RESEARCH ARTICLE

Behavioral color vision in a cichlid fish: *Metriaclima benetos*

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ABSTRACT

Color vision is the capacity to discriminate color regardless of brightness. It is essential for many fish species as they rely on color discrimination for numerous ecological tasks. The study of color vision is important because it can unveil the mechanisms that shape coloration patterns, visual system sensitivities and, hence, visual signals. In order to better understand the mechanisms underlying color vision, an integrative approach is necessary. This usually requires combining behavioral, physiological and genetic experiments with quantitative modeling, resulting in a distinctive characterization of the visual system. Here, we provide new data on the color vision of a rock-dwelling cichlid from Lake Malawi: *Metriaclima benetos.* For this study we used a behavioral approach to demonstrate color vision through classical conditioning, complemented with modeling of color vision to estimate color contrast. For our experiments we took into account opsin coexpression and considered whether cichlids exhibit a dichromatic or a trichromatic visual system. Behavioral experiments confirmed color vision in *M. benetos*; most fish were significantly more likely to choose the trained over the distracter stimuli, irrespective of brightness. Our results are supported by visual modeling that suggests that cichlids are trichromats and achieve color vision through color opponency mechanisms, which are a result of three different photoreceptor channels. Our analyses also suggest that opsin coexpression can negatively affect perceived color contrast. This study is particularly relevant for research on the cichlid lineage because cichlid visual capabilities and coloration patterns are implicated in their adaptive radiation.

KEY WORDS: Fish vision, Color opponency, Trichromacy, Classical conditioning

INTRODUCTION

Animals vary greatly in color pattern, with coloration often playing an important role in speciation. Evolutionary biology aims to understand the selective mechanisms shaping the form and perception of color patterns by conspecifics and heterospecifics. Animals' visual perception of such patterns depends on the detectability of these color signals (Cheney et al., 2013), which can be shaped by the light environment where animals live, the color properties of the signaler and the visual sensitivities of signal receivers (Endler, 1992, 1993; Lythgoe, 1979). However, in order to understand how color patterns evolve, we must first study the ultimate mechanisms underlying color vision. Color vision is the ability to discriminate color regardless of brightness. In vertebrates,

color vision is achieved through color opponency, by which spectrally opponent channels produce a signal from spectrally distinct cone photoreceptors (Bowmaker and Hunt, 2006; Jacobs and Rowe, 2004; Kelber et al., 2003). Therefore, color vision requires at least two spectrally distinct types of photoreceptors operating in a similar intensity range (Kelber, 2016).

In the retinas of most vertebrates, photoreceptors are classified as rods and cones. Rods function under dim light conditions, whereas cones function in daylight and are responsible for color vision (Baylor, 1996; Bowmaker and Hunt, 2006; Yau, 1994). In fish, cone photoreceptors are usually arranged in a highly organized manner, the retinal mosaic. Fish exhibit great variation in the number of different cone types that they possess, with some species having only one type of cone with a single visual pigment (monochromatic) and others having four spectrally distinct types of cones (tetrachromatic) (Douglas and Partridge, 1997; Lythgoe and Partridge, 1989; Marshall et al., 2003a; Neumeyer, 1992). Cone photoreceptors also exhibit morphological differences and can be classified as single or double cones. Double cones are two fused cones that are found in the eyes of most fish species and in several vertebrates (Ebrey and Koutalos, 2001). It has been suggested that, in some species, double cones are electrically coupled (Marchiafava, 1985) and that they play a role in luminance detection (Marshall and Vorobyev, 2003; Marshall et al., 2003b; Siebeck et al., 2014). This is based on the 'summation hypothesis', which states that signals of double cones are summed in the retina, conveying a single signal to the brain (Marshall and Vorobyev, 2003; Marshall et al., 2003a). This is particularly true in birds, in which double cones detect luminance and multiple types of single cones discriminate color (Lind et al., 2014; Maier and Bowmaker, 1993). However, fish often have only one type of single cone, with single and double cones each contributing to color discrimination (Pignatelli et al., 2010).

Among teleosts, Cichlidae is one of the largest families, with approximately 2000 species widely distributed across ecosystems from Africa and South Asia to Central and South America (Friedman et al., 2013; Turner et al., 2001; www.fishbase.org). Cichlids are also diverse in their visual tasks as species forage on different foods, and vary in mating systems and parental care. The colorful body patterns of cichlids can be sexually dimorphic and are likely important for species recognition, mate choice and speciation (Price et al., 2008; Seehausen et al., 2008; Selz et al., 2014). Thus, visual communication is essential for cichlid behavior. Vision research on cichlid flocks from the African Great Lakes has identified the genetic basis of their visual sensitivities: seven spectrally distinct cone opsins and a rod opsin gene (Carleton, 2009). The cone opsins belong to four cone opsin classes, including UV sensitive (SWS1), short-wavelength sensitive (SWS2A, SWS2B), rhodopsin-like (RH2Aα, RH2Aβ, RH2B) and long-wavelength sensitive (LWS) (Carleton et al., 2016).

Although much is known regarding the visual system of African cichlids, it is unclear whether cichlids possess chromatic discrimination. Demonstrating color vision requires other approaches, including behavioral methods (Douglas and

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List of symbols and abbreviations	
$A(\lambda)$	wavelength-dependent absorbance of the photoreceptor, normalized to a peak of 1
F_{abs}	quantum catch absorption coefficient
i	receptor type
JND	just noticeable differences
k	absorption coefficient of the photoreceptor at the peak absorption wavelength
K	von Kries factor for receptor i
L	long-type photoreceptor
LWS	long-wavelength sensitive opsin
M	medium-type photoreceptor
MSP	microspectrophotometry
N	absolute quantum catch
Q	quantum catch
R	the sensitivity (opsin absorbance template) of receptor
RH2Aα	Aα rhodopsin-like gene
RH2Aβ	Aβ rhodopsin-like gene
RH2B	B rhodopsin-like gene
RNL	receptor noise-limited model
S	short type photoreceptor
SWS1	UV-sensitive opsin
SWS2A	short-wavelength sensitive opsin
SWS2B	short-wavelength sensitive opsin
T_{50}	wavelength at which 50% transmission is reached
Δf	contrast in a receptor channel
ΔS	chromatic distance between two colors, measured in JNDs
τ	summation time
ω	Weber fraction

Hawryshyn, 1990). Data on photoreceptor spectral sensitivities, behavioral experiments and physiological models combined provide a unique opportunity to study the neural interactions underlying color vision. Testing for chromatic discrimination in fish is beneficial for vision research because it provides insight about how photoreceptor signals might be processed by the rest of the retina. Visual discrimination experiments in teleosts have elucidated how different photoreceptors are used for chromatic and achromatic tasks (Neumeyer, 1984; Neumeyer et al., 1991; Pignatelli et al., 2010; Siebeck et al., 2014; Smith et al., 2012). Therefore, quantitative modeling could suggest how photoreceptor signals are combined and compared in the process of discriminating spectrally different stimuli (Kelber et al., 2003).

The aggressive behavior and territoriality of species from the genus *Metriaclima* makes them an ideal system to test hypotheses through behavioral approaches. *Metriaclima benetos* Stauffer, Bowers, Kellogg and McKaye 1997 is a rock-dwelling cichlid from Lake Malawi and its color vision has been characterized through microspectrophotometry (MSP) and opsin gene expression (Carleton, 2009; Carleton et al., 2008; Jordan et al., 2006). *Metriaclima benetos* spectral sensitivities are based on a 'short' opsin palette expressing three spectrally distinct opsins: SWS1 (379 nm), RH2B (489 nm) and RH2Aα (522 nm) (Hofmann et al., 2009; Jordan et al., 2006). However, we still do not know whether there is color opponency in the cichlid retina that enables chromatic discrimination.

In this exploratory research we wanted to know whether cichlids are able to discriminate between colors regardless of brightness. This would give us insight into how photoreceptors interact in the retina and its implications in color discrimination. In this study, we took into account how spectrally different photoreceptors are stimulated by different colors, and the role of opsin coexpression and photoreceptor noise in color discriminability.

MATERIALS AND METHODS
Data measurements
We trained cichlids to recognize the color blue as our main stimulus. Blue is the primary body coloration of male *M. benetos*. Blue has also been used multiple times in color vision experiments (Cheney et al., 2013; Neumeyer, 1992; Pignatelli et al., 2010; Siebeck et al., 2008).

We measured stimuli reflectance, the illuminating light quantified from side-welling irradiance and lens transmission. Stimuli were made by printing colored circles of 1.5 cm diameter on standard paper and then laminating them. In order to create darker and lighter shades of each color, black or white was added using Adobe Illustrator. Stimuli reflectance was measured using a fiber-optic spectrometer based on an Ocean Optics USB2000 (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). Side-welling irradiance was measured inside the tanks under fluorescent lights with a 1000 µm fiber fitted with a cosine corrector (CC-3). Finally, lens transmission was measured by placing the isolated cichlid lens on a UV-transparent coverslip, which was illuminated from above by a fiber-optic cable attached to a pulsed xenon light source (PX-2, Ocean Optics) 15 mm above the lens. Another fiber-optic cable was placed 5 mm directly under the specimen and delivered the signal to the spectrometer. Transmission was measured by comparing measurements with and without the lens. An *xy* stage was used to center the lens and maximize the transmission. Three replicate measurements were made of each lens of four fish. The resulting spectral scans were normalized to 100% transmission at 700 nm. Finally, we quantified the T_{50} values (which represent the wavelength at which 50% transmission is reached).

Behavioral approach
We used a similar approach to classic 'gray card' experiments where bees were trained to associate a reward with a specific color and thereby could be tested for how well they could discriminate the trained color from others (von Frisch, 1914). We tested the ability of *M. benetos* individuals to choose blue over distracter stimuli. For this, we trained fish to blue through classical conditioning and subsequently tested them when offered two or more choices. The same seven fish were used for all tests. Although only males were used, we have never found differences in male and female sensitivities (although see Sabbah et al., 2010).

Fish training
In order to train the fish, a feeding apparatus consisting of a plastic feeder tube (5 mm diameter and 20–30 cm long) was attached to a 3 ml syringe filled with a mix of fish flakes and water. The amount of food available to the fish was manually controlled and could be adjusted by varying the pressure applied to the syringe (Fig. S1B). In this way, different amounts of food could be delivered to the fish. The food was delivered at the front of the aquarium. Because we wanted to train fish to touch a specific stimulus (blue) with their mouth [referred as 'taps' (Siebeck et al., 2008)], initially fish were fed through the feeder tube alone. Once the fish learned to bite/tap the tube, a colored flat disc was attached to the end of the tube helping the fish learn to tap the color stimuli. The fish were then introduced to a laminated card with the stimuli at the center (the feeder was not inside the tank at this point). As fish learned to associate the color with reward, they started to tap the colored stimuli on the card and consequently were rewarded (Fig. S1C,D).

Fish were trained only for blue and not for light blue or dark blue. In order to make sure fish could see all stimuli before choosing, fish

were lured towards the posterior section of the tank while the color cards were placed in the front of the tank. The feeder was removed and the fish turned in order to make a choice. The experimenter was able to see which color stimulus fish tapped with a mirror placed above the tank. The fish never saw the experimenter or other fish during tests. Fish were tested approximately 10 or 20 times to confirm that they could discriminate colors; when they succeeded 75% of the time, testing started. Seven male fish were trained within a 2-month period, in which some individuals learned faster than others. This seemed to be related to the different levels of confidence that each individual exhibited. Indeed, one of the most difficult steps in the training process was to convince the fish to approach the feeder tube or color card in the presence of the experimenter.

Fish care
Fish were held individually in 26×50 cm tanks with a common recirculating system (Fig. S1A), and were fed daily during training and testing periods. All fish were managed under the guidelines of the University of Maryland Institutional Animal Care and Use Committee protocol (#R15-54). Fish were tested from November to March in 2015–2016 at the Tropical Aquaculture facility at the University of Maryland, USA.

Experiment 1: binary choice
The first experiment consisted of a binary choice test where fish chose between two cards with one color circle each, the trained blue stimulus was presented with yellow and gray as distracters. As soon as the fish tapped one of the two cards, the cards were removed, the fish was rewarded if it chose correctly and the trial ended. In order to avoid bias against a specific side of the tank, the same color was not presented on the same side more than two times in a row. Furthermore, if the fish did not show a response to the stimuli for more than 2 min, the fish was not rewarded and the trial was not counted. To ensure that fish were not selecting stimuli based on luminance, the trained and distracter stimuli varied in three levels of brightness. In total we tested seven fish to assess whether they had the ability to detect chromatic differences between colored stimuli (blue, yellow and gray).

Experiment 2: multiple-choice gray
To further confirm our results in the first experiment, we used a multiple-choice discrimination test. Cards contained eight color stimuli of which one was blue and the rest were multiple shades of gray. Stimuli were arranged in two horizontal rows with four circles of color each. Five cards were designed with different combinations (Fig. S1E), and were presented to the fish in a random fashion during testing. As in the previous experiment, we added luminance noise to make brightness an unreliable cue for blue; therefore, blue in the cards varied between three levels of brightness. For experiments 2 and 3, each fish was tested five times for each combination card.

Experiment 3: multiple-choice color
Finally, for the third experiment, we wanted to know whether fish could discriminate blue from several different wavelengths and whether there was a bias against a specific color. Six cards were designed containing different stimuli of which one was blue and the rest were different colors (black, brown, violet, pink, red, yellow and green) (Fig. S1E,F). Because brightness bias was already tested in the two previous experiments, luminance noise was not introduced in this experiment. As in experiment 2, the different color card combinations were presented in a random fashion to the fish.

Data analysis
For experiments 1, 2 and 3, a one-tailed binomial test was used to calculate whether the fish could distinguish the trained from distracter stimuli. For this, the number of correct trials was compared to the distribution of taps if fish were choosing randomly (50% of the time for the experiment 1 and 12.5% for experiments 2 and 3). Confidence intervals were calculated assuming a binomial distribution. All binomial tests and visual modeling calculations were done in the statistics package of R software for each fish in each experiment (www.r-project.org/).

Differential interference contrast (DIC) images of *M. benetos* cone photoreceptors
One fish was sacrificed with an overdose of MS-222, the eyecup was removed and the eye was dissected under a stereoscope. 1× hyaluronidase/collagenase was placed into the open eyecup and incubated for ~45 min, adding more if needed. The vitreous humor was removed and the retina gently dissected away from the retinal pigment epithelium (RPE) by flushing with copious cold phosphate-buffered saline. As soon as the retina was separated from the RPE and the vitreous humor, it was pinned in an agar plate where it was fixed in 4% paraformaldehyde. Photographs were taken with a Leica DM5500 microscope (Leica Microsystems).

Visual modeling
To quantify how colors stimulate the visual sensitivities of photoreceptors, we calculated quantum catches (Q), which represent the number of incident photons that are captured by visual pigments in each photoreceptor (Hárosi, 1996). Therefore, estimating quantum catches allows us to examine how spectrally different colors stimulate different cichlid photoreceptors. These calculations include: (1) the spectrum of environmental light, (2) the reflectance spectrum of an object (e.g. stimuli), (3) the lens transmission and (4) the spectral sensitivities of photoreceptors (Dalton et al., 2010; Kelber et al., 2003).

Quantum catches are based on the seven opsins present in cichlids, and use the MSP spectral sensitivities of *M. benetos* (SWS1, RH2B and RH2Aα) and of the closely related species *Metriaclima zebra* (SWS2B, SWS2A, RH2Aβ and LWS). Genetic analyses show that the opsin sequences of *M. zebra* and *M. benetos* do not differ significantly, having identical amino acids in the retinal-binding pocket sites (Smith and Carleton, 2010). Hence, the visual sensitivities of *M. benetos* should be, if not equal, highly similar to *M. zebra*. We further consider the possibility of opsin coexpression in single cones and double cones, which has been demonstrated in *M. zebra* (Dalton et al., 2014, 2015, 2016). This coexpression varies across the retina and seems to be minimal within the area centralis (believed to be used in high-visual-acuity tasks); however, there is significant variation between individuals. Opsin coexpression can have different effects on color vision because two opsins in the same cone would shift its peak absorbance. Therefore, we estimated quantum catches based on pure opsin expression (SWS1, RH2B, RH2Aα) and on coexpressed opsins (SWS1/SWS2B, RH2B/RH2Aβ, RH2Aα/LWS) in a single photoreceptor. Because opsins can be differentially coexpressed, we considered four combinations of opsin coexpression that have been found to encompass the variation in *M. zebra* (Table S1A) (Dalton et al., 2014, 2016). We used different spectral sensitivities based on reported coexpression combinations in order to calculate quantum catches.

Quantum catches (Q) were calculated for the short, medium and long (denoted by subscript S, M and L, respectively, in the equations)-wavelength sensitive cones for each color using Eqn 1

(below), where R_i is the sensitivity (opsin absorbance template) of receptor i, L is the lens transmittance, S is the surface reflectance (color stimuli), I is the illuminant and K_i is the von Kries factor for receptor i (Table S1):

$$Q_i = K_i \int R_i(\lambda)L(\lambda)S(\lambda)I(\lambda). \qquad (1)$$

The opsin absorbance template (R_i; Eqn 2) is derived from the quantum catch absorption coefficient (F_{abs}), which represents the fraction of photons entering a photoreceptor that are actually absorbed (Johnsen, 2012). Here, k is the absorption coefficient of the photoreceptor at the peak absorption wavelength (the peak absorbance determined by MSP in units of μm^{-1}), $A(\lambda)$ is the wavelength-dependent absorbance of the photoreceptor, normalized to a peak of 1, and l in the length of the outer segment in μm:

$$R_i = F_{abs} \propto \int_{300}^{750} (1 - e^{-kA(\lambda)l}). \qquad (2)$$

The von Kries factor (Eqn 3) is derived from von Kries' color constancy model in which receptors adapt independently to the background illumination (Dalton et al., 2010; Endler and Mielke, 2005; Kelber et al., 2003):

$$K_i \propto \frac{1}{\int R_i(\lambda)L(\lambda)S(\lambda)I(\lambda)}. \qquad (3)$$

In order to test whether double cones are involved in color vision (color opponency), we modeled quantum catches both separately for each cone member (M and L) and for the combined double cone (DC) with Eqn 4 (Pignatelli et al., 2010):

$$Q_{DC} = \frac{Q_M + Q_L}{2}. \qquad (4)$$

Quantum catches also allow us to calculate the contrast between the tested colors to the cichlid eye. For this we use the receptor noise-limited (RNL) model (Vorobyev and Osorio, 1998). Briefly, we used quantum catches of each cone class (i) to calculate contrast between pairs of colors, Δf_i (Siddiqi et al., 2004):

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

Color discrimination is also determined by receptor noise. Relative receptor noise (ν) is related to the Weber fraction (ω) for a single photoreceptor (i) by: $\omega_i = \nu_i / \sqrt{n_i}$, where n is the number of receptors of i type (Vorobyev et al., 1998, 2001). Further, we followed Koshitaka et al. (2008) in assigning a receptor noise for each cone class (Eqn 6) (Koshitaka et al., 2008). In our calculations, the long (L) receptor is assumed to have a noise value of 0.1 (see Discussion), and the noise values for the short (S) and medium (M) cone classes were calculated using their relative abundance in the retinal mosaic. *Metriaclima benetos* has a square mosaic like its close relative *M. zebra* (Dalton et al., 2014), where the S:M:L cones ratio is 1:2:2 (Fig. S1G,H). This gives us a relative noise value of 0.14 for S cones, and 0.1 for M and L cones:

$$\omega_i = 0.1 \sqrt{\frac{n_L}{n_i}}. \qquad (6)$$

Following Vorobyev and Osorio (1998), we can compute the distance between two colors (ΔS). To further test whether cichlids achieve color opponency either through stimulation of each photoreceptor or combining signals from double cones, we

computed ΔS for a dichromatic (S, DC; Eqn 7) and a trichromatic (S, M, L; Eqn 8) visual system as follows:

$$\Delta S = \sqrt{\frac{(\Delta f_{DC} - \Delta f_S)^2}{\omega_S^2 + \omega_{DC}^2}}, \qquad (7)$$

$$\Delta S = \sqrt{\frac{\omega_S^2(\Delta f_L - \Delta f_M)^2 + \omega_M^2(\Delta f_L - \Delta f_S)^2 + \omega_L^2(\Delta f_S - \Delta f_M)^2}{(\omega_S \omega_M)^2 + (\omega_S \omega_L)^2 + (\omega_M \omega_L)^2}}. \qquad (8)$$

ΔS is the chromatic distance of two colors in the photoreceptor space and its units are 'just noticeable differences' (JND). Values <1 JND indicate that the two colors are indistinguishable, whereas values above 1 JND indicate that two colors can be distinguished (Siddiqi et al., 2004).

Because we performed these experiments under fluorescent lights that do not emit short wavelengths (Fig. 1), to which *M. benetos* are sensitive, and because, at lower light intensities, photon-shot noise can affect color discrimination (Olsson et al., 2015), we wanted to calculate absolute spectral sensitivity, $R_i(\lambda)$, for each photoreceptor type as:

$$R_i(\lambda) = \nu\tau \left(\frac{\pi}{4}\right)^2 \left(\frac{d}{f}\right)^2 D^2 (1 - e^{-kA(\lambda)l}), \qquad (9)$$

where ν is the number of cones per receptive field and τ is the summation time; d/f is the acceptance angle of a cone [d is the diameter of the receptor and f is the lens focal length (2.5 mm, calculated from the lens radius multiplied by Matthiessen's ratio)] and D is the pupil diameter. When $R_i(\lambda)$ is included in Eqn 1, it can give us absolute quantum catches, N. We can then include photon-shot noise into the RNL model in Eqns 3, 5 and 6 by further substituting the noise term from Eqn 10. In this way, we can analyze how spectral sensitivities change with and without the von Kries normalization:

$$\omega_{i,\text{photon}} = \frac{\sqrt{\omega_i^2 N_i^2 + N_i}}{N_i}. \qquad (10)$$

For these calculations, ν and d were determined from the retinal mosaic (Fig. S1G,H), and D was measured from five fish. l was obtained from Carleton et al. (2000) and Dalton et al. (2014). k and peak wavelengths to estimate $A(\lambda)$ were obtained from Carleton (2009) and Jordan et al. (2006). To our knowledge, τ has not been measured for cichlids, so we use 40 ms, as estimated for coral reef fish (Champ et al., 2016).

RESULTS
Stimuli, illumination and visual system properties
Color stimuli were designed and their spectral reflectance quantified. Similarly, side-welling irradiance was measured in the tanks. Lens transmission yielded a T_{50} of 370 nm (Fig. 1).

Experiment 1: binary choice
For this experiment, a total of 3353 tests were performed (n_{fish}=7; $n_{color-pairs}$=18). This experiment showed that each fish could easily discriminate between blue and yellow and blue and gray. Most fish were more likely to choose the trained (blue) over the distracter (yellow or gray) stimuli, irrespective of brightness (Fig. 2). On average, fish tapped correctly 99.75% (96–100%) of the trials against yellow and 91% (48–100%) against gray (Fig. 2, Movie 1). Two fish failed to discriminate light blue and dark gray, and one fish
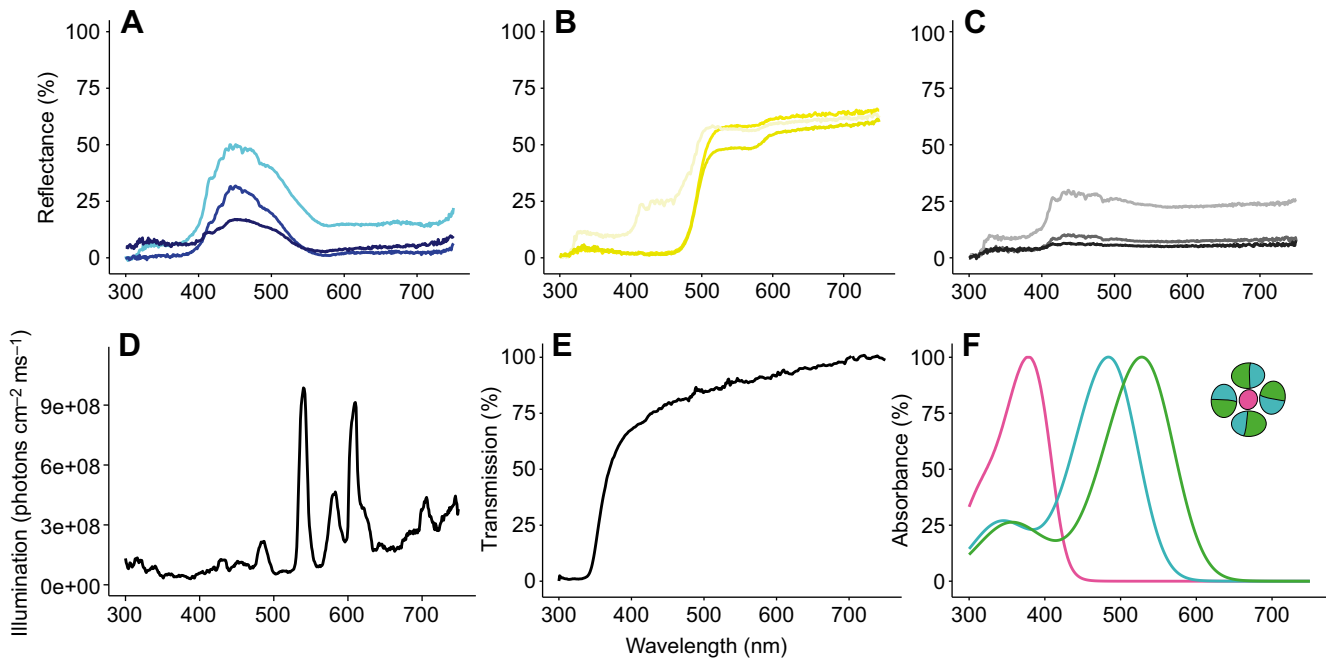
Fig. 1. Reflectance spectra, side-welling irradiance, lens transmission and cone sensitivities. (A–C) Reflectance spectra of blue, yellow and gray targets, respectively, with stimuli varying in brightness. (D) Lab illumination measured from side-welling irradiance in absolute photons. (E) Lens transmission spectra. (F) Color vision plot with spectral sensitivity of the short-, medium- and long-wavelength pigments. A typical square retinal mosaic is shown in the inset: the short-wavelength sensitive opsin (SWS1; pink) is expressed in single cones, whereas medium- and long-wavelength pigments [RH2B (blue-green) and RH2A (green), respectively] are expressed in double cones.

failed in discriminating light blue and gray. Five fish achieved correct-choices significance in all conditions (Table S2A).

Experiments 2 and 3: multiple choice

The same fish from the binary-choice experiment were used in the multiple-choice test. In experiment 2 (525 tests), fish were more likely to choose blue over the different shades of gray (Fig. 3A). Fish tapped correctly blue, light blue and dark blue, 89% (88–96%), 76% (60–96%) and 88% (76–96%) of the time, respectively, as compared to the different shades of gray (Movie 1). Similarly, in experiment 3 (205 tests), all fish were more likely to choose blue over different colors, and fish tapped correctly 74% (70–80%) of the time (Fig. 3B, Movie 1). All fish achieved significant results in experiments 2 and 3 (Table S2A). Interestingly, in experiment 3, we noticed that, of the few mistakes fish made (20–30%), i.e. tapping another color instead of blue, most of the mistakes (78%) were with the color purple. Hence, this suggests that these fish have difficulty discriminating blue from purple (Table S2A).

Quantum catches

We estimated quantum catches by two approaches, one considering the spectral absorption (referred simply as 'quantum catch') and the second considering absolute spectral sensitivity (referred to as 'absolute quantum catch'). We first considered the simplest case, a dichromatic visual system with pure opsin expression. Here, color pairs like blue–yellow differ in the signals from single cones and from summed signals of double cones (Table S2B, Fig. 4A). Thus, yellow and blue would be discriminated on the basis of spectral differences between both single and double cones. By contrast, blue and gray have similar quantum catches for single and double cones (Table S2B; Fig. 4A); hence, this could potentially preclude *M. benetos* from discriminating between these colors.

We next considered the trichromatic case, which assumes that the three cone types independently contribute to color vision. Quantum catch calculations suggest that the short, medium and long (S, M and L) cones are differentially stimulated for each color target (Fig. 4C,E, Table S2B). Furthermore, because fluorescent lights do not emit UV light, absolute quantum catch calculations resulted in essentially zero stimulation for single cones (Table S2B).

We next considered coexpression for both di- and trichromacy. We found that photoreceptors are differentially stimulated as compared with pure opsin expression. In dichromats, signals from single and double cones change for blue–gray comparisons, with blue shifting away from gray but purple is more similar to blue. Yellows also appear to be more different when there is opsin coexpression (Fig. 4B, Fig. S2). For a trichromatic visual system, differential stimulation from each photoreceptor is maintained (Table S2B, Fig. 4D,F), although blue and purple seem to generate similar signals (Fig. 4F, Fig. S2). Finally, as a consequence of low stimulation of the S photoreceptors, absolute quantum catches suggest that color vision under our experimental scenarios primarily relies on stimulation of the two double cone members, the M and L cones (Fig. S3).

Chromatic distance

Color distance (ΔS) analysis, in a dichromatic and trichromatic visual system, provided two main outcomes for colors used in experiments 1, 2 and 3. First, these analyses suggest that, for the cichlid visual system, yellow distracters exhibit greater ΔS than gray distracters when compared to shades of blue. Second, our results show that opsin coexpression can increase, maintain or decrease ΔS for blue compared with different colors (Figs 5 and 6, Table S2C,D).

In determining whether cichlids are dichromats or trichromats, we note that for, a dichromat, ΔS between blue and gray is below 1 JND when there is pure opsin expression (Table S2D). Therefore, in a
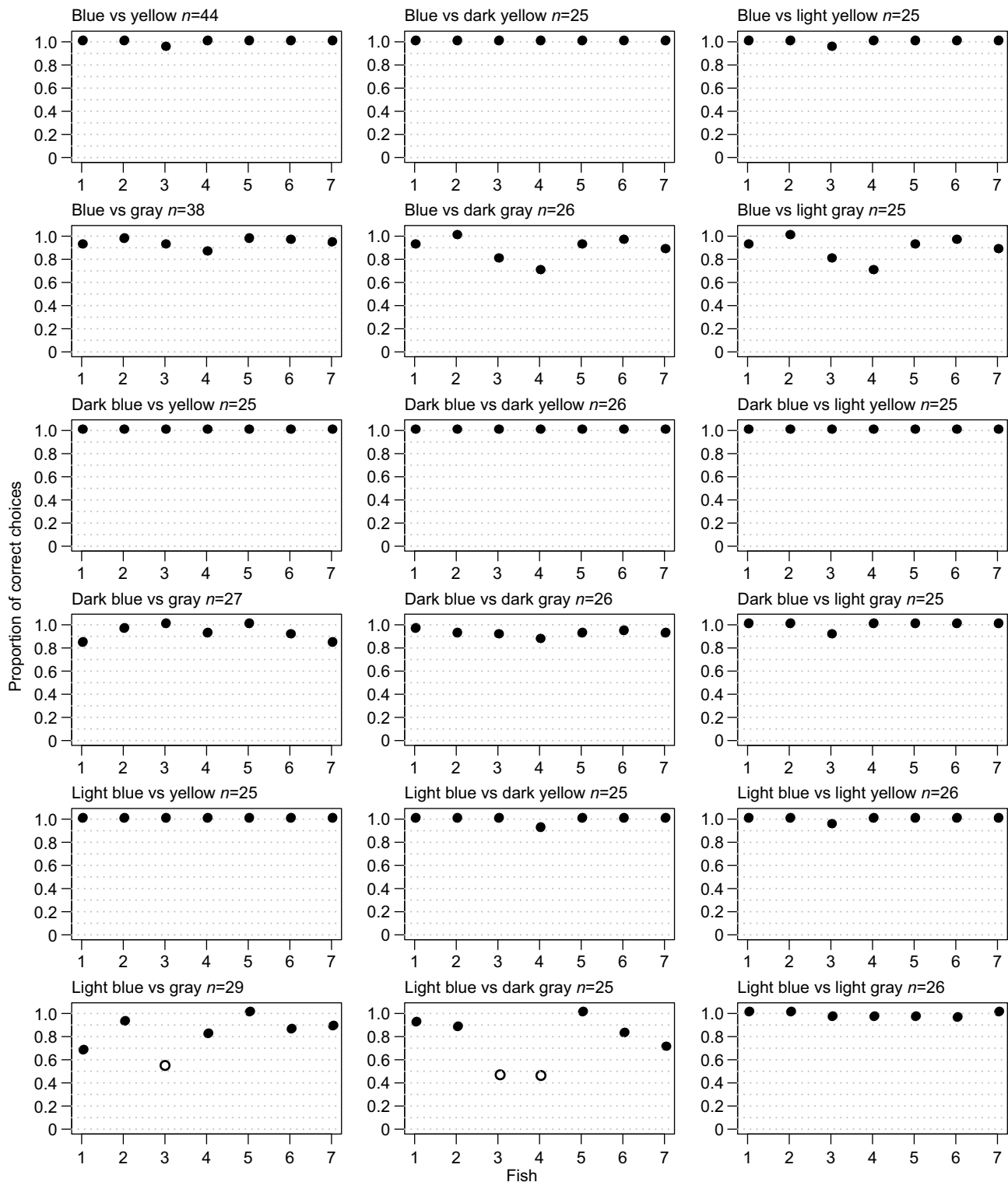
Fig. 2. Proportion of times the stimuli were chosen correctly in the first binary-choice experiment. Each treatment and the number of trials (n) are specified. Numbers on the x-axis specify each individual fish, whereas the proportion of correct choices are specified on the y-axis. Empty symbols denote when the binomial test was not significant (P>0.05).

dichromatic visual system, cichlids would not be able to discriminate blue from gray. Although opsin coexpression does increase blue/gray chromatic distance, our previous studies suggest that most individuals utilize pure opsins in the area centralis (Dalton et al., 2016). By contrast, in a trichromatic visual system, blue/gray

chromatic distance is greater than 4 JND; hence, cichlids could potentially discriminate blue from gray regardless of opsin coexpression (Fig. 5). This suggests that cichlids must be trichromatic if they successfully distinguish blue from gray, independent of brightness.
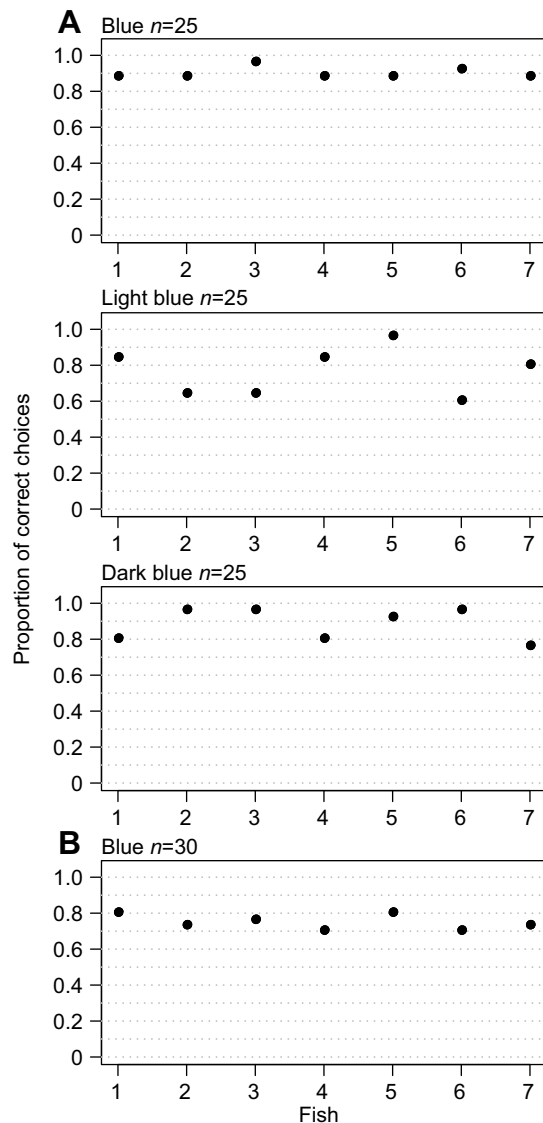
Fig. 3. Proportion of times the stimuli were chosen correctly for multiple-choice experiments. (A) Experiment 2 for blues versus multiple grays and (B) experiment 3 for blue versus multiple colors. The number of trials are specified (*n*). Numbers on the *x*-axis specify each individual fish whereas the proportion of correct choices is specified on the *y*-axis. All binomial tests were significant (*P*<0.05).

Overall, for both types of visual systems, comparisons of pure pigments and opsin coexpression yielded similar or higher ΔS for blues against shades of gray with all four coexpression combinations (Table S2C,D). However, there are some exceptions. In a dichromat, opsin coexpression increased ΔS between light blue and shade of grays, but it negatively affected ΔS between light blue and dark gray (Fig. 5B).

We find similar results for the broad range of colors used in experiment 3. Our results suggest that there is great variation in ΔS with color stimuli, opsin coexpression and visual systems. For example, when compared to blue, ΔS for brown, orange, green and yellow increases with all coexpression combinations in both dichromats and trichromats (Fig. 6) (Table S2C,D). However, ΔS varies for blue versus red and pink, with both increases and decreases. Overall, most ΔS exceeded 1 JND in a pure opsin expression scenario (except for brown in dichromats), and these

results were highly similar when ΔS was calculated with or without photon-shot noise (Table S2D).

One important comparison is for blue and purple, where ΔS is above 1 JND for pure pigments but small in all four coexpression combinations, particularly for a dichromatic visual system, with $\Delta S \leq 0.8$ JNDs (Fig. 6B, Table S2D). Combining these results with those for blue/gray, we find that, although coexpression increases blue/gray discrimination, it makes blue/purple discrimination worse. Therefore, coexpression cannot compensate for dichromacy in all tested scenarios. This further supports that cichlids must be trichromatic to successfully perform all visual tasks.

DISCUSSION
The results of this study show that *M. benetos* has color vision. Fishes were able to distinguish blue from other colors regardless of brightness in all the experiments. Our behavioral results also imply that cichlids are trichromats in which the three types of photoreceptors (S, M and L) are necessary for color discrimination. This is in agreement with von Kries corrected quantum catches calculations, which suggest that each photoreceptor is differentially stimulated by each color. Thus, we suggest that double cone members (M and L) provide opponent spectral channels used for color vision, because modeling in which double cones are summed together suggests that multiple colors would equally stimulate the photoreceptors. The unique contributions of double cones are further supported by quantum catch calculations without von Kries correction. Under these conditions, there is very low stimulation of the single cones by the lighting in these experiments, such that double cones could mostly mediate color discrimination. Furthermore, our visual modeling suggests that quantum catches and chromatic distance can be affected by opsin coexpression.

Cichlid behavior
Cichlid vision has been extensively studied using a variety of behaviors associated with visual cues. Cichlids are quite adaptable and several species seem amenable to training under laboratory conditions. Cichlids have shown that they are able to recognize facial cues between conspecifics (Satoh et al., 2016), and Lake Malawi *Metriaclima* species have been used for shape discrimination, object categorization and symmetry perception tasks (Schluessel et al., 2012, 2014, 2015).

Here, we provide some of the first behavioral evidence that the Lake Malawi cichlid, *M. benetos*, possesses color vision. The potential ability for color vision in cichlids has previously been suggested using molecular and MSP methods. Those data show that *M. benetos* rely on three visual pigments resulting from expression of three different cone opsin genes. However, the current study is the first behavioral evidence of their chromatic discrimination capabilities. These behavioral experiments confirm that, within weeks, *M. benetos* can be trained to perform visual tasks based on color cues alone. These results rely on classical conditioning using color choice (Kelber et al., 2003), as have been used in previous studies on fish color vision (Cheney et al., 2013; Neumeyer, 1992; Pignatelli et al., 2010; Risner et al., 2006; Siebeck et al., 2008).

Color opponency and opsin coexpression
Our visual modeling assuming a dichromatic visual system (based on double cone summation) predicted that cichlids would not be able to discriminate blue from gray. However, in our behavioral results, all fish successfully distinguished blue from gray regardless of
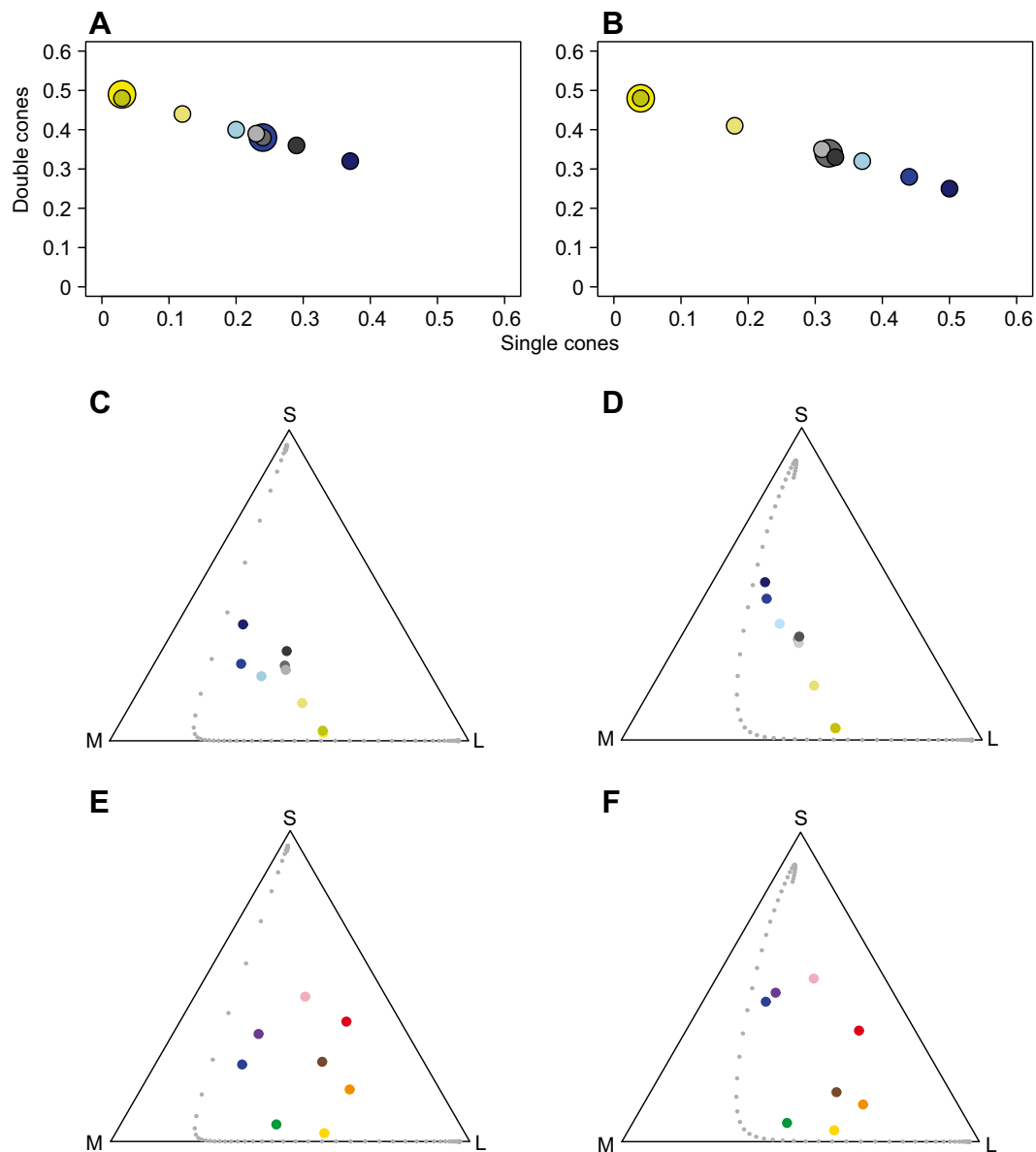
Fig. 4. Normalized quantum catch of colors in experiments 1, 2 and 3. In a dichromatic visual system, photoreceptor stimulations are plotted as signals in single cones (x-axis) and as summed signals in double cones (y-axis). Quantum catches were normalized using Eqns 1 and 2 for short-, medium- and long-wavelength sensitive cones. Combined signal stimulation (summed) of double cones was calculated with Eqn 4. (A) The quantum catch when there is pure opsin expression. (B) Opsin coexpression combination 1. Bigger circles are used to reveal overlapping data points in the single-cone–double-cone space. (C-F) For a trichromatic visual system, photoreceptor stimulations are plotted in chromaticity diagrams with target colors plotted in the color receptor space of *M. benetos* for experiments 1, 2 and 3. Each axis corresponds to the quantum catches of short/UV (S), medium (M) and long (L) sensitive photoreceptors. Monochromatic loci at 5 nm intervals are represented by gray dots. (C,E) These plots are based on a pure-opsin-expression visual system (SWS1, RH2B, RH2Aα); (D,F) correspond to opsin coexpression combination 1 (SWS1/SWS2B, RH2Aβ/RH2B, LWS/RH2Aα). Only coexpression combination 1 is displayed because it is the one that has been reported in the area centralis.

of luminance noise. This suggests that cichlids have a trichromatic visual system where color vision is based on the differential stimulation of each photoreceptor (S, M and L).

Based on our visual modeling and behavioral results, we suggest that *M. benetos* achieves color vision probably through color opponency mechanisms. This agrees with the assumption that, in double cones, spectrally opponent channels exist between each cone member, producing a signal that is the result of differences between spectrally distinct cones (Pignatelli et al., 2010) (Fig. 4C–F). This hypothesis is supported because, in our experiments, fish were able to differentiate blue from gray regardless of the similarities of

quantum catches of single cones and the summed signals of double cones (Fig. 4A). Therefore, each double cone member is likely generating a different signal, causing spectral differences that would be registered by ganglion cells in a trichromat.

In this study, we are assuming that color opponency is the product of two different cone photoreceptor sensitivities that are being compared by ganglion cells. Nevertheless, the retina neural circuit can be highly complex. For example, bipolar and horizontal cells have been shown to receive feedback from three to four spectral types of cones in cyprinids (De Aguiar et al., 2006; Klaassen et al., 2016; Li et al., 2012). Morphological and physiological studies
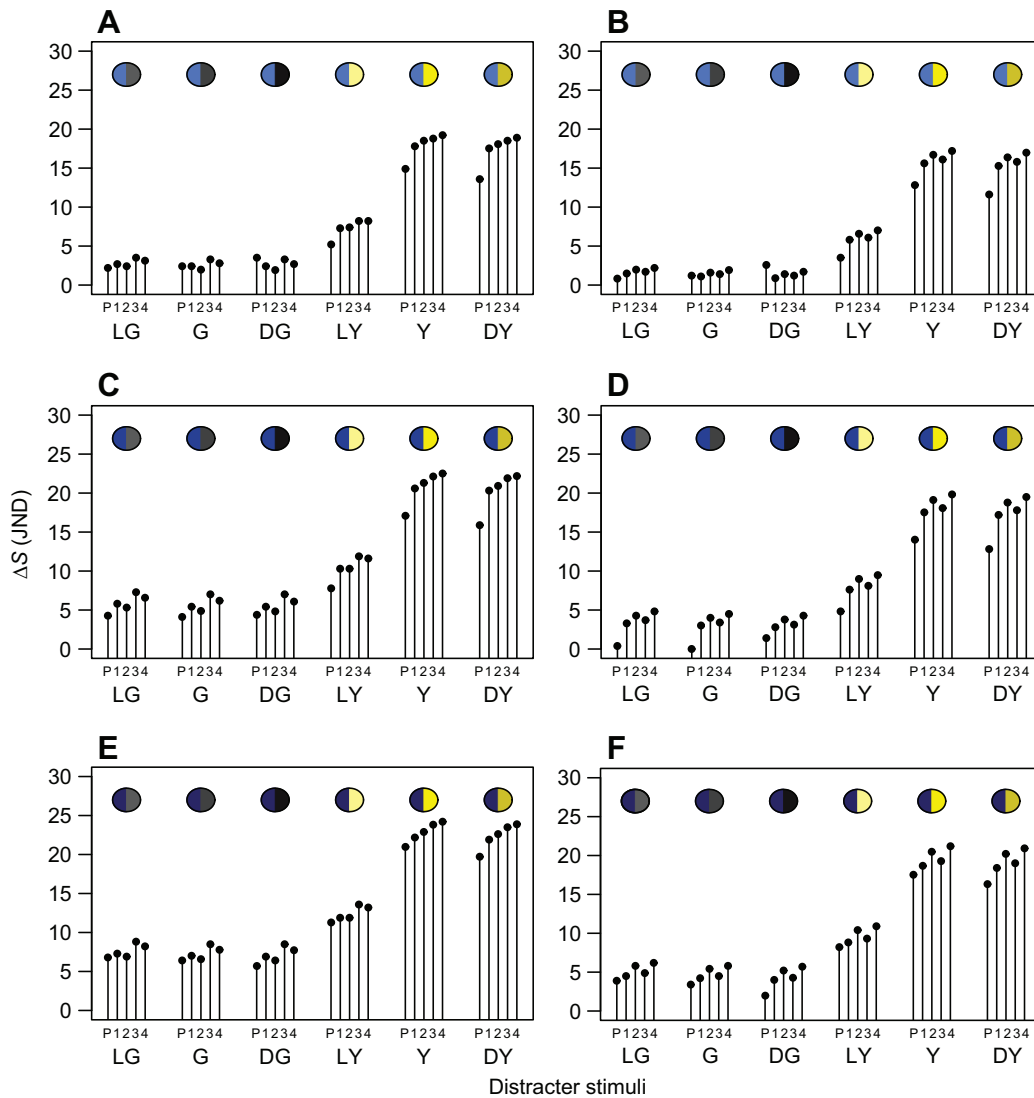
Fig. 5. Chromatic distances (ΔS) of colors in experiments 1 and 2. Chromatic distances between stimuli for experiments 1 and 2 are given for light blue (A,B), blue (C,D) and dark blue (E,F), against distracter stimuli (G, gray; LG, light gray; DG, dark gray; LY, light yellow; Y, yellow; DY, dark yellow). (A,C,E) ΔS for a trichromatic visual system; (B,D,F) ΔS for a dichromatic visual system. Pure (P) and coexpression combinations (1–4) are specified for each color pair.

analyzing the neural network of the cichlid retina are needed in order to better understand how color opponency takes place.

Color opponency needs at least two different spectral channels whose quantum catches are compared (Bowmaker and Hunt, 2006). In cichlids, the presence of multiple cone types to produce these different spectral channels is supported by our previous *in situ* labeling of different opsins to examine the spatial distribution of cone types in the closely related Malawi cichlid, *M. zebra* (Dalton et al., 2014, 2015). In those studies, we found a highly organized retinal mosaic with single and double cones. Both single and double cones contain spectrally different opsins, including unique opsins in opposite members of double cones. More interestingly, Dalton et al. (2016) showed that *M. zebra* has an area centralis in the retina close to the optic nerve, with high densities of both photoreceptors and ganglion cells, and minimal opsin coexpression. This suggests that this region in the retina provides high acuity for visual tasks, including color discrimination. We further found that the spatial patterns of opsin coexpression vary between individuals, with at least one of six individuals showing coexpression in the area centralis (Dalton et al., 2016).

In our visual modeling, opsin coexpression increases ΔS for some colors but decreases ΔS for others (Figs 5 and 6, Table S2C,D). Interpretation of the size of JNDs should be done with caution because, even though chromatic distance is an indicator of color discriminability, it does not assess perceptual similarity from highly discriminable stimuli (Kelber et al., 2003). Large ΔS values do not necessarily mean that some colors are more discriminable than others; instead, this suggests that color discrimination is preserved over longer distances because water acts as an attenuating medium making colors more achromatic over larger distances (Champ et al., 2016). This effect is irrelevant in our experiment because fish were very close to the stimuli. Even though we have not confirmed opsin coexpression in *M. benetos*, qPCR data suggest that, because of the expression of multiple single cone opsins, it is likely (Carleton et al., 2008; Hofmann et al., 2010). However, coexpression is less common in the area centralis, where fish would be viewing objects of interest. We suggest that opsin coexpression might have negatively affected discrimination between specific colors where coexpression decreased ΔS (e.g. blue versus purple), and this is in concordance with our behavioral evidence. Furthermore, some
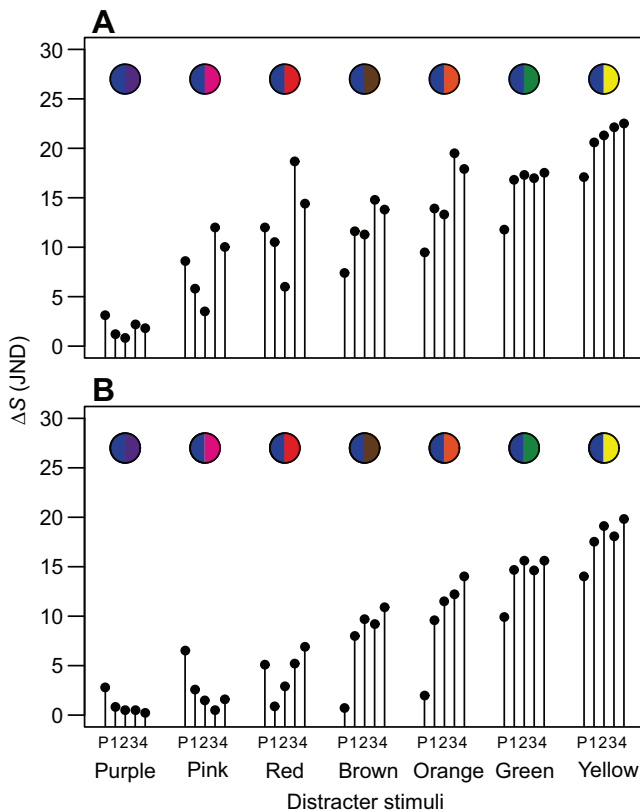
Fig. 6. Chromatic distances (ΔS) of colors in experiment 3. Chromatic distances between blue and colors used in experiment 3. (A) Trichromatic visual system; (B) dichromatic visual system. Pure (P) and coexpression combinations (1–4) are specified for each color pair.

individuals show more difficulty with discrimination than others, and this might occur in the few individuals with increased coexpression. This would require further genetic testing.

Color discrimination and the Weber fraction
In this study, we wanted to take the first step in understanding vision in cichlids and to know whether they have true color vision. Further studies are needed to dissect the cichlid visual system as well as its adaptations and limitations but, given our visual modeling and behavioral results, we can infer the physiological characteristics of the cichlid retina. Initially, based on previous fish-vision studies applying the RNL model, we assumed a Weber fraction of 0.05 (Champ et al., 2016; Cheney et al., 2009; Dalton et al., 2016; Wilkins et al., 2016) for the LWS channel. However, in order to better predict cichlid performance, we adjusted this to a higher Weber fraction of 0.1. We did this because, even though we are not evaluating color discrimination thresholds, increasing the Weber fraction, and hence lowering chromatic distance, would partially explain the mistakes fish made (Figs 2 and 3). In general, a Weber fraction of 0.05 is accepted for most animals (Vorobyev and Osorio, 1998) but it is known that predicted thresholds can disagree with experimental data (Avarguès-Weber and Giurfa, 2014; Olsson et al., 2015).

There were a few color combinations where fish were more likely to make mistakes. Fish more often made mistakes with discriminating blue from purple. This might be explained by the similarities in the reflectance profiles of these colors (Fig. S1F). Mistakes of blue versus purple might also be explained by opsin coexpression. With opsin coexpression combination #2, ΔS is low

(<1 JND) (Fig. 6A, Table S2C), thus making discrimination 'harder' for the fish. In contrast, mistakes by several of the individuals between light blue and grays are not explained by either opsin coexpression, similarities in quantum catches or low JND. Light blue and grays exhibit a ΔS above 1 JND in most coexpression combinations in both dichromatic and trichromatic visual systems. One possibility is that the receptor noise is greater than 0.1, resulting in lower ΔS. Indeed, if we increase the noise ratio to 0.3, ΔS between light blue and grays decreases proportionately by a factor of 3 (0.7–1.1 JND). Higher levels of noise could be a consequence of the low stimulation of the short-sensitive cone (which is UV sensitive in *M. benetos*). This is supported by absolute quantum catches calculations (Table S2B), which remove the von Kries correction and better account for the fact that the light environment in which fish were tested lacked UV light (Fig. 1D), thus affecting the stimulation of single cones (Fig. S3). Hence, the low stimulation of single cones and high levels of photoreceptor noise might explain fish mistakes in discriminating between light blue and grays.

Overall, only trichromacy would allow fish to successfully choose blue over gray because these colors are only discriminable in a trichromatic visual system (Fig. 5C). In a dichromat, blue and gray would only be discriminated when there is opsin coexpression (Fig. 5D); however, we have shown that coexpression is uncommon in the area centralis (Dalton et al., 2016). Further, if cichlids are trichromats, with or without coexpression, they would be able to discriminate blue and purple (Fig. 6A). In contrast, dichromats with coexpression would not be able to choose blue over purple (Fig. 6B), which was not the case: all fish significantly chose blue over purple.

These mistakes fit with the RNL model predictions, where chromatic discrimination is only plausible under bright illumination and not in low-illumination conditions because achromatic mechanisms may become important (Kemp et al., 2015; Vorobyev, 2003; Vorobyev and Osorio, 1998). This is because the Weber law suggests that, in bright light, photoreceptor noise is independent of the signal, whereas, in dim light, chromatic discrimination is affected by both internal photoreceptor noise and fluctuations in the number of absorbed photons. Thus, the Weber law is no longer valid (Schaefer et al., 2007). It is remarkable that, even with very little stimulation of single cones when photon-shot noise is considered (Table S2B), *M. benetos* succeeded in discriminating color stimuli, reinforcing the assumption that there is color opponency between members of double cones.

Lastly, there might be a behavioral component we are overlooking causing fish to make mistakes. It is noteworthy that, in spite of their mistakes, most fish succeeded in choosing the trained stimuli over the distracter in all tests. Likely, *M. benetos* is achieving color vision using their area centralis, where color discrimination would be at its best. In addition, in bright environments like Lake Malawi, there is significantly more UV light to stimulate single cones so that photoreceptor shot noise would be smaller and color discrimination better.

Color vision and its relationship with cichlid ecology and evolution
Our results are particularly relevant for the study of evolution in the cichlid lineage because the exploitation of color vision allows cichlids to use communication channels that can be subject to variation. Subsequently, this organismal variation in sensory cues can lead to bursts of signaling evolution, where male secondary sexual characteristics diversify leading to cladogenic events (Kocher, 2004; Streelman and Danley, 2003). This is likely important for *M. benetos* because its sympatric close relatives,

M. zebra and *Metriaclima sandaracinos*, differ mainly in coloration patterns (Albertson et al., 1999; Stauffer et al., 1997).

In the wild, *M. benetos* would benefit from color vision. Similar to many Malawi cichlids, this species relies heavily on visual cues for foraging and mating. *M. benetos* would use color vision to identify conspecifics and to discriminate between dominant and subordinate individuals. This has been suggested because male *M. benetos* are especially UV reflective in the dorsal fin and flanks, which are displayed in mating and social-rank signaling (Jordan et al., 2003, 2004a). Chromatic discrimination would also benefit females because they would be able to choose between conspecific males. Male coloration pattern is important for African cichlids' assortative mating and this has been studied behaviorally (Seehausen and van Alphen, 1998; Seehausen et al., 1997; Selz et al., 2014) because visual signals are likely the first step in the multimodal courtship (Escobar-Camacho and Carleton, 2015).

Color vision would also facilitate foraging tasks for *M. benetos* because rock-dwelling cichlids are notorious omnivores with a broad spectrum of feeding habits and items, ranging from scraping algae to zooplanktivory (Genner et al., 1999; Mckaye and Marsh, 1983; Reinthal, 1990). Color discrimination would enable cichlids to tell apart specific food items from a variety of different objects and backgrounds. Indeed, UV sensitivity improves foraging efficiency in *M. benetos* (Jordan et al., 2004b); however, this discrimination could rely on contrast as well as color vision.

More experiments are needed to further test cichlids' chromatic discrimination capabilities. In this study, fish were trained exclusively for blue but we do not know whether cichlids exhibit bias towards specific colors. There is the possibility that *M. benetos*, owing to its coloration pattern and its main sensitivity to the 'short' range of the wavelength spectrum, succeeded in discriminating blue because of a preexisting preference towards this wavelength. Color bias has been reported in teleosts before. Picasso triggerfish (*Rhinecanthus aculeatus*) seems to avoid yellow and blue, and both the Picasso triggerfish and the lunar wrasse (*Thalassoma lunare*) seem to prefer green and red (Cheney et al., 2013). Preference towards specific colors in assortative mating could also underlie a sensory bias. This has been reported for the stickleback's preference for red (Smith et al., 2004) and the guppy's preference for orange (Rodd et al., 2002). Because male *M. benetos* exhibit a pale-blue nuptial coloration (Stauffer et al., 1997), sensory bias studies are needed to test whether there is a preexisting preference for blue.

Conclusions

In this study, we have shown that cichlids can be trained through classical conditioning in order to perform color discrimination tasks. Cichlids successfully discriminated blue from gray as well as from several different color targets. Our visual modeling and behavioral results suggest that cichlids have color vision, probably through color opponency mechanisms produced by neural interactions of three different photoreceptor spectral channels. Furthermore, we suggest that opsin coexpression can vary in its effects on color perception towards specific wavelengths and, hence, in color discrimination power. The capability of color discrimination in cichlids can have a big impact in understanding the natural history of this speciose clade because cichlids' visual capabilities and coloration patterns have been associated with their adaptive radiation and evolutionary success.

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