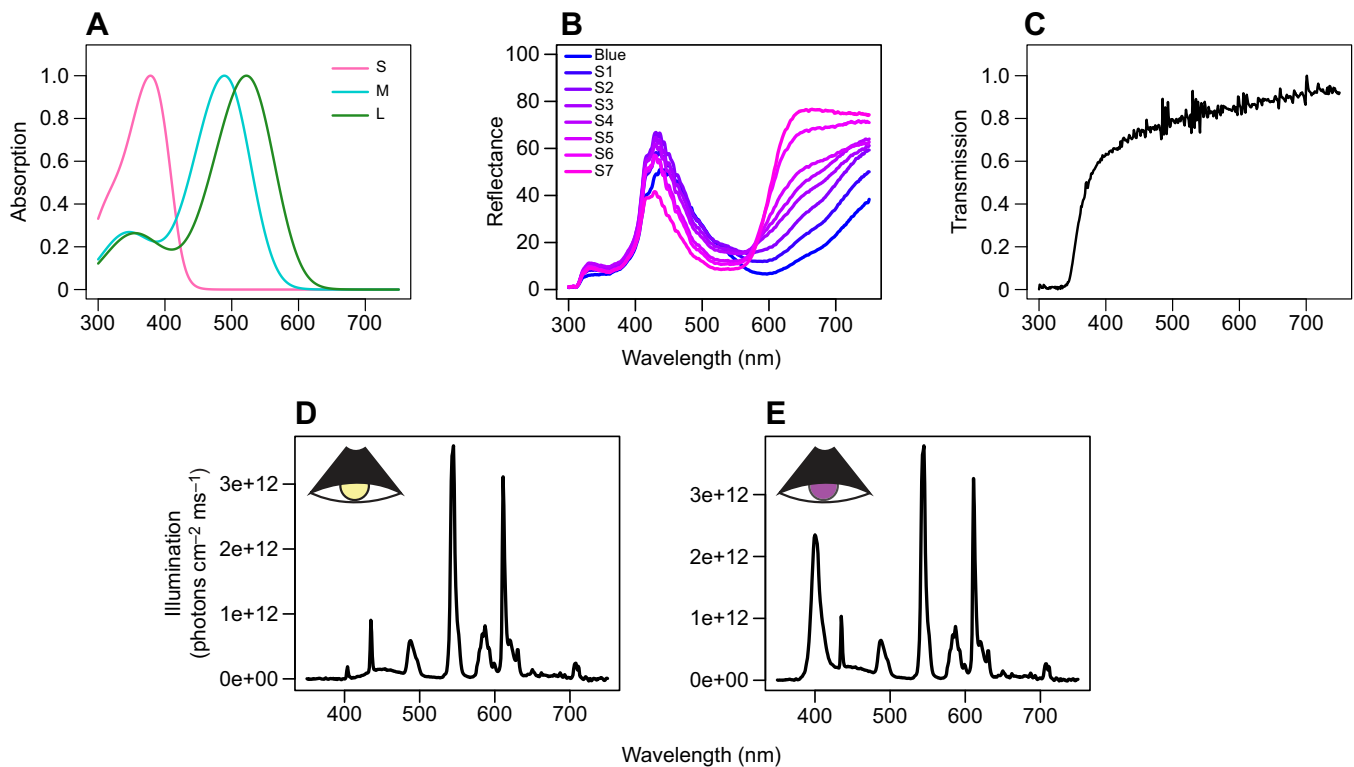


Fig. 1. Spectral parameters for color discrimination threshold experiments. (A) Spectral sensitivity of the short, medium and long (S, M and L) wavelength pigments present in single and double cones of *Metriaclima benetos* (Jordan et al., 2006). (B) Reflectance spectra of blue and distracter stimuli (S1–S7). (C) Normalized *M. benetos* lens transmission spectra (Escobar-Camacho et al., 2017) (Table S1C). (D,E) Aquarium side-welling irradiance of fluorescent (D) and violet lights (E). These were measured with white paper attached to the sides and front of the tank.

Quantum catch allowed us to calculate chromatic contrast between the different colors for which fish were tested. For this, we applied the RNL model (Vorobyev and Osorio, 1998). Therefore, we used quantum catch of each cone class (i) to calculate contrast between pairs of colors Δf_i (Siddiqi et al., 2004) (Eqn 5):

$$\Delta f_i = \ln \left[\frac{Q_i(\text{color 1})}{Q_i(\text{color 2})} \right]. \quad (5)$$

Furthermore, color discrimination is determined by receptor noise. The standard deviation of the noise in a single cone channel (v) is related to the Weber fraction (ω) for each photoreceptor type (i) by $\omega = v/\sqrt{n_i}$, where n is the number of receptors of type i . We can assign receptor noise for each cone class (Eqn 6) by first assigning the noise value to the long (L) receptor and then calculating the noise values of the short (S) and medium (M) cone classes based on their relative abundance in the retinal mosaic. For *M. benetos*, the S:M:L ratio is 1:2:2 (Escobar-Camacho et al., 2017). Even though estimates of noise can vary among animals (Olsson et al., 2018), in this study we set the standard deviation of noise (v) of the L receptor to 0.05 because it has been used in previous color-vision research studying several organisms (Koshitaka et al., 2008; Schaefer et al., 2007; Vorobyev, 2003), including fish (Champ et al., 2016). This gives us a relative noise value of 0.07 for S cones and 0.05 for M and L cones:

$$\omega_i = 0.05 \sqrt{\frac{n_L}{n_i}}. \quad (6)$$

Photoreceptor noise determines the smallest chromatic contrast (ΔS) that can be detected between two colors (Eqn 7) in units of JND. ΔS represents the chromatic distance of two colors in the perceptual color space where values below 1 JND are indicative of colors that cannot be discriminated:

$$\Delta S = \sqrt{\frac{\omega_S^2(\Delta f_L - \Delta f_M)^2 + \omega_M^2(\Delta f_L - \Delta f_S)^2 + \omega_L^2(\Delta f_S - \Delta f_M)^2}{(\omega_S \omega_M)^2 + (\omega_S \omega_L)^2 + (\omega_M \omega_L)^2}}. \quad (7)$$

We calculated the chromatic distance of each of the distracter stimuli versus rewarded blue stimulus. Then, we behaviorally determined the color discrimination threshold when fish chose the rewarded stimulus more than the distracter stimuli (at 65% correct choices, see 'Data analysis', below). Finally, as ω is inversely related to ΔS , we can also experimentally estimate individual receptor noise by fitting behavioral color discrimination thresholds between a series of stimuli where ΔS is 1 JND.

Calibration of colored stimuli and quantum catch

To calibrate visual stimuli used in this experiment, we first measured the reflectance of several colors with different RGB values that were printed on multipurpose recycled paper (Eagle Office 30, brightness 92) and were subsequently laminated. We then selected a series of colors that gradually moved away from blue in color space. Color cards were made by printing single colored circles (~1.5 cm diameter). Distracter colors were designed so that they would differentially stimulate the short and long wavelength-sensitive cones of *M. benetos* (Fig. 1A). To do this, we increased red and decreased blue intensity using Adobe Illustrator. Reflectance of colored stimuli was measured using a fiber-optic attached to a Flame spectrometer (Ocean Optics, Dunedin, FL, USA) fitted with a 400 μm fiber and calibrated with a NIST (National Institute of Standards and Technology) traceable tungsten halogen lamp (LS-1, Ocean Optics) (Table S1A). Side-welling irradiance was measured inside the tanks under fluorescent and fluorescent plus violet LED light with a 1000 μm fiber fitted with a cosine corrector (CC-3, Ocean Optics) (Fig. 1D,E; Table S1B). Side-welling irradiance was measured with white paper attached to the sides and front of the tank to match conditions during behavioral trials. For the rest of this article, we will refer to light environments as fluorescent and violet.

Quantum catch calculations suggest that the S, M and L cones are differentially stimulated by blue and purple colors (Table 1, Fig. 2; Fig. S2A–D). Distracter stimuli move across the perceptual color space, starting with pink and becoming progressively more similar to blue, with S7 being the most different to blue and S1 being the most similar (Fig. 2). This successive pattern can be observed in the

Table 1. Quantum catch corresponding to rewarded and distracter stimuli and for white background under different illuminations

Color	Light	Von Kries			ΔS (JND)	Without Von Kries			ΔS (JND)
		Q_S	Q_M	Q_L		Q_S	Q_M	Q_L	
	White								
B		36.26	25.08	17.20		2.94e+03	9.50e+05	1.31e+06	
S1		42.44	26.48	19.39	1.28	3.45e+03	1.00e+06	1.47e+06	1.28
S2		47.98	27.90	20.95	2.07	3.90e+03	1.06e+06	1.59e+06	2.07
S3		45.75	25.76	20.15	2.57	3.71e+03	9.76e+05	1.53e+06	2.57
S4		45.19	23.98	19.15	3.22	3.67e+03	9.08e+05	1.46e+06	3.22
S5		40.51	19.59	15.90	4.20	3.29e+03	7.42e+05	1.21e+06	4.20
S6		40.92	18.06	15.23	5.26	3.32e+03	6.84e+05	1.16e+06	5.26
S7		31.74	13.97	12.80	5.64	2.58e+03	5.29e+05	9.73e+05	5.64
W		88.62	93.58	90.52	8.97	7.19e+03	3.54e+06	6.88e+06	8.97
	Violet								
B		21.05	25.81	18.01		5.23e+04	1.32e+06	1.59e+06	
S1		24.80	27.71	20.30	1.11	6.16e+04	1.42e+06	1.80e+06	1.11
S2		27.65	29.54	21.97	1.61	6.87e+04	1.51e+06	1.94e+06	1.61
S3		25.78	27.38	21.04	1.83	6.41e+04	1.40e+06	1.86e+06	1.83
S4		27.37	26.00	20.15	2.97	6.80e+04	1.33e+06	1.78e+06	2.97
S5		23.37	21.59	16.80	3.28	5.81e+04	1.10e+06	1.49e+06	3.28
S6		24.22	20.41	16.19	4.33	6.02e+04	1.04e+06	1.43e+06	4.33
S7		21.30	16.06	13.58	5.63	5.29e+04	8.21e+05	1.20e+06	5.63
W		77.10	91.65	89.92	5.13	1.92e+05	4.69e+06	7.96e+06	5.13

Q denotes quantum catch from the short, medium and long cone (S, M and L, respectively). Chromatic contrast (ΔS) between blue and different colors was calculated assuming a noise standard deviation (v) of 0.05. JND, just noticeable difference; B, blue stimulus; W, white background.

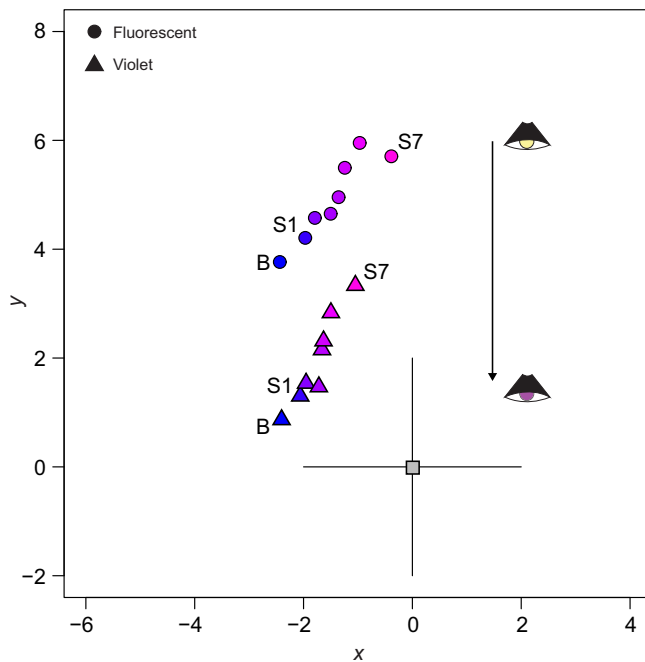


Fig. 2. Chromaticity diagram of the color space of *M. benetos* based on the receptor noise-limited (RNL) model. Color stimuli presented to the fish showing their relative positions in the color space under fluorescent and violet light with respect to the white background of the color cards (gray square). The color space here is represented as a two-dimensional color opponent diagram from the RNL model where the x- and y-axis are defined in eqn 4 of Hempel De Ibarra et al. (2001), but based on the spectral sensitivities of *M. benetos*. Axes are related to receptor signals. Here, $f_i = \ln(q_i)$, where q_i is the quantum catch ratio of the color to the background, $x = A(f_L - f_M)$ and $y = B[f_S - (af_L + bf_M)]$, where:

$$A = \sqrt{\frac{1}{\omega_M^2 + \omega_L^2}},$$

$$B = \sqrt{\frac{\omega_M^2 + \omega_L^2}{(\omega_S \omega_M)^2 + (\omega_S \omega_L)^2 + (\omega_M \omega_L)^2}},$$

$$a = \frac{\omega_M^2}{\omega_M^2 + \omega_L^2},$$

$$b = \frac{\omega_L^2}{\omega_M^2 + \omega_L^2}.$$

An arrow indicates the shift of the loci of colors when illuminated under different light.

cichlid perceptual color space with both fluorescent and violet light (Fig. 2). In our modeling, we noticed that the location of all stimuli shifted toward the achromatic point in the color space when we included violet light (Fig. 2). Furthermore, ΔS calculations confirmed our previous observations of stimuli in the perceptual color space, with stimulus S1 being closest to blue and S7 being the most distant (Fig. S2E–H). Our model shows that the distracter stimuli distances gradually increased in the color space from the blue reward stimulus (Fig. 2).

Experiment 1: color discrimination threshold between blue and purple

This experiment consisted of an array of binary choice tests where fish had to choose between two cards with one color circle on each (Fig. S1D, Movie 1). Fish ($n=7$) had to choose between the rewarded blue stimulus and a series of purple–violet distracter stimuli. As soon as the fish tapped one of the color cards, both were

removed and the fish was rewarded if it chose correctly, and the test ended. In order to avoid bias for one side of the tank, the same color was not presented on the same side more than 2 times in a row. If the fish did not respond to the stimuli for 2 min, it was not rewarded and the test was not counted. For testing, the sequence of presentation was from the most chromatically distant distracter stimulus (S7 versus blue) and finished with the chromatically closest distracter stimulus (S1 versus blue). This was split into two testing periods. We first performed 25 tests for all the fish and all the colors, starting with S7 and working through the color distracters to S1. Each fish completed 25 tests with a given color before proceeding to the next color. Then, we performed an additional 15 tests, again starting with S7 and proceeding to S1. In this way, we completed a total of 40 tests for each fish with each distracter color paired with blue.

Experiment 2: multiple choice with brightness variation

Color stimuli in experiment 1 were located along a line in SML color space, but were not controlled for brightness. To make sure that fish were discriminating target blue from distracter purples based on chromatic cues alone and not luminance, we added luminance noise to experiment 2. The threshold interval (when fish significantly discriminated blue from purple) in experiment 1 was between distracter stimuli S3, S4 and S5. We introduced luminance noise by using a multiple-choice test where fish were asked to choose blue from seven distracters on an eight-choice colored card (Fig. S1H). This color card contained blue, one of the distracter stimuli from the threshold interval (S3, S4, S5) and six other distracter stimuli (Fig. S1E,H, Movie 1). The six distracter stimuli were all similar in ΔS (within 0.5 JNDs) relative to S3, S4 and S5 but differed in brightness (Fig. S3). The brightness based on the luminance channel was calculated as the average quantum catch from the M and L cones (Fig. S3G–I) (Escobar-Camacho et al., 2017; Pignatelli et al., 2010). Cards in this experiment contained eight colors each and four card arrangements were designed for each of the threshold interval colors. Each of these color card arrangements was presented to the fish six times in a random fashion, adding up a total of 24 tests per distracter stimulus (S3, S4 and S5, respectively) (Fig. S1E,H) (Escobar-Camacho et al., 2017). The sequence of presentation was from the most chromatically distant (S5) to the chromatically closest stimulus (S3) with all tests for a given stimulus completed before proceeding to the next.

Experiment 3: color discrimination threshold with different background illumination

This experiment was similar to experiment 1, but quantified the color discrimination threshold under violet light. We performed this experiment to examine whether color discrimination thresholds changed when stimulation of the short wavelength (UV)-sensitive cone was increased. Therefore, experimental procedures were the same as in experiment 1, but fish were tested under both LED black lights (OPPSK 27 W 9 LED UV) and fluorescent lights to produce our violet treatment (Movie 1). The sequence of presentation of stimuli was the same as for experiment 1, where we first completed a set of 25 tests followed by 15 additional tests.

Cichlid visual modeling in Lake Malawi

To consider the implications of cichlid color discrimination, we used the RNL model to calculate color discrimination of the blue of *Metriaclima* species versus a variety of backgrounds and other cichlid colors. ΔS between blue and different spectra were calculated employing our new noise estimations obtained from experiment 1. Cichlid color reflectance data were taken from previous

measurements (Dalton et al., 2010). Briefly, fish color reflectance was measured from live specimens where fish were illuminated at 45 deg relative to their surface with a pulsed xenon lamp (PX2, Ocean Optics). Reflected spectra were collected with a bifurcated optical fiber (Ocean Optics) where light was collected at the same 45 deg angle as the illuminant (Dalton et al., 2010).

For this study, substrate background spectra for grasses, sand, rocks and mud were measured using Sub-spec II, a submersible fiber-optic spectrometer based on an Ocean Optics USB2000, fitted with a 50, 100 or 400 μm fiber, and calibrated with a tungsten halogen lamp (LS-1, Ocean Optics). Substrate reflectance spectra were obtained by measuring the substrate reflectance compared with that of a white Teflon standard placed at each sampling site. Either down-welling light or a high-intensity quartz halogen lamp (Light and Motion, Monterey, CA, USA) was used to illuminate substrates. These measurements were taken at two locations: the south side of Thumbi West Island near Mitande Point (latitude 14°1'23"S, longitude 34°49'27"E) and the east side of Otter Point (latitude 14°2'17"S, longitude 34°49'22"E). All reflectance spectra are given in Table S1D (Fig. S4). Finally, we also compared cichlid colors against background space light. Sidewelling radiance near Otter Island and the Mawlamba Bay on the north side of Thumbi West Island was obtained from Sabbah et al. (2011) and S. Sabbah, personal communication (Table S1D, Fig. S4C).

For our modeling with Lake Malawi data, we measured the blue reflectance of *M. benetos* in the lab, following the same methods as for quantifying reflectance of color targets, and we calculated ΔS between blue and other fish colors and environmental light spectra.

Quantum catch for different cichlid colors and different substrates was calculated using Eqn 1 but with the illuminant (I) being the Lake Malawi side-welling irradiance (data from Thumbi West Island at a depth of 3 m from Dalton et al., 2010, 2017; Table S1D; although previously published, the data here are presented on an absolute scale). For the color blue viewed against the natural space light, we calculated the quantum catch of side-welling radiance as one of the targets. In that case, the quantum catch is calculated where ($S \times I$) is the side-welling radiance. These quantum catches were then used in Eqn 7 to determine the ΔS between blue and several colors.

Data analysis

For experiments 1, 2 and 3, a one-tailed binomial test was used to calculate whether fish discriminated the rewarded from the distracter stimuli. The number of correct tests was compared with the distribution of taps if fish had chosen randomly (50% of the time for experiment 1 and 3, and 12.5% for experiment 2). Therefore, the threshold discrimination was established at 65% of correct choices ($n=40$, $P<0.05$, one-tailed binomial test) for experiments 1 and 3, and at 29% of correct choices ($n=24$, $P<0.05$, one-tailed binomial test) for experiment 2. Confidence intervals were calculated assuming a binomial distribution. Finally, we fitted our behavioral results with psychometric curves, where a generalized linear model (GLM) is applied to the dataset to fit a logistic regression, to see the relationship between ΔS and the proportion of correct choices (Champ et al., 2016; Olsson et al., 2015). All visual modeling calculations and binomial tests were done in R; in addition, we used 'psyphy' and 'modelfree' packages for estimating psychometric functions (www.r-project.org/)

RESULTS

Experiment 1: color discrimination threshold between blue and purple

For this experiment, a total of 1960 tests were performed ($n=7$ fish, $n=7$ color pairs, $n=40$ tests per color card). In general, all fish

exhibited significant discrimination of blue over colors S5, S6 and S7, with proportions of correct choices of 70%, 80% and 90%, respectively ($n=40$, $P<0.008$, $P<0.001$ and $P<0.001$, respectively) (Fig. 3A). There was considerable variation among individuals (Fig. S5B,C), and not all were able to significantly discriminate blue from stimuli S3 and S4, with a wide range of correct choice frequencies (55–72% and 50–70% for S3 and S4, respectively) (Table S1E). All fish failed to discriminate blue from colors S1 and S2, with correct choice frequencies of 55% ($P<0.317$, $n=40$) for both stimuli.

The stimulus closest to each fish's behavioral threshold (when fish choice frequency of blue over distracter stimuli reached statistical significance) was S4. With a standard deviation of noise (ν) of 0.05, this behavioral threshold had a ΔS of 3.4 ± 0.52 (Fig. 3A).

Experiment 2: multiple choice with brightness variation

For this experiment, a total of 432 tests were performed using the 8-choice color card ($n=6$ fish, $n=12$ color card arrangements, $n=6$ tests per color card). Fish were more likely to choose blue versus distracters S4- and S5-like stimuli with correct choice frequencies of 35–40% ($n=24$, $0.006<P<0.0001$) but they failed to choose blue over distracter stimuli similar to S3 (correct choice frequency of 24%, $n=24$, $P=0.07$) (Fig. 3B) (Table S1F). This confirmed our results from experiment 1, which suggested the color discrimination threshold occurs between colors S3 and S4 (Fig. 3A). Furthermore, these results show that fish are not relying on brightness for discriminating blue versus distracter stimuli in experiment 1.

Experiment 3: color discrimination threshold with different background illumination

For this experiment, a total of 1680 pairwise tests were performed ($n=6$ fish, $n=7$ color pairs, $n=40$ tests per color card). Surprisingly, in this experiment, most fish failed to discriminate blue from any distracter stimuli, S1–S7, with correct choice frequencies ranging from 48% to 62% (Fig. 3C). We again observed individual variation where some fish significantly discriminated blue from stimuli S3, S4, S6 and S7 yet not with high significance overall (Table S1G). Our results from this experiment show that fish cannot optimally discriminate colors under violet illumination (Figs 3C and 4). These results also suggest that either the behavioral chromatic threshold would occur at greater ΔS as predicted by the psychometric functions or that photoreceptor noise may be greater with this type of violet illumination.

Cichlid visual modeling in Lake Malawi

Chromatic distance for cichlid blue versus the different substrates (Fig. 5A) and different cichlid colors (Fig. 5B) suggests that blue has high chromatic contrast with space light and long wavelength colors. There were some cichlid colors where cichlid blue had low color contrast, including black, blue–green, green and white. The cichlid blue spectrum was quite broad and not a very saturated color, making it difficult to distinguish from other broad color hues (Fig. S4). However, it did show high contrast with colors such as yellow, orange and red.

DISCUSSION

Color discrimination thresholds and receptor noise

In this study, we confirmed that *M. benetos* possess color vision because fish were able to discriminate the rewarded from the distracter stimuli regardless of brightness. Our results also allowed us to compare the predictions of the RNL model with our behavioral discrimination assays. We used a standard deviation of noise (ν) of

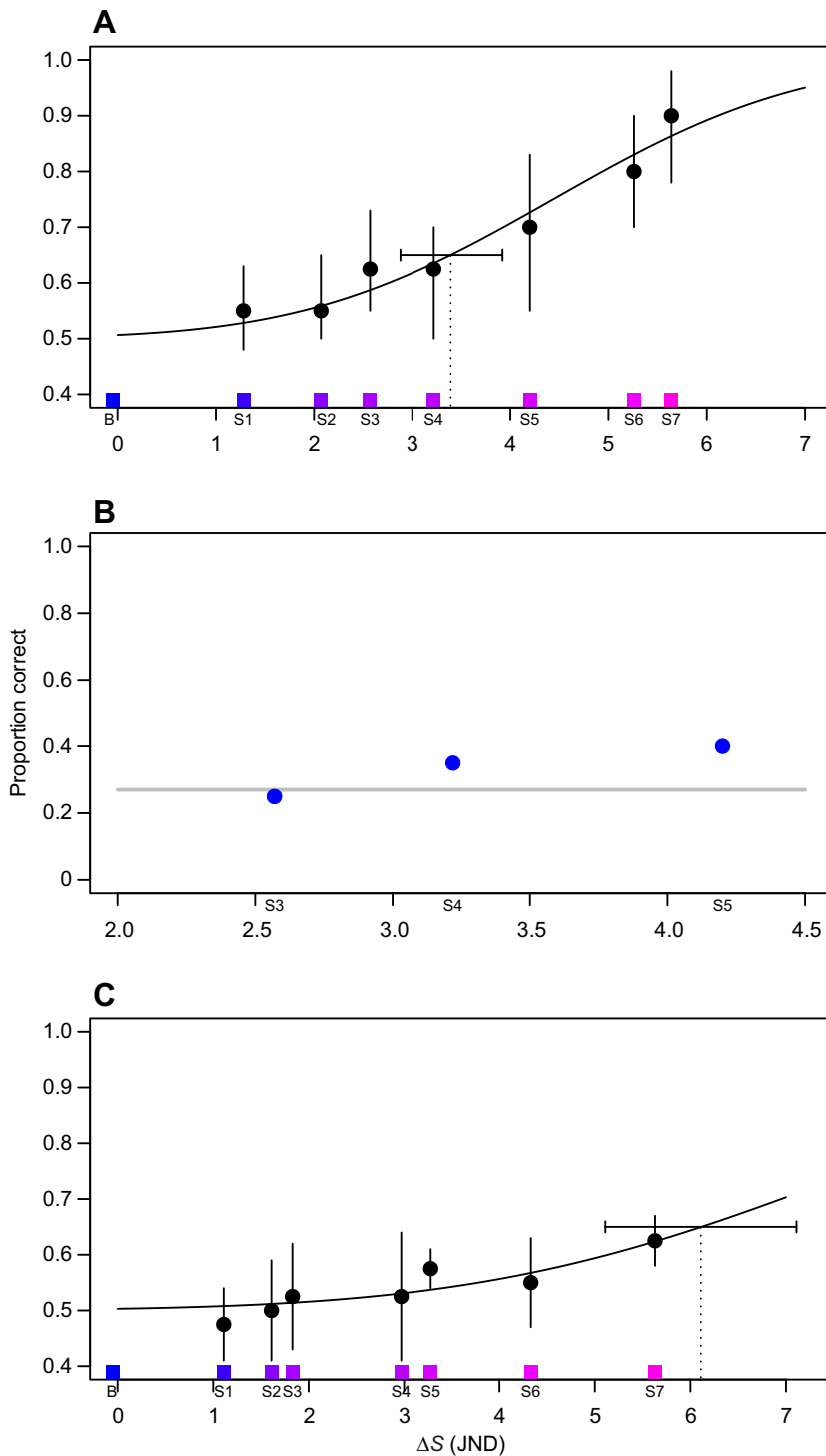


Fig. 3. Color discrimination in the three experiments. (A) Color discrimination thresholds under fluorescent light (experiment 1) where the proportion of correct choices is shown as a function of chromatic contrast (ΔS) (just noticeable differences, JNDs). ΔS was estimated using a standard deviation of noise (v) of 0.05. The x-axis represents ΔS between colors. Filled circles denote the average fraction of correct choices made by fish for stimulus S1–S7, and colored inserts represent the appearance of the respective distracter stimuli. The continuous line denotes the psychometric curve showing the relationship between ΔS and the proportion of correct choices. The dotted line denotes where the threshold value is at 65% correct choices. Bars indicate the variation (standard deviation) of the correct choices made by fish and at the behavioral threshold. (B) Results of brightness tests (experiment 2) showing the average fraction of correct choices (blue over distracter stimuli) for the different distracter stimuli groups in the 8-choice test (S3, S4 and S5). The gray line indicates the threshold of significance. (C) Results of color discrimination thresholds under violet light (experiment 3). ΔS was estimated using a standard deviation of noise (v) of 0.05.

0.05 for the long wavelength-sensitive (LWS) channel, as suggested from previous studies, which, coupled with our behavioral results, suggests that color discrimination thresholds arise when $\Delta S=3$, which would be equivalent to 1 JND (Fig. 3A). This differs from previous studies suggesting that a ΔS of 1 is enough for color pairs to become discriminable (Siddiqi et al., 2004). However, our results are similar to previous studies that measured color discrimination thresholds in triggerfish and found thresholds arise at ΔS greater than 1 (Champ et al., 2016; Cheney et al., 2019).

As the RNL model did not fit with our behavioral data using previous parameters, this implies that the cichlid visual system has higher receptor noise levels. The best fit of the behavioral performance of our fish would require increasing the standard deviation noise value (v) for the LWS channel from 0.05 to 0.16. This would increase the Weber fraction (w) (0.22 for S and 0.16 for M and L cones) such that the behavioral discrimination threshold would arise at a minimum ΔS of 1, which would correspond to 1 JND (Fig. S5A) in agreement with the RNL model predictions.

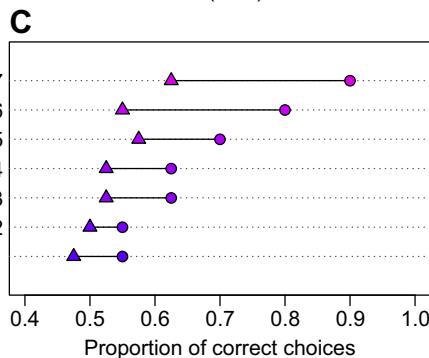
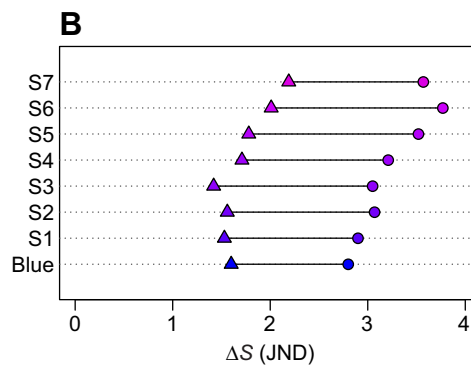
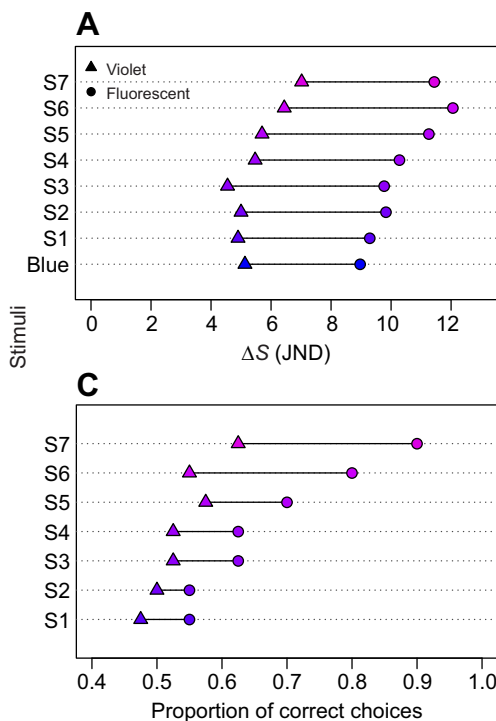


Fig. 4. Variation under different light illumination of ΔS between colors versus background and correct choice frequency. (A,B) The shift in ΔS between the color stimuli and the background when illuminated under violet and fluorescent light. ΔS was estimated using a standard deviation of noise (v) of 0.05 (A) and 0.16 (B). (C) The proportion of correct choices (blue over distracter stimuli) when colors were presented in different light environments.

Noise has been shown to vary in animals. For example, in insects, direct-noise measurements have been reported to be 0.12 (Vorobyev et al., 2001), and in birds, behavioral noise estimates vary from 0.06 to 0.105 (Lind et al., 2014; Olsson et al., 2015, 2018). In addition, the RNL model predicts the discriminability of colors taking into account several visual parameters (e.g. visual pigment sensitivity, lens transmission, environmental light) (Vorobyev and Osorio, 1998). However, the RNL model does not take into account information about photoreceptor interactions in the retina or central color processing, which are currently unknown in cichlids but essential in understanding their color vision. More morphological and physiological experiments analyzing the cichlid retina and their color processing are needed for a better understanding of the relationship between photoreceptor noise and color discrimination.

Furthermore, our results are strictly based on laboratory assays in which the light environment is dimmer than the fish's natural habitat, Lake Malawi. Light intensity can have an effect on the performance of the fish and also on our visual modeling because one of the RNL requirements is that experiments must be executed under bright illumination (Vorobyev and Osorio, 1998). Bright illumination is ideal in experiments as it suppresses the contribution of the achromatic channel in color discrimination. Although these experiments were well above the photon-shot noise limit (Escobar-Camacho et al., 2017), there could still be some effect of the light levels being a factor of 10 lower than those of the natural environment.

Finally, fish in this study were trained for a visual task discriminating two color elements. However, we cannot exclude the possibility that our results could be the product of cichlids performing artificial tasks in an artificial setting, or because fish lost motivation owing to long periods of time without testing. Conversely, the results could be different if cichlids performed other tasks such as species recognition or mate selection in their natural habitat.

Color discrimination under different background illumination

Experiment 3 shows that cichlids exhibit a limited color constancy under these experimental conditions, because fish made more errors when discriminating blue from distracter stimuli under violet light. This contradicts our calculations with the von Kries correction that predicts similar ΔS between blue and distracter stimuli for both light environments (Table 1; Fig. S2A–D). However, most of our fish were unable to discriminate between blue and purple under violet light. This decrease in the performance due to changes in the light environment has also been reported in experiments with bees, fish and birds (Dörr and Neumeyer, 2000; Dyer, 1999; Dyer and Chittka, 2004; Lind, 2016; Neumeyer et al., 2002; Olsson et al., 2015, 2016) and suggests that cichlids have limitations in their color constancy, like other organisms. Additionally, our experiments only tested color discrimination in a specific area of cichlid color space (blues and purples). Previous studies have shown that color constancy correction varies in different areas of an animal's color space (Dörr and Neumeyer, 2000; Dyer and Chittka, 2004; Olsson et al., 2016) and that it can be 'poorer' for objects reflecting shorter wavelengths (Dyer and Chittka, 2004). The visual system of *M. benetos* is not unlike that of bees in utilizing UV, blue–green and green photoreceptor channels. This suggests that our experiments may have been particularly hard for the fish because the tested colors (rewarded and distracter) were similar in the short wavelength part of the spectrum but differed in the long wavelength part of the spectrum. As these cichlids usually do not express a LWS visual pigment, they may not be sensitive to these long wavelength spectral differences.

To understand why the discrimination tasks were harder for fish under violet lighting, we first analyzed the stimulation of photoreceptors under different light environments. We noticed that, under violet light, the quantum catch of the S cones increased by at least 10-fold without the von Kries normalization (Table 1). More importantly, when we used the RNL model to calculate ΔS between color stimuli and the 'white' background of the color cards

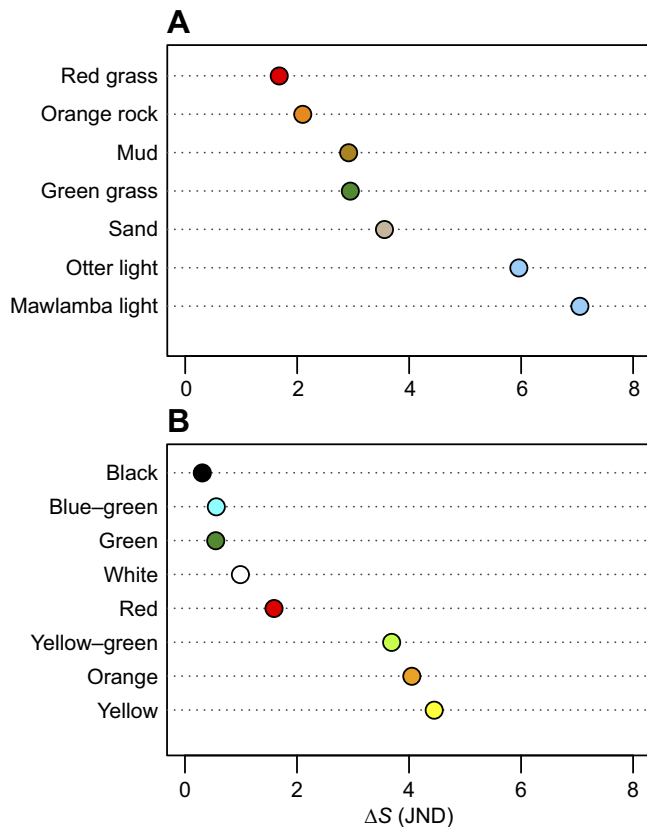


Fig. 5. Variation of ΔS of cichlid blue versus background and other cichlid colors. (A) ΔS between blue and background colors of Lake Malawi. (B) ΔS between blue and cichlid colors. ΔS was estimated using a Weber fraction of 0.16 for the long wavelength-sensitive channel.

in our experiments, we noticed that ΔS of all tested stimuli and the white background decreased significantly under violet light (Fig. 4A). This likely caused color discrimination to become more difficult for the fish because the ΔS of blue and the background under violet light decreased compared with the ΔS of blue and the background under fluorescent light (Fig. 4A,B). However, ΔS between the stimuli and background was greater than 1, even when using the larger noise value of $v=0.16$, which suggests stimuli should still be ‘discriminable’.

The discordance between visual modeling and our behavioral thresholds can again be better explained by assuming a higher noise level. By using a noise level of 0.16 in the LWS channel, ΔS between blue and the background under violet light decreased to ~ 1.6 , which is close to threshold (Fig. 4B). Greater noise would decrease the chromatic contrast of the rewarded stimuli and the background and it would help explain why our fish could not discriminate between two colored stimuli under violet light. As light illumination changed, blue was harder to distinguish from the background and, hence, fish made more mistakes (Fig. 4C). The overall shift in chromatic distance due to different light environments can also be observed in the cichlid perceptual color space (Fig. 2), where all tested colors were closer to the white background under violet light than when modeled with fluorescent light. This suggests that fish have less chromatic information for chromatic contrast detection.

Therefore, we suggest that the inability of cichlids to discriminate blue and purple stimuli under violet illumination is a product of the higher-order processing of color and that it cannot be explained

solely by photoreceptor channels. Thus, our results show cichlids exhibit only approximate color constancy in experiment 3 and that color discrimination thresholds strongly depend on the chromatic contrast between tested stimuli and the background (Lind, 2016; Olsson et al., 2016).

Individual performance variation

During our experiments, we observed individual variation in fish performance, which affected the results of both experiment 1 and experiment 3 (Table S1E–G, Fig. S5B,C). Some fish exhibited color discrimination thresholds at lower ΔS , suggesting ‘better’ color discrimination skills than others. For example, in experiment 1, one fish remarkably chose blue over S2 with high frequency despite their small ΔS . Similarly, in experiment 3, one fish was able to discriminate blue from S3 and S4 and three were able to discriminate blue from S7 (Fig. S5C, Table S1G).

This variation in performance among individuals could be the product of different experiences during training and testing and it provides insight into the heterogeneity that is present in Lake Malawi wild populations. This variable capacity in discriminating colors during several behaviors would predict that cichlids exhibit a continuous range in color discrimination thresholds in the wild, suggesting that some cichlids would be better at discriminating colors than others and, hence, may outperform other fish in several visually mediated tasks.

Visual ecology in Malawi

The study of color vision in cichlids is important because cichlids communicate through colorful visual signals that can be subject to variation and ultimately sexually selected (Kocher, 2004; Strelman and Danley, 2003). Our results can potentially inform the study of visual ecology of Malawi cichlids to start thinking about their color perception in the wild and their ability to perform different tasks. For example, color discrimination analyses of blue versus background spectra suggest greater ΔS between blue and space light backgrounds than between blue and orange rock backgrounds (Fig. 5A). Furthermore, ΔS values are greater between blue and yellow cichlid colors than between blue and green (Fig. 5B). Large ΔS values are not an indicator of how discriminable color pairs are; they are indicative of how much color discrimination would be preserved over longer distances as water attenuation would gradually make colors more achromatic (Wilkins et al., 2016). Therefore, ΔS is informative about which colors would remain discriminable with increasing distance. Our measurements of noise levels will help inform those estimates.

Behavioral observations in the field are in agreement with the importance of estimating ΔS of some color–background pairs. In Malawi, female *M. benetos* swim above the rocks of male territories, with males then swimming up to them to perform their courtship display, trying to lead them back to their territories for mating and spawning (K. L. Carleton, unpublished observations). Hence, the capacity of discriminating between blue and either the rocks or the space light would be necessary in identifying conspecific males and their visual signals first against the rocks and then in the water column. Furthermore, the large difference in ΔS between blue and yellow would be useful in several behaviors in the maternal mouth-brooding cichlids, including during courtship, where females peck male egg-spots and then pick up eggs after spawning (Hert, 1989), and for discriminating between species that exhibit blue and/or yellow in their nuptial coloration. Blues and yellows are the main nuptial colorations present in Lake Malawi and they have evolved

repeatedly within the lake, suggesting that these color signals are favored by sexual selection (Allender et al., 2003).

Future directions

Although we are starting to learn more about cichlid color vision, several more experiments are needed in order to understand the dimensionality of the cichlid visual color space. In our color discrimination threshold experiments, we analyzed color discrimination in only one region of the color space (blue and purple). Ongoing research suggests that color discrimination thresholds can differ between different directions of the perceptual color space (Cheney et al., 2019). This would imply that animals' visual systems could be wired to be more sensitive to changes in some colors than in others. This is relevant to cichlids because they exhibit great variation in visual sensitivities and spectral tuning mechanisms (Carleton, 2009; Carleton et al., 2016). Future experiments should test for color discrimination on a wide range of colored stimuli in different regions of color space.

Additionally, more studies on cichlid color constancy are needed because in this research we only used two different light treatments. Future experiments should consider using light environments that would vary in chromatic hue because fish have been shown to remain 'more' color constant under some colors than others (Dörr and Neumeyer, 2000). Color constancy can also be influenced by different backgrounds (e.g. gray and black) (Dörr and Neumeyer, 2000; Neumeyer et al., 2002); hence, future experiments should also consider manipulating color card backgrounds during behavior assays. Finally, future color constancy experiments should consider using light illumination intensities relevant to those in their natural habitat. This would help to elucidate whether cichlid color constancy depends on light levels. This is relevant to cichlid vision because in Lake Malawi, some cichlids can inhabit depths from 1 to 20 m, where light is gradually attenuated with increasing depth (Sabbah et al., 2011; Smith et al., 2011).

Conclusion

In this study, we have shown that cichlids can be trained to perform color threshold discrimination experiments, controlling for brightness and chromatic cues. Cichlids successfully discriminated the rewarded stimuli from a series of distracter stimuli that varied in hue. We further confirmed that cichlids discriminated these colors regardless of brightness by combining luminance noise tests with multiple-choice assays. This study also shows that by using the RNL model, we can successfully determine behavioral color discrimination thresholds between different colors. However, our results only meet the RNL model assumptions if we increase the Weber fraction, which suggests that cichlids have higher receptor noise, at least in lab conditions, than we have previously predicted. However, this is similar to the estimates found for other animals. Furthermore, we show that under some conditions, cichlids have limited color constancy, such that their ability to discriminate colors decreases under a different light illumination. This likely happened because changing the illuminating light decreased chromatic contrast of the background and the tested colors. Continued research into cichlid color vision is needed as it could help us understand more about the role of vision in cichlid ecology, sexual selection and ultimately cichlid speciation.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: D.E.-C., M.A.T., K. L. Carleton; Methodology: D.E.-C., M.A.T., K. L. Carleton; Software: D.E.-C., K. L. Carleton; Validation: D.E.-C., K. L. Carleton; Formal analysis: D.E.-C., M.A.T.; Investigation: D.E.-C., M.A.T., K. L. Cheney, N.F.G., J.N.M., K. L. Carleton; Resources: D.E.-C., J.N.M., K. L. Carleton; Data curation: D.E.-C., M.A.T.; Writing - original draft: D.E.-C.; Writing - review & editing: D.E.-C., K. L. Cheney, N.F.G., J.N.M., K. L. Carleton; Visualization: D.E.-C., M.A.T., K. L. Cheney, N.F.G., J.N.M., K. L. Carleton; Supervision: D.E.-C., K. L. Cheney, N.F.G., J.N.M., K. L. Carleton; Project administration: D.E.-C., K. L. Carleton; Funding acquisition: D.E.-C., K. L. Carleton.

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Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.201160.supplemental>

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Supplementary Information Supplementary Figures

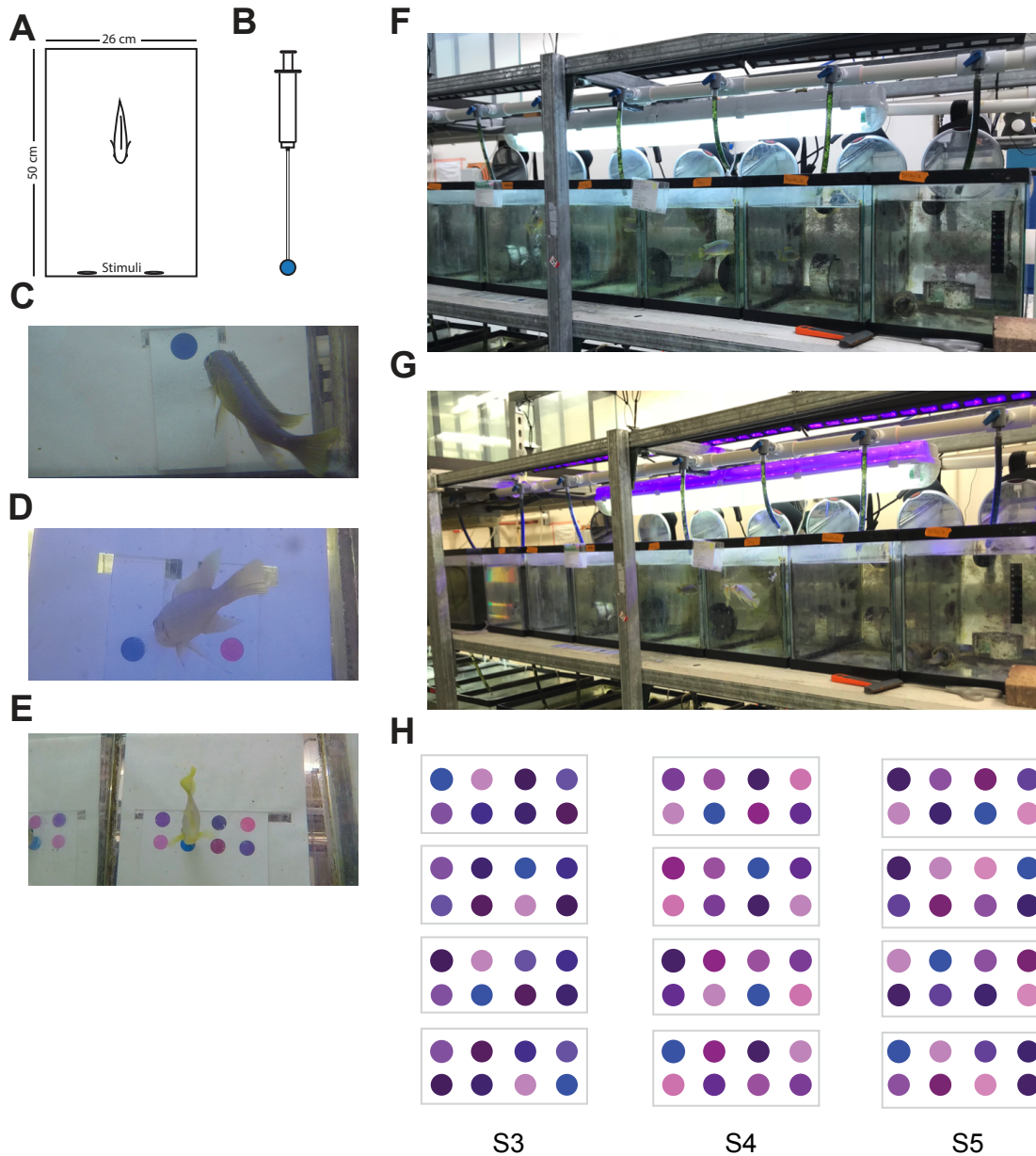


Figure S1. Training and testing set up, modified from Escobar-Camacho et al., 2017. (A) House hold tanks. (B) Feeding apparatus for training. (C) Photograph of fish during training displaying a color card with the blue rewarded stimulus. (D) Photograph during a binary (two-alternative) choice test, experiment 1 and 3. (E) Photograph of a fish during testing, experiment 2. (F-G) Lightning set up with fluorescent lights (F) and violet lights (G). (H) Multiple choice color cards used in Experiment 2.

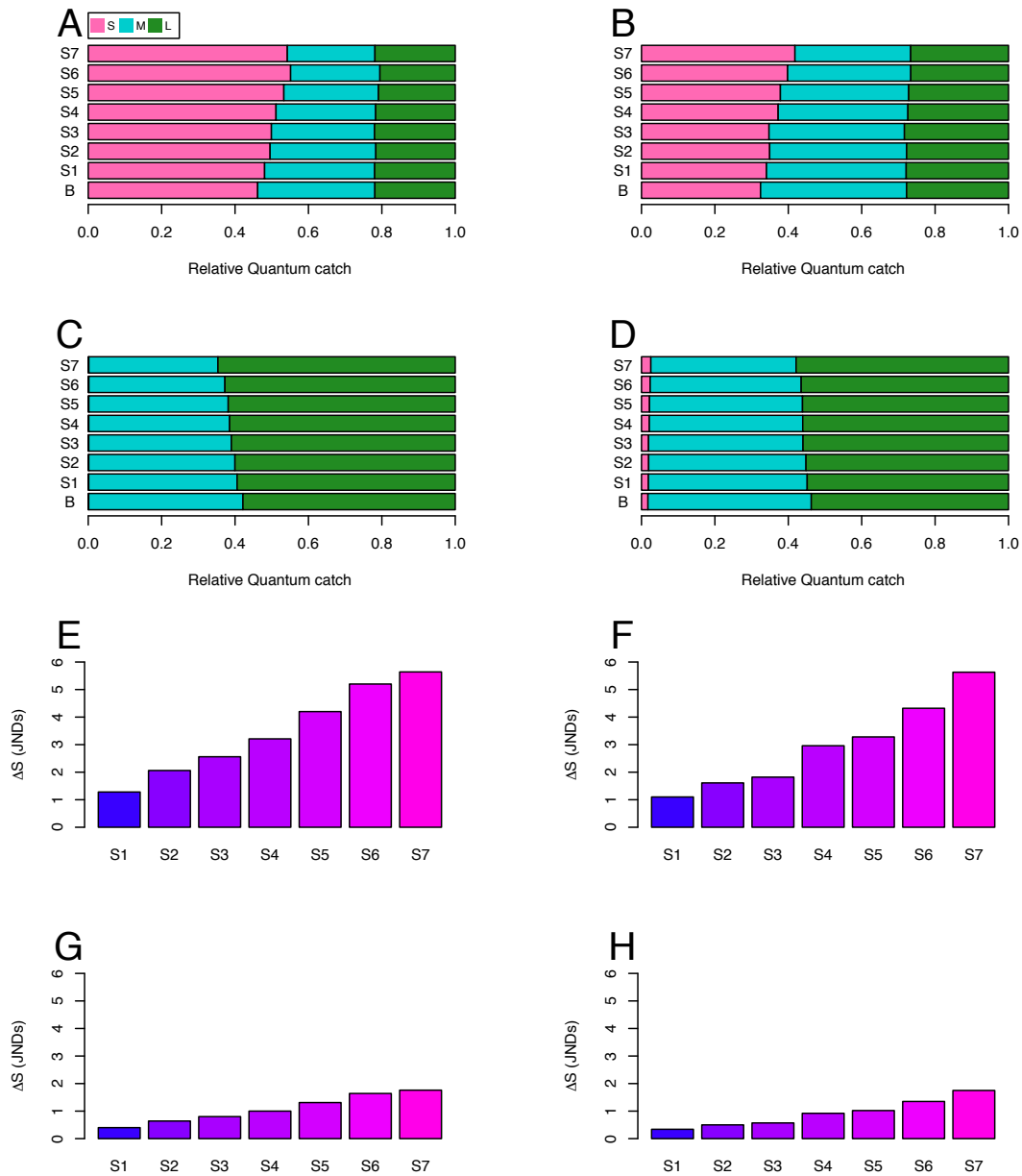


Figure S2. Normalized quantum catches of color stimuli calculated with (A & B) and without (C & D) the von Kries correction. (A & C) were calculated with fluorescent light whereas (B & D) with violet light. (E - H) is ΔS of distracter stimuli calculated with a standard deviation noise value (v) for the LWS channel of 0.05 (E & F) and 0.16 (G & H). (E & G) were calculated with fluorescent light whereas (F & H) with violet light.

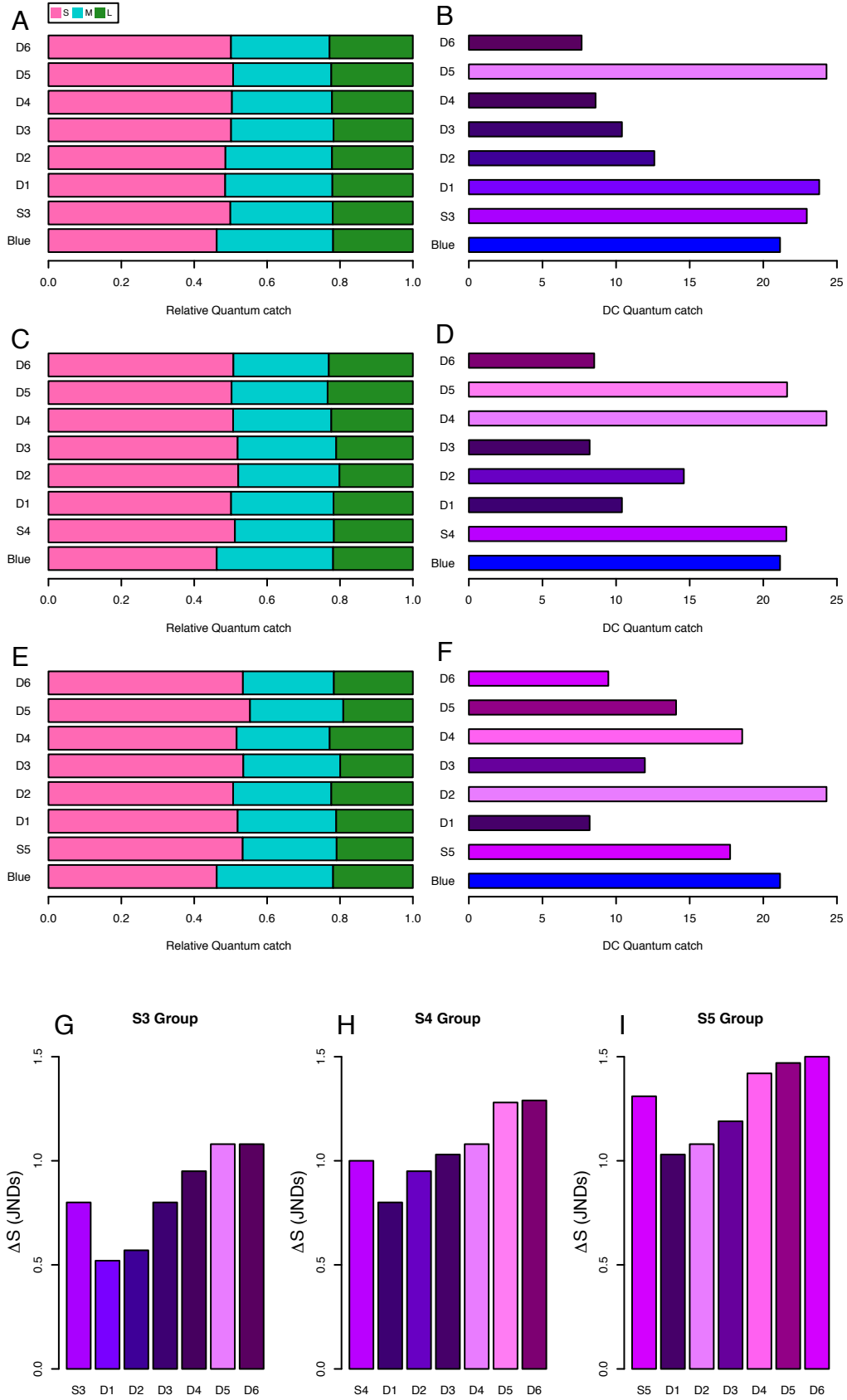


Figure S3. Quantum catches of color stimuli presented in multiple-choice test color cards under fluorescent light in Experiment 2. (A-F) denote quantum catches of blue, the distracter stimuli (from the threshold interval of Experiment 1 S3, S4, and S5) and the respective distracter stimuli from each group (D1-6). (A, C & E) denote normalized quantum catches of each color present in the multiple-choice test color cards (S3, S4 and S5 respectively) with their respective distracter colors (D1-6). Stacked bars show the stimulation for each type of photoreceptor (S, M and L) for each color. (B, D & F) show the relative quantum catch for the Double Cones (DC) luminance channel, for each color as calculated with Equation 3. (G, H & I) show ΔS of distracter stimuli (for each color group S3, S4 and S5 respectively) from rewarded stimuli (blue) using a standard deviation noise value (ν) for the LWS channel of 0.16.

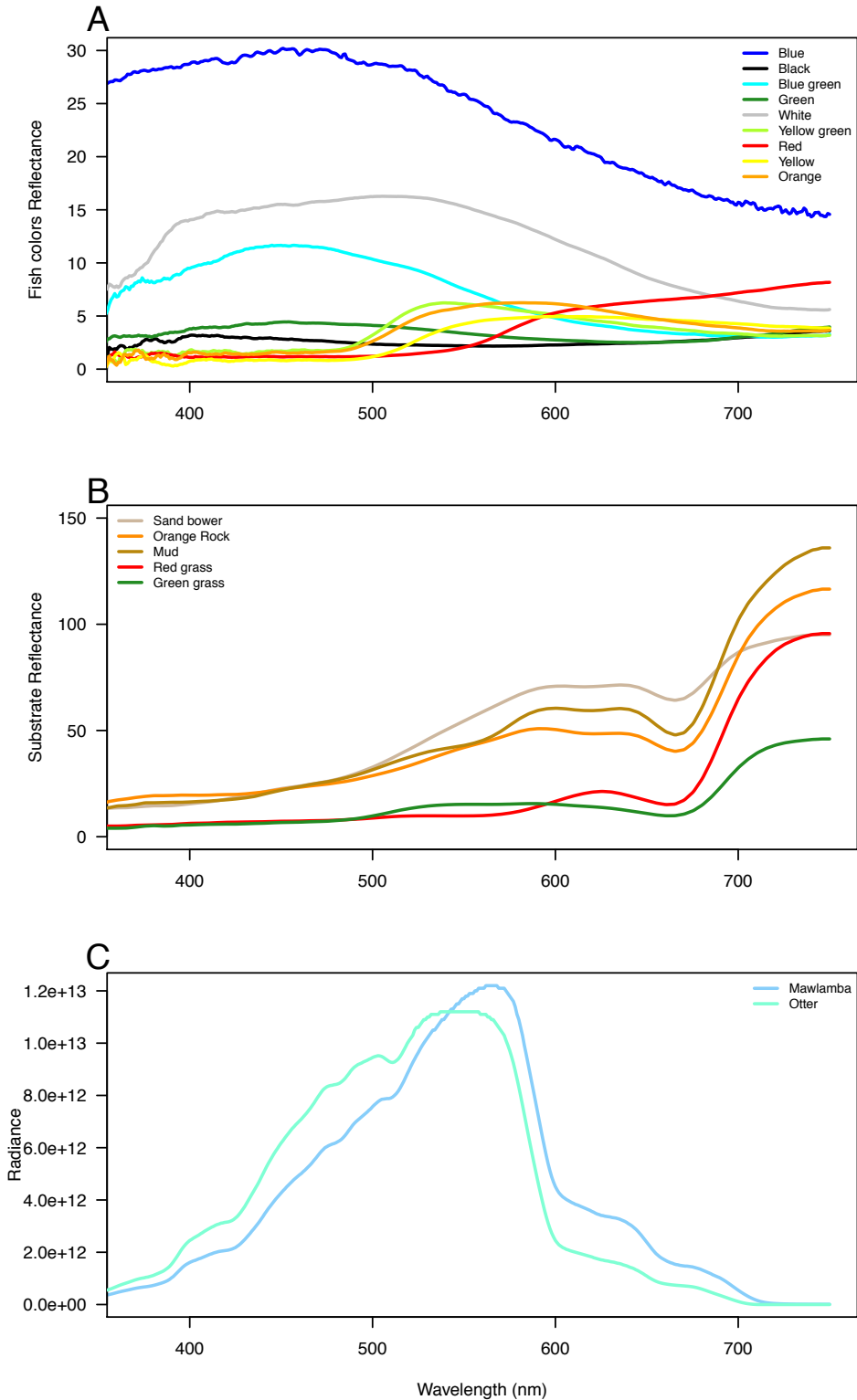


Figure S4. Spectral measurements from Lake Malawi. (A) Denotes the reflectance from cichlids' colors, data from Dalton et al., 2010. (B) denotes reflectance from background substrates, this study. (C) Denotes side-welling radiance at 3 meter depth from Mawlamba Bay and Otter Point, data from Sabbah et al., 2011.

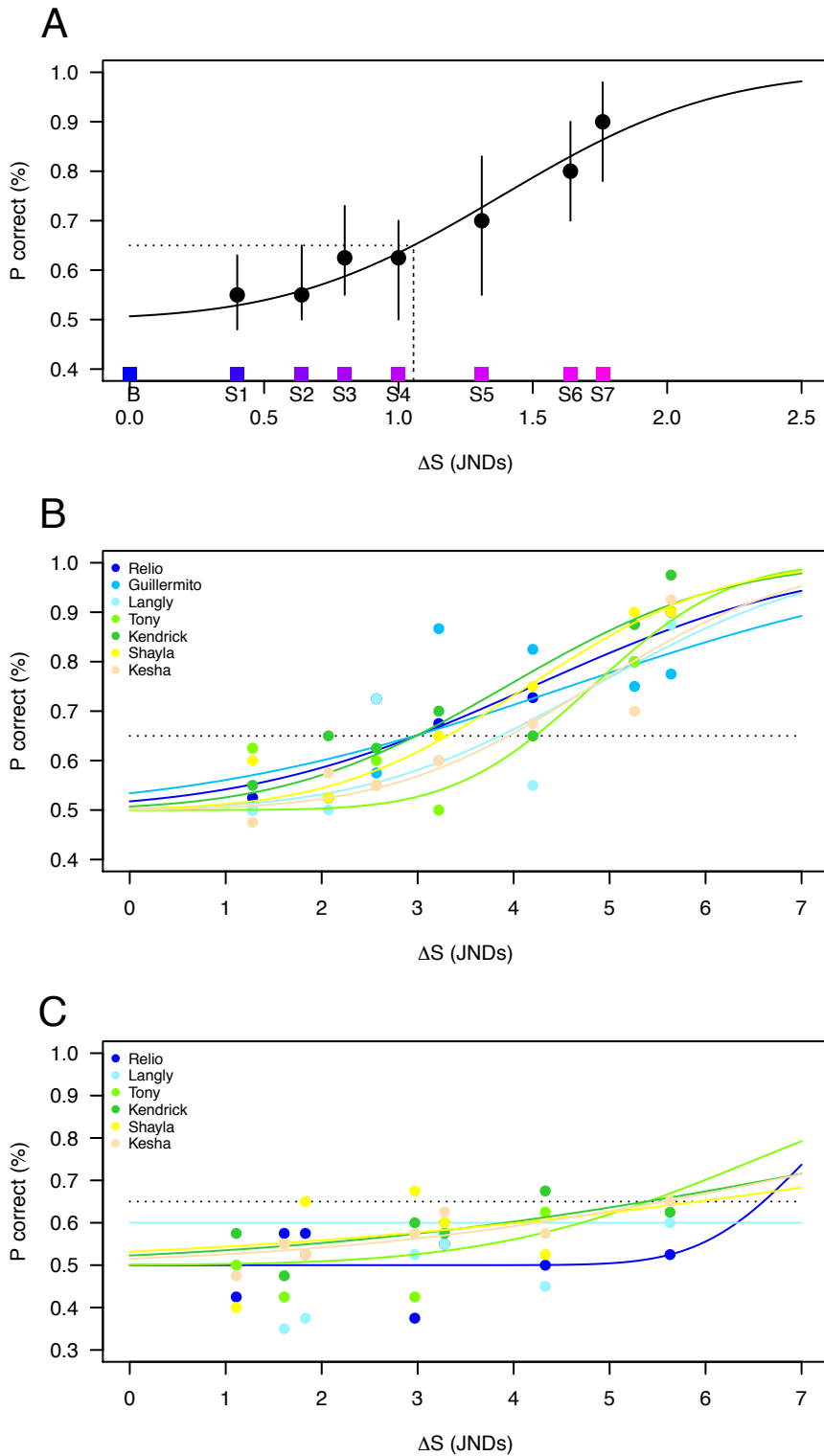
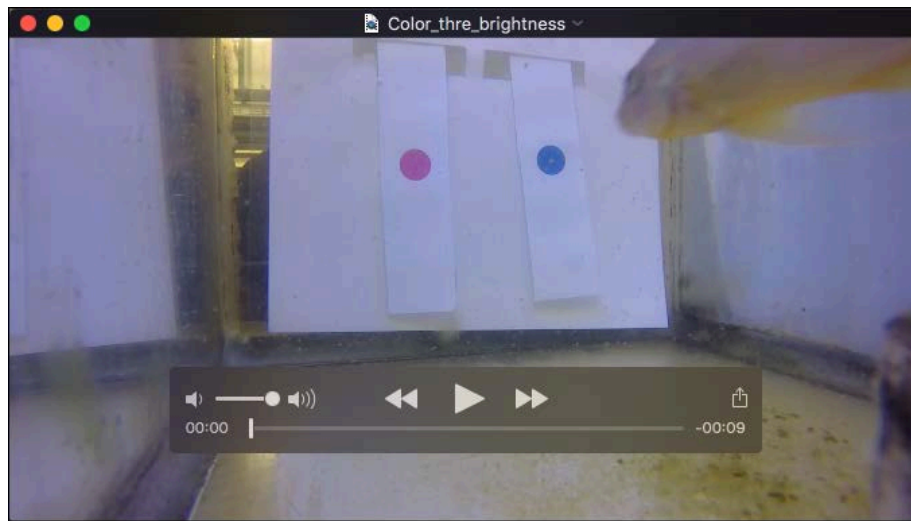


Figure S5. (A) Color thresholds under fluorescent light where proportion of correct choices is shown as a function of ΔS (JNDs). The x-axis represent ΔS in JNDs. Filled circles denote the fraction of correct choices made by fish for stimulus S1-S7, and colored inserts represent the respective distracter stimuli

appearances. Chromatic distance was estimated using a standard deviation noise value (ν) for the LWS channel of 0.16. (B & C) Individual variation of behavioral color thresholds expressed as Proportion of correct choices as a function of ΔS under fluorescent (B) (Experiment 1) and violet light (C) (Experiment 3). The x-axis represents ΔS . Filled colored circles denote the fraction of correct choices made by each fish for stimulus S1-S7. ΔS was estimated using the standard deviation noise value (ν) for the LWS channel of 0.05



Movie 1. Experimental setup showing *Metriaclima benetos* selecting color stimuli.

Table S1: A. Rewarded and distracter stimuli; B. Tanks Sidewelling Irradiance ; C. Lens transmission of *M. benetos*; D. Lake Malawi data; E. Results from experiment 1; F. Results from experiment 2; G. Results from experiment 3.

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