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RESEARCH ARTICLE

An Ishihara-style test of animal colour vision

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ABSTRACT

Colour vision mediates ecologically relevant tasks for many animals, such as mate choice, foraging and predator avoidance. However, our understanding of animal colour perception is largely derived from human psychophysics, and behavioural tests of non-human animals are required to understand how colour signals are perceived. Here, we introduce a novel test of colour vision in animals inspired by the Ishihara colour charts, which are widely used to identify human colour deficiencies. In our method, distractor dots have a fixed chromaticity (hue and saturation) but vary in luminance. Animals can be trained to find single target dots that differ from distractor dots in chromaticity. We provide MATLAB code for creating these stimuli, which can be modified for use with different animals. We demonstrate the success of this method with triggerfish, *Rhinecanthus aculeatus*, which quickly learnt to select target dots that differed from distractor dots, and highlight behavioural parameters that can be measured, including success of finding the target dot, time to detection and error rate. We calculated discrimination thresholds by testing whether target colours that were of increasing colour distances (ΔS) from distractor dots could be detected, and calculated discrimination thresholds in different directions of colour space. At least for some colours, thresholds indicated better discrimination than expected from the receptor noise limited (RNL) model assuming 5% Weber fraction for the long-wavelength cone. This methodology could be used with other animals to address questions such as luminance thresholds, sensory bias, effects of sensory noise, colour categorization and saliency.

KEY WORDS: Visual ecology, Colour vision assessment, Animal behaviour, Colour measurement, Spectrophotometry

INTRODUCTION

Over recent years, studies of animal colour vision have focused on the identification of physiological mechanisms, including photopigment and photoreceptor spectral sensitivities and neurons coding for opponency mechanisms, and on theoretical models to predict colour discrimination from this information (e.g. Partridge, 1989; Vorobyev and Osorio, 1998; Shapley and Hawken, 2002; Porter et al., 2012). Such data and models cannot replace behavioural tests of colour perception and of the role of colour in animals' daily lives. Behavioural investigations have tested discrimination thresholds (e.g. Wright, 1972; Vorobyev et al., 2001; Thoen et al., 2014, Olsson et al., 2015; Champ et al., 2016) and higher-order neural processes, such as colour constancy (e.g. Olsson et al., 2016; Wilkins et al., 2016), generalization (e.g. Baddeley et al., 2001; Kitschmann and Neumeyer, 2005; Scholtyssek et al., 2016) and categorization (e.g. Jones et al., 2001; Hanley et al., 2017; Caves et al., 2018). However, the underlying mechanisms of these processes remain poorly understood, even in primates (Kelber, 2016). In part, this is because behavioural tests of visual processes with non-human animals are challenging and time consuming. Therefore, novel methods for testing animal colour vision, quickly and with naturalistic behaviour, would be very useful for our understanding of ecological and evolutionary processes.

Working with honeybees, von Frisch (1914) conducted the first behavioural demonstration that non-human animals could identify coloured targets, independent of reflectance intensity. Bees were trained to receive a food reward associated with a blue coloured card and then continued to select the blue card even when the food reward was omitted and the blue card was presented among grey cards of similar achromatic ('brightness') cues. Many subsequent studies have trained animals to a rewarded colour or pattern (Olsson et al., 2015; Champ et al., 2016; Newport et al., 2017), often using operant conditioning and pairwise or multiple-choice discrimination tests. In these experiments, subjects learn a specific colour to receive the food reward. To achieve this, memory of the absolute colour is required, as the animal has to recall the colour learnt in a previous test to distinguish it from the more or less similar alternatives. Also, these methods limit the number of colours that can be examined within a reasonable time (Goldsmith and Butler, 2003; Olsson et al., 2015; Champ et al., 2016), and are particularly restrictive in animals that are challenging to train. For example, Champ et al. (2016) took 4 months to train fish to conduct a pairwise discrimination test, and only three out of seven individuals learnt the task well enough to continue to the testing phase. We have also experienced similar difficulties training fish using a paired-choice test methodology. Some animals may also learn the relationship between presented colours in a paired-choice test rather than a specific colour; for example, in Hemmi (1999), wallabies learnt to choose the colour with the longest wavelength, which enabled the testing of multiple colour combinations in the experiment. Other methodologies, including the spontaneous pecking of dots that varied in size and colour (as per Osorio et al., 1999, with chicks) have been used. However, there is no training to the test stimulus in such studies; therefore, it is potentially difficult to disentangle innate sensory bias or prior experience with colour discrimination abilities. During training, it is possible to test whether preferences for particular colours exist.

Here, we introduce a new method for testing animal colour vision, which is inspired by Ishihara tests used to identify colour

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vision deficiencies in humans (Ishihara, 1917; Fig. 1A). We used stimuli that display a single (or more) target dot(s), which differs from the distractor dots in chromaticity. Animals learn to identify and approach the odd coloured dot to receive a food reward. This offers three very significant advantages over most existing methods. First, the task itself does not require memory of the colour, and consequently more closely resembles most methods used to test human colour thresholds, which are based on simultaneous comparisons of adjacent colours (MacAdam, 1942). Second, one can add uninformative variation (noise) in any direction of choice in the animal's colour space, which can be used to control task difficulty or to investigate neural mechanisms such as opponent channels. Here, we added luminance noise to the distractor colours to confirm that the fish were using chromatic signals. Third, it is easy to collect at least two separate psychometric measures: accuracy (or error rate) and latency (time) to find the target dot, which can be useful for example in evaluating responses to suprathreshold colour differences. In addition, the method makes it easy to test multiple colours in quick succession without retraining, which is a highly efficient experimental design. Finally, we will see that, at least for the triggerfish, this task seems to evoke normal foraging behaviour, making it easy to run and giving some confidence that performance is ecologically relevant.

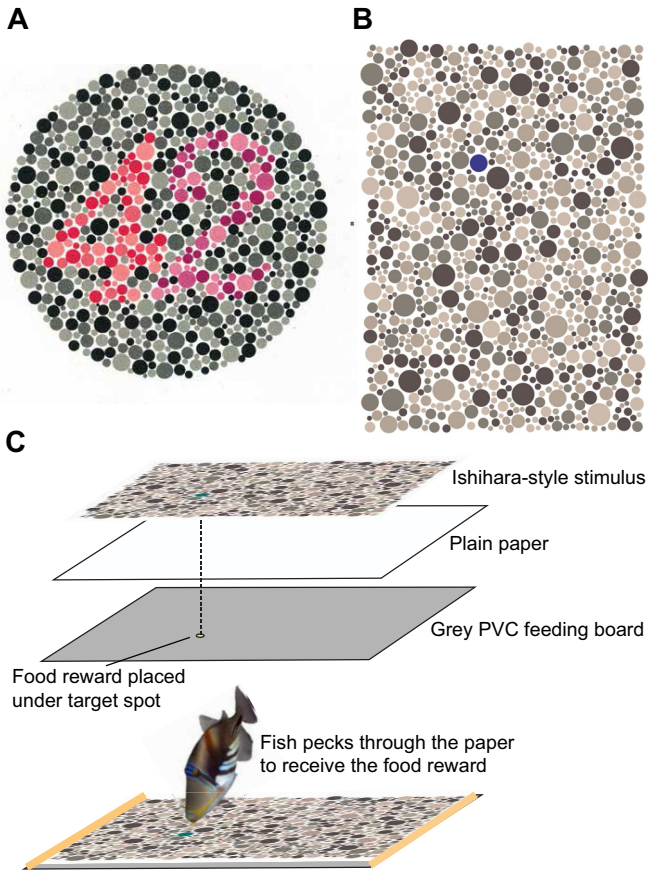

Fig. 1. Ishihara tests and experimental setup. (A) An example of an Ishihara pseudoisochromatic colour plate (plate 23 of 38 in Ishihara, 1917). The distractor dots vary in luminance and are achromatic, whereas chromatic dots make up the numerical symbols and can be detected because of changes in hue/saturation. Those with normal colour vision will read 42, whereas individuals with strong protanopia (less sensitivity to red light) will read 2, and those with strong deuteranopia (less sensitivity to green light) will read 4. (B) An example of our colour stimuli used to test discrimination thresholds in fish. The distractor dots only vary in luminance and are achromatic to triggerfish when calibrated and printed on laserjet copy paper. The target dot (in this example, blue) is chosen randomly and varies in hue and/or saturation but is within the luminance range of the distractor dots. (C) Food is placed on a grey feeding board directly under the location of the target dot. A second piece of paper is placed in between the board and the stimulus to ensure no discernible bump is left by the food that can be detected by the fish. Fish are trained to find and peck through the papers at the target dot to receive the food reward underneath. Elastic bands hold the stimulus in position on the feeding board.

Ishihara plates comprise an array of dots that vary in colour (i.e. chromaticity), brightness and size. The original Ishihara tests of human colour vision exploit the ability of the visual system to segregate elements of an image into figure (object) and ground (background) based on their sharing some common feature, with the colours and brightness of the Ishihara dots being designed so that subjects group dots of (approximately) equal chromaticity, which is dependent on the type of colour vision (Fig. 1A). Our tests do not examine visual grouping by colour (although they could easily be modified to do so; Mitchell et al., 2017; Siniscalchi et al., 2017). Instead, the animal learns to find a single target dot that differs in hue or saturation from the distractor dots that only vary in luminance (Fig. 1A) and tap at it to receive a food reward. We found that the triggerfish, *Rhinechanthus aculeatus*, seems not to learn a particular colour, but instead recognizes the target dot that differs in chromaticity. This has the major practical advantage of allowing multiple colours to be tested without retraining fish. We provide a MATLAB code that produces these stimuli and randomizes the location of the target dot.

To demonstrate the performance of the fish in these tests, we provide data to calculate colour thresholds of triggerfish and compare these with predictions of the receptor noise-limited (RNL) model, which is widely used to predict colour discrimination thresholds (just noticeable differences, JNDs) in non-human animals (Vorobyev and Osorio, 1998; Vorobyev et al., 2001).

MATERIALS AND METHODS

Study species

Rhinecanthus aculeatus (Linnaeus 1758) lives on sub-tidal reef flats throughout the Indo-Pacific and is a generalist omnivore, feeding predominantly on molluscs and crustaceans. Individuals are easily trained, and perform well in behavioural tests of colour vision (Pignatelli et al., 2010; Cheney et al., 2013; Champ et al., 2016; Simpson et al., 2016; Newport et al., 2017). Fish (n=8; total length 6–16 cm; age and sex could not be determined) were collected from shallow reefs around Lizard Island using hand nets, and then shipped to The University of Queensland. Here, they were housed in individual aquaria (60×40×30 cm deep) with running seawater from a reservoir (sump) tank and adequate aeration. Tanks were illuminated using KR 96-K36B LED 35 W lights (Ecolamps Inc., Nivelles, Belgium; Fig. S4). Experiments were conducted in February–April 2016. Fish were collected under a Queensland General Fisheries Permit (no. 161624) and a Great Barrier Reef Marine Parks Authority Permit (no. G12/35688). This research was conducted in accordance with approval granted by the University of Queensland's Animal Ethics Committee (SBS/111/14/ARC).

Rhinecanthus aculeatus was the first species known to use double cone members independently in colour vision (Pignatelli et al., 2010), and has trichromatic vision based on one type of single cone containing short-wavelength visual pigment (photoreceptor λ_{max}=413 nm); and a double cone, with one member containing middle-wavelength pigment (photoreceptor λ_{max}=480 nm) and the other member containing long-wavelength pigment (photoreceptor λ_{max}=580 nm) (Cheney et al., 2013). *Rhinecanthus aculeatus* has a

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yellow corneal pigment (Siebeck and Marshall, 2001; Fig. S1), the density of which increases during the day (our unpublished data). Because these fish are diurnally active, we therefore modelled the photoreceptor spectral sensitivities with the corneal pigment filtering the incident light. Luminance signals are assumed to be encoded either by both members of the double cone or by the long-wavelength photoreceptor alone (Wild, 2011). In behavioural tests, this species has a visual acuity of 1.75 cycles per degree, similar to that of goldfish (Champ et al., 2014). In a previous study (Newport et al., 2017), triggerfish were able to resolve a pattern of 2 mm (diameter) dots from a control when stimuli were placed at a similar distance from the fish to that in this study (20 cm or less). The dots in our patterns ranged from 3 to 16 mm in diameter, and therefore all dots were visible to the fish at their attack distance (<20 cm). In our code, the size of the dots can be altered to make the pattern more suitable for larger or smaller species and/or those with different visual acuity.

Creating and measuring colours

To calibrate and select our distractor and target colours, we first created matrices of colours with a range of RGB values using our MATLAB code GetRGBcombinations.m (Fig. 2). These colours were then printed using a Canon LaserJet Pro 400 printer (Canon, Melville, NY, USA) on Steinbeis TrendWhite A4 recycled 80 g unbleached white copy paper (Steinbeis Papier GMbH, Glückstadt, Germany). Printing with a laserjet printer ensured the pigment (actually melted plastic) did not run or change over time when immersed for <5 min, and no chemicals or dye was released into the water. We chose this paper as it has lower fluorescence than most common brands of bleached printer paper, allowing us to print

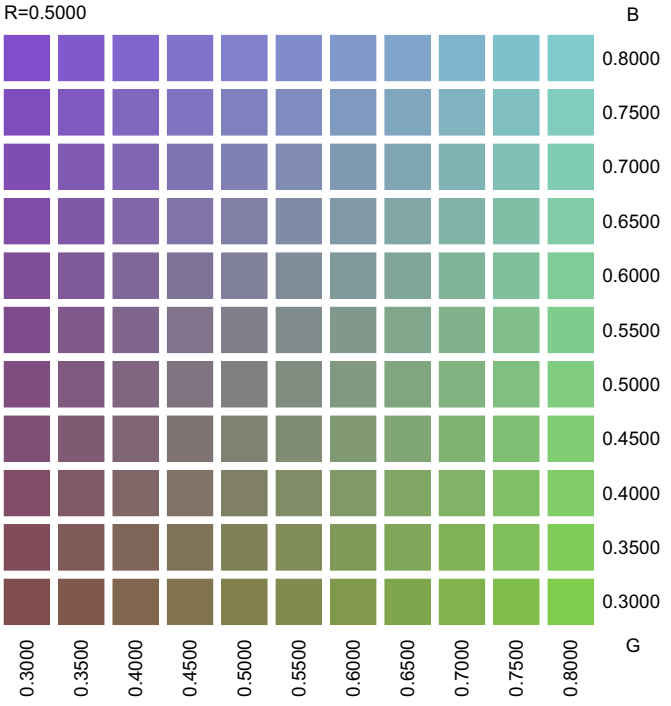

Fig. 2. Colour matrix with specific RGB values created using the GetRGBcombinations.m code. Colours were printed with a laserjet printer and the spectral reflectance of each colour combination was measured with a spectrophotometer. All the colours in this matrix have an R value of 0.5. The G and B values are shown on the *x*-axis and *y*-axis, respectively. We made additional sample colours with other R values ranging from 0 to 1 to make the gamut of experimental colours.

colours close to the achromatic point, as modelled with the visual system of the triggerfish (Fig. 3). After printing, the paper was then briefly soaked in water, as the paper would be wet during experiments, removed and the spectral reflectance of each colour was measured in air relative to a Spectralon white standard with an Ocean Optics USB2000 spectrophotometer and a desktop computer running OceanView software (Ocean Optics, Winter Park, FL, USA). Measurements were made using a 200 µm diameter, bifurcated cable, which was also connected to a PX-2 pulsed xenon light source (Ocean Optics). For accurate measurements, the fibre was held 1 mm above the paper at a 45 deg angle with an RPA-SMA Fiber Holder Arm and shielded from stray light. To ensure colours produced by the printer were consistent, each colour was printed on different days and measured on at least five separate occasions (mean±s.d. difference from first printed colour: ΔS=0.38 ±0.17, well below the putative threshold of 1.0). Variation was greatest when ink levels of the printer were low; therefore, we limited printing of test stimuli to when levels were sufficient. We also used the Colour Calibration and Head Cleaning utility in the printer's menu regularly to maintain consistency. The MATLAB code (MathWorks, Natick, MA, USA) used to create the colour matrices and stimuli was written by J.A.E. and is available from the Dryad Data Repository (doi:10.5061/dryad.gr38v6r), in addition to detailed guidelines for using this code. For researchers without access to MATLAB or one of its free mimics, we also provide information on code in R, which creates similar stimuli.

Visual modelling and selection of target colours

Chromaticity of distractor and target colours was specified by the estimated excitations of triggerfish photoreceptors (quantum catch; Table S1), as quantified using photoreceptor spectral sensitivity of triggerfish, and illumination and reflectance spectra of printed colours (Figs S1, S2, S4; as per eqn 1 in Vorobyev and Osorio, 1998). A von Kries correction for light adaption was applied using the average spectral reflectance of the distractor dots and background paper between the dots. Calculations were conducted in the R package colourvision (Gawryszewski, 2018).

Colour distances ΔS between colours were modelled using the trichromatic photopic RNL model (Vorobyev and Osorio, 1998; Kelber et al., 2003), which assumes that colour discrimination is dependent on chromatic signals and limited by noise originating in the receptors. Colours were also plotted in an RNL chromaticity diagram defined in eqn 4 of Hempel de Ibarra et al. (2001). We used the Weber fraction to estimate noise in the photoreceptors because there are no direct measurements of receptor noise in this species (Kelber et al., 2003). Following evidence from other vertebrates (Vorobyev and Osorio, 1998; Olsson et al., 2015), this model assumes that spatial summation reduces receptor noise, and that noise in each receptor mechanism can be estimated based on the relative abundance of photoreceptor types in the retina, which is a ratio of 1:2:2 (short:middle:long, S:M:L). The long-wavelength sensitive (LWS) noise threshold was set at 0.05 and therefore estimated noise in each photoreceptor was: S, 0.07; M, 0.05; L, 0.05.

Nine reflectance spectra that were very close to the triggerfish achromatic point were chosen as the distractor colours (Fig. 3; Figs S2, S3). Twenty-nine target colours (RGB values used for printing are shown in Table S1) were chosen based on their positions in several radial lines away from the achromatic point (Fig. 3); all colours along the same line were similar in hue (line angle from the origin or achromatic point) but varied in chromaticity (distance from achromatic point). On four lines or colour sets ('Brown', 'Green', 'Pink' and 'Blue') we used six target colours,

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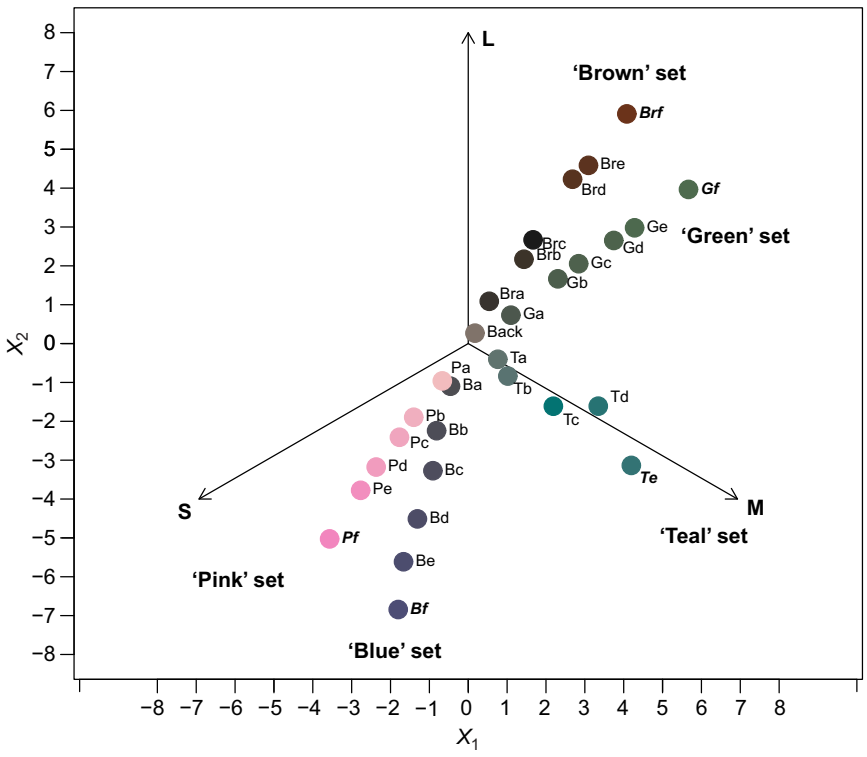

Fig. 3. Chromaticity diagram with colour of target dots corresponding to the receptor noise-limited (RNL) model. X_1 (X) and X_2 (Y) are defined in eqn 4 of Hempel de Ibarra et al. (2001) based on spectral sensitivities of triggerfish *Rhinecanthus aculeatus*. Colours with names in bold-italic (Brf, Gf, Te, Bf, Pf) were used for training and for reinforcement during testing. Distractor ('Back') denotes the cluster of greys used as the distractor dots. This plot was produced using the R colour vision package (Gawryszewski, 2018). We refer to colours approximately on the same line radiating from the achromatic point as colour sets. M, middle wavelength; L, long wavelength; S, short wavelength.

but on our 'Teal' set we used only five target colours because of the limitations of laserjet reflectance spectra. All colours were tested in February–April 2016 with eight fish.

Experimental setup

During training and testing, tanks were divided into two halves with an opaque partition, which included a door that could be opened by sliding a board upwards. This enabled one side of the tank to be the test arena, where the stimulus could be set up without the fish seeing that area. Printed A4 stimuli were placed on a grey A4-sized plastic (PVC) feeding board and secured in place with two light brown elastic bands at each end, which were ignored by fish during testing. We first trained fish with a plain grey background on which there was only one dot, which was one of the following five colours: Brf, Gf, Te, Bf, Pf (Fig. 3). During the first 2–4 sessions (with six trials per session), we placed the food reward (small pieces of squid) on top of the dot to encourage fish to approach and peck at the dot. Once fish began to associate the dot with a reward, the food was then placed on the PVC board, underneath the paper and directly located under the target dot (Fig. 1C). We also placed an additional, plain piece of paper (same stock) under the printed stimuli to ensure that the colour of the stimuli was not altered by the grey PVC board and that no marks or impressions were left by the food. Fish quickly learnt to peck through the paper to create a hole, obtain the food, and spit out the paper. Movie 1 is a video of a fish performing this behaviour. Each fish completed a further 5 training sessions with food underneath the dot.

Fish then progressed to a second stage of training during which we used Ishihara-style stimuli, which had one target dot that was deemed easily detectable by the fish (Brf, Gf, Te, Bf, Pf; Fig. 1B). Within a few days, fish learnt to find the dot that differed in hue or saturation (chromaticity) from the distractor and then peck the target dot to obtain food (squid) placed underneath it (Fig. 1C). During this stage, consisting of 12 sessions, fish were randomly presented with the five training colours to ensure they did not learn that the food was rewarded from a particular colour. After six training sessions, we conducted a generalized linear mixed model using the glmer function in the lme4 package (Bates et al., 2015) in R (http://www.R-project.org/) with colour as a fixed factor, fish ID as a random factor and time to detection as the response variable. We found that there was no success rate bias for different training colours (glmer, $z=-1.36$, $n=224$, $P=0.17$).

During testing, trials commenced when the door within the partition was removed, located approximately 30 cm from the stimulus, and the fish swam through to the test arena. Fish were given 30 s to find the target dot and peck through it to receive the food reward (Fig. 1C). Each test session consisted of five trials, and 1–2 sessions were conducted per day. The order in which the 29 colours were presented, the size of the dot and position of the target dot were all randomized. In total, we conducted 906 individual trials in March–April 2016 and each target colour was presented to each fish between 2 and 11 times (mean±s.d.: 3.91±1.56).

During each trial, we recorded: (1) whether the fish was successful in pecking the target dot within 30 s of entering the test arena; (2) if so, the time taken from entry to pecking at the target (latency to find the dot); and (3) the number of dots that were pecked incorrectly before the target was pecked. Interestingly, the fish always pecked directly on a dot and not in between dots or elsewhere on the paper. After the target dot had been pecked or 30 s had elapsed, the fish were gently encouraged with a net to swim out of the test arena and back through the door, and the stimulus was removed.

Throughout the experiment, we also randomly conducted 120 control trials (15 per fish) in which there was no differently coloured target dot, i.e. they were all distractor dots. Food was still placed under one randomly selected dot to ensure that fish were using visual information and not olfactory or other cues other than differences in hue/saturation to detect the target. The mean success rate for control trials was low (3.3%), indicating that, although this was greater than chance (there are approximately 180 of the largest

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three dots on each stimulus; which gives a chance level of 0.6%), it was very unlikely that fish used predominantly olfactory or other cues (such as a mark or blister on the paper created by the squid) to find the food.

Statistical analyses

To model the probability of success for each colour, cumulative Gaussian curves were fitted to the data (Wichmann and Hill, 2001) using the quickpsy R package (Linares and López-Moliner, 2016). Deviance values were very similar to curves fitted with a logistic curve function. The ΔS at which the probability of success was 50% was calculated for each colour set. In previous studies that used paired choice tests, discrimination thresholds were often modelled at 75% correct choices to be statistically above a 50% random choice threshold (Vorobyev et al., 2001). Because of our experimental design, we reduced our threshold to 50% given the number of dots being presented to the fish and the 3.3% success rate in our control trials; however, this could be modified depending on the research question. In trials, dot size did not significantly impact the chance of finding the target dot (z=−1.19, n=906, P=0.24), as expected.

RESULTS

For each target colour, the mean success rate at which fish located the target dot ranged from 0% and 100%. For each fish and colour set, the probability of success fitted a normal cumulative distribution function (deviance <6.31, P>0.31), with the exception of Fish O-Teal and Fish L-Green, which both exhibited an abrupt step function (Fig. 4). Teal had the lowest 50% ΔS threshold (mean±s.d.: 0.69±0.30) and Pink had the highest (2.87±0.66). Fish discrimination thresholds for the other colour sets were: Brown 2.33±0.35, Blue 2.63±0.72, Green 1.39±0.57.

In successful trials (n=699), fish took between 1.17 and 29.91 s (mean±s.d.=7.04±6.43 s) to find the target dot and fish made between 0 and 8 (0.55±1.10) incorrect pecks. During unsuccessful

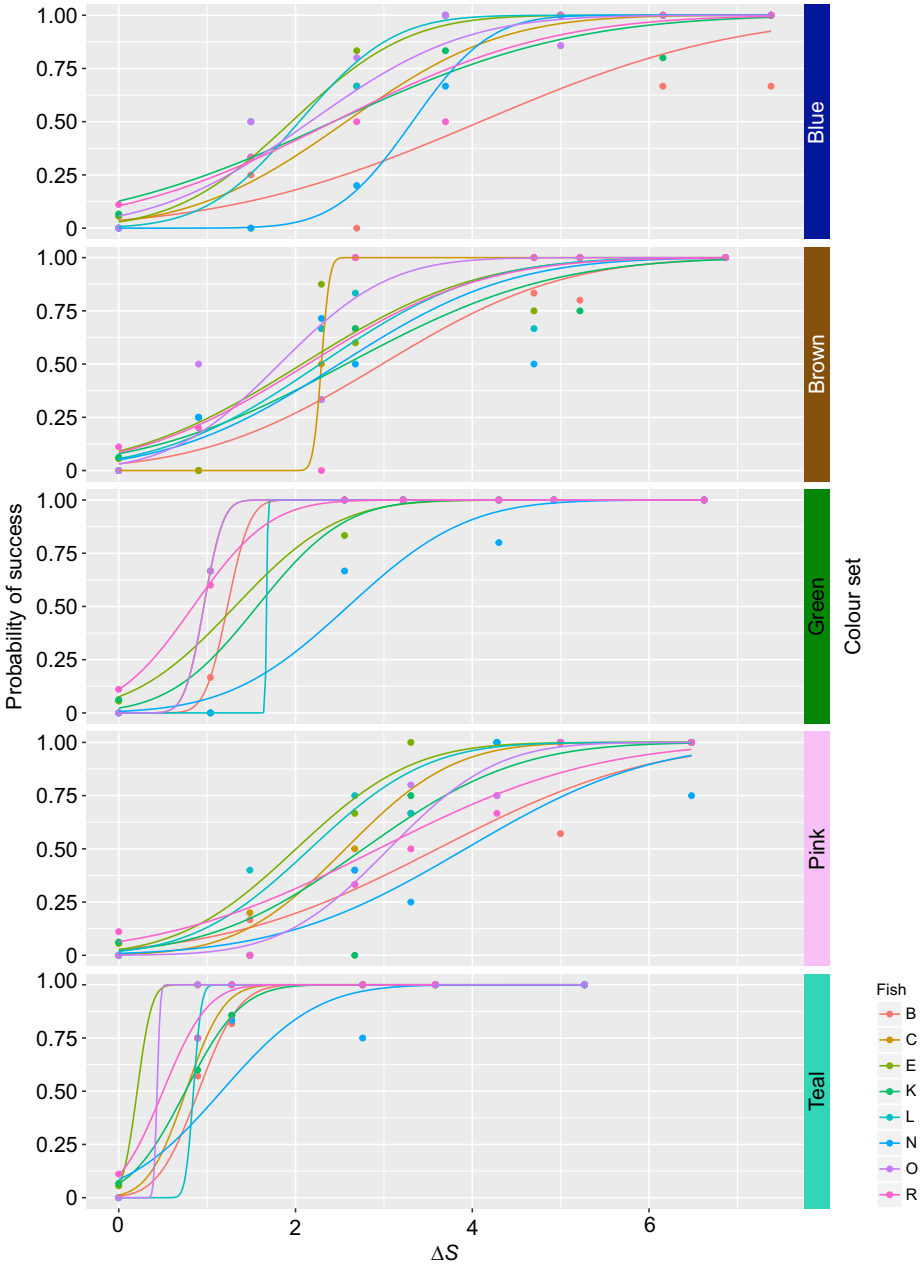

Fig. 4. The probability of success in detecting each of the 29 target colours separated by fish and colour set. Psychophysical cumulative Gaussian curves were fitted for each fish with the quickpsy package in R (see Statistical analyses for details). A greyscale version of this figure is available in the supplementary information (Fig. S4).

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trials (n=207), fish made between 0 and 7 incorrect pecks (3.05±1.91). The average number of incorrect pecks and the time taken to find the target dot decreased with increased ΔS in a non-linear manner (Fig. 5). Further analysis of specific threshold data in relation to the fishes' visual mechanisms is currently being undertaken and will be published in due course.

DISCUSSION

We have presented a modification of the Ishihara colour vision test method for animals and demonstrated how it can be used to assess colour discrimination thresholds of a teleost. We have provided MATLAB code and other resources so that this method may be used with other animals. The method differs fundamentally from most other tests of animal colour vision, which are based on memory, and as such our method is much more similar to the methods used to test human colour thresholds and the role of colour in visual search. Fish learnt the task quickly, and multiple colours could be tested concurrently because of the fishes' ability to learn to find the dot that differed in terms of chromaticity, avoiding the need to train them separately to each rewarded colour. Therefore, at a more practical level, many colours can be tested rapidly and concurrently, making it possible to investigate colour discrimination throughout

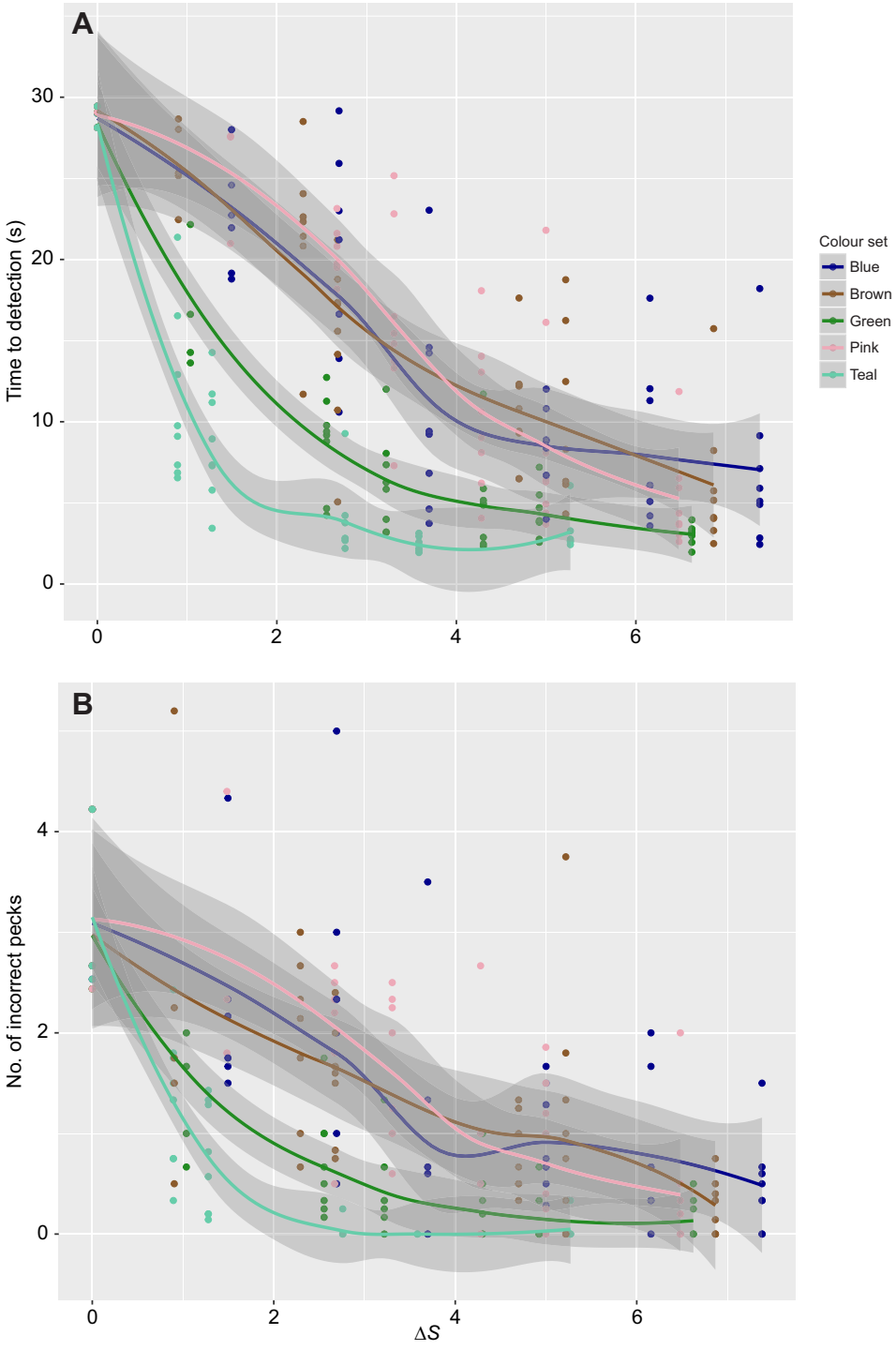

Fig. 5. Time taken to detect target dot and number of incorrect dots chosen. Plots of (A) mean time to detection as a function of ΔS for each coloured target/fish during successful trials and (B) number of incorrect pecks during trials. Lines were plotted with LOESS (local polynomial regression fitting) and shaded areas indicate confidence intervals. Additional figures have been provided in the supplementary information suitable for black and white printing.

colour space in more detail than has hitherto been possible in non-human animals (our unpublished data). This method was chosen to resemble natural foraging behaviour of the triggerfish, i.e. pecking at objects on the substrate, rather than selecting a specific spectral stimulus to receive a food reward, which we expect to be reproducible in other species with comparable foraging ecologies. Indeed, this method could be used to test the visual capabilities of other fish species, lizards, birds and mammals, including standard laboratory model organisms such as rodents and zebrafish. We would also be interested to see whether this method works with invertebrates, such as bees. One disadvantage of this method is the significant cost involved in printing large quantities of stimuli using a laserjet printer and slight variation in printed colours over time. We believe some animals could be trained to instead peck at laminated stimuli or tap at a screen (even a touchscreen) displaying the stimuli to receive a food reward from above, which could be more cost effective and limit the time taken to produce the stimuli. Indeed, our triggerfish have since been trained to tap on Ishihara stimuli displayed on an iPad screen placed in an underwater housing.

Our fish were able to find a dot that differed from the distractor dots in terms of chromaticity, regardless of the particular hue. In further experiments, we have found that fish also perform this task when the distractor dots are coloured, rather than achromatic grey (our unpublished data). The test stimuli feature many distractor dots, which is expected to improve the ease with which animals can perform this task, by increasing recognition of the relationship between distractors, and because they produce a 'pop out' effect in which the odd stimulus stands out from the rest. In addition, many vertebrates, for example guppies (Eakley and Houde, 2004) and birds in urban areas (Tryjanowski et al., 2016), have shown an innate preference for novel items, which may attract them to an odd coloured dot. Colour and contrast are crucial cues in animal learning (Newport et al., 2017; Osorio et al., 1999) and so we anticipate that learning to find an odd colour may be easier than other tasks, such as learning the odd pattern. Our method is indeed advantageous because animals learn the task rather than a particular colour and readily generalize to other colours. We envisage that a number of other animals will also be able to perform this behaviour with relative ease.

We are mindful that our methodology may be prone to false negatives: if the animals do not respond to the target dot, they may still be able to discriminate it from distractor dots but other factors may influence their decisions. However, our fish were very motivated to perform the task to receive a food reward and made very few mistakes when making a correct choice; therefore, we do not believe that false negatives significantly impacted our results but perhaps this should be considered if this method is used with other animals.

To enable accurate calibration of coloured dots, it would be necessary to have spectral sensitivity measurements of cone photoreceptors using microspectrophotometry from the animal being tested, in addition to information on spectral filters, including cornea, lens and oil droplets. Detailed information on how achromatic signals are processed may not be essential if the distractor dots cover a luminance range that encompasses the brightness of the target colours, as modelled with a range of probable luminance channels, i.e. combined quantum catch of the double cone versus quantum catch of the long-wavelength receptor alone.

Using this methodology, our study demonstrated that discrimination thresholds varied according to the direction of colour space tested, compared with the theoretical prediction (Vorobyev and Osorio, 1998). Discrimination thresholds ranged from 0.7 ΔS (Teal) to 2.9 ΔS (Pink). Some of this variation in thresholds may indicate that noise levels, as calculated by the Weber fraction and the ratio of different photoreceptor types, were incorrect. Measurements of noise within individual photoreceptors is available for very few species, namely honeybees (Vorobyev et al., 2001), so it would be of great value to measure photoreceptor noise in other species. Other factors that may influence thresholds include co-expression of opsin genes in particular parts of the retina (Dalton et al., 2014), background colour (adaptation), and temporal and spatial effects or prior experience leading to positive or negative associations with certain colours, all of which require further investigation. Using pairwise tests, discrimination thresholds were measured in our model species *R. aculeatus*, as approximately 2 ΔS (Champ et al., 2016) for blue colours; therefore, the two methods appear to give similar results, but this latter experiment only measured thresholds in one area of colour space.

We also recorded time to detection and number of incorrect choices, which can be used to measure the detection of suprathreshold colours and colour saliency. With minor modifications, this methodology could explore grouping of stimuli using achromatic and chromatic cues (as per Mitchell et al., 2017), or investigate the impact of sensory noise on signal detection by designing distractor dots so they differ in achromatic and/or chromatic noise. This code also enables more than one target dot to be created and therefore could also be used to examine other questions such as colour categorization and sensory bias.

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Competing interests
The authors declare no competing or financial interests.

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

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Data availability
Data and MATLAB code are available from the Dryad Digital Repository (Cheney et al., 2018): dryad.gr38v6r

Supplementary information
Supplementary information available online at
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References
Baddeley, R., Osorio, D. and Jones, C. D. (2001). Colour generalisation by domestic chicks. *Behav. Brain Sci.* **24**, 654-654.

Bates, D., Mächler, M., Bolker, B. and Walker, S. (2015). Fitting linear mixed-effects model using lme4. *J. Stat. Softw.* **67**, 1-48.

Caves, E. M., Green, P. A., Zipple, M. N., Peters, S., Johnsen, S. and Nowicki, S. (2018). Categorical perception of colour signals in a songbird. *Nature* **560**, 365-367.

Champ, C., Wallis, G., Vorobyev, M., Siebeck, U. E. and Marshall, N. J. (2014). Visual acuity in a species of coral reef fish *Rhinecanthus aculeatus*. *Brain Behav. Evol.* **83**, 31-42.
Champ, C. M., Vorobyev, M. and Marshall, N. J. (2016). Colour thresholds in a coral reef fish. *R. Soc. Open Sci.* **3**, 160399.
Cheney, K. L., Newport, C., McClure, E. C. and Marshall, N. J. (2013). Colour vision and response bias in a coral reef fish. *J. Exp. Biol.* **216**, 2967-2973.
Cheney, K. L., Green, N., Vibert, A., Vorobyev, M., Marshall, J. N., Osorio, D. C. and Endler, J. A. (2018). Data from: An Ishihara-style test of animal colour vision. *Dryad Digital Repository*. https://doi.org/10.5061/dryad.gr38v6r
Dalton, B. E., Loew, E. R., Cronin, T. W. and Carleton, K. L. (2014). Spectral tuning by opsin coexpression in retinal regions that view different parts of the visual field. *Proc. R. Soc. Lond.* **281**, 20141980.
Eakley, A. L. and Houde, A. E. (2004). Possible role of female discrimination against 'redundant' males in the evolution of colour pattern polymorphism in guppies. *Proc. R. Soc. Lond. B* **271**, S299-S301.
Gawryszewski, F. M. (2018). Color vision models: some simulations, a general n-dimension model, and the *colourvision* R package. *Ecol. Evol.* **8**, 8159-8170.
Goldsmith, T. H. and Butler, B. K. (2003). The roles of receptor noise and cone oil droplets in the photopic spectral sensitivity of the budgerigar, *Melopsittacus undulatus*. *J. Comp. Physiol. A.* **189**, 135-142.
Hanley, D., Grim, T., Igic, B., Samaš, P., López, A. V., Shawkey, M. D. and Hauber, M. E. (2017). Egg discrimination along a gradient of natural variation in eggshell coloration. *Proc. R. Soc. Lond. B.* **284**.
Hemmi, J. M. (1999). Dichromatic colour vision in an Australian marsupial, the tammar wallaby. *J. Comp. Physiol. A.* **185**, 509-515.
Hempel de Ibarra, N., Giurfa, M. and Vorobyev, M. (2001). Detection of coloured patterns by honeybees through chromatic and achromatic cues. *J. Comp. Physiol. A.* **187**, 215-224.
Ishihara, S. (1917). *Tests for color-blindness*. Tokyo: Handaya, Hongo Harukicho.
Jones, C. D., Osorio, D. and Baddeley, R. J. (2001). Colour categorization by domestic chicks. *Proc. R. Soc. Lond. B* **268**, 2077-2084.
Kelber, A. (2016). Colour in the eye of the beholder: receptor sensitivities and neural circuits underlying colour opponency and colour perception. *Curr. Opin. Neurobiol.* **41**, 106-112.
Kelber, A., Vorobyev, M. and Osorio, D. (2003). Animal colour vision - behavioural tests and physiological concepts. *Biol. Rev. Camb. Philos. Soc.* **78**, 81-118.
Kitschmann, M. and Neumeyer, C. (2005). Generalization and categorization of spectral colors in goldfish I. Experiments with one training wavelength. *J. Comp. Physiol. A.* **191**, 1025-1036.
Linares, D. and López-Moliner, J. (2016). quickpsy: an R Package to fit psychometric functions for multiple groups. *R. Journal* **8**, 122-131.
MacAdam, D. L. (1942). Visual sensitivies to colour differences in daylight. *J. Opt. Soc. Am. A.* **32**, 247-274.
Mitchell, L., Cheney, K. L., Cortesi, F., Marshall, N. J. and Vorobyev, M. (2017). Triggerfish uses chromaticity and lightness for object segregation. *Royal Soc. Open Sci.* **4**, 171440.
Newport, C., Green, N. F., McClure, E. C., Osorio, D. C., Vorobyev, M., Marshall, N. J. and Cheney, K. L. (2017). Fish use colour to learn compound visual signals. *Animal Behav.* **125**, 93-100.
Olsson, P., Lind, O. and Kelber, A. (2015). Bird colour vision: behavioural thresholds reveal receptor noise. *J. Exp. Biol.* **218**, 184-193.
Olsson, P., Wilby, D. and Kelber, A. (2016). Quantitative studies of animal colour constancy: using the chicken as model. *Proc. R. Soc. Lond. B* **283**, 20160411.
Osorio, D., Miklósi, A. and Gonda, Z. (1999). Visual ecology and perception of coloration patterns by domestic chicks. *Evol. Ecol.* **13**, 673-689.
Partridge, J. C. (1989). The visual ecology of avian cone oil droplets. *J. Comp. Physiol. A.* **165**, 415-426.
Pignatelli, V., Champ, C., Marshall, J. and Vorobyev, M. (2010). Double cones are used for colour discrimination in the reef fish, *Rhinecanthus aculeatus*. *Biol. Lett.* **6**, 537-539.
Porter, M. L., Blasic, J. R., Bok, M. J., Cameron, E. G., Pringle, T., Cronin, T. W. and Robinson, P. R. (2012). Shedding new light on opsin evolution. *Proc. R. Soc. Lond. B* **279**, 3-14.
Scholtyssek, C., Osorio, D. C. and Baddeley, R. J. (2016). Color generalization across hue and saturation in chicks described by a simple (Bayesian) model. *J. Vis.* **16**, 8.
Shapley, R. and Hawken, M. (2002). Neural mechanisms for color perception in the primary visual cortex. *Curr. Opin. Neurobiol.* **12**, 426-432.
Siebeck, U. E. and Marshall, N. J. (2001). Ocular media transmission of coral reef fish–can coral reef fish see ultraviolet light? *Vis. Res.* **41**, 133-149.
Simpson, E. E., Marshall, N. J. and Cheney, K. L. (2016). Coral reef fish perceive lightness illusions. *Sci. Rep.* **6**, 35335.
Siniscalchi, M., D'Ingeo, S., Fornelli, S. and Quaranta, A. (2017). Are dogs red–green colour blind? *Royal Soc. Open Sci.* **4**, 170869.
Thoen, H. H., How, M. J., Chiou, T. H. and Marshall, J. (2014). A different form of color vision in mantis shrimp. *Science* **343**, 411-413.
Tryjanowski, P., Møller, A. P., Morelli, F., Biaduń, W., Brauze, T., Ciach, M., Czechowski, P., Czyz, S., Dulisz, B., Golawski, A. et al. (2016). Urbanization affects neophilia and risk-taking at bird-feeders. *Sci. Rep.* **6**, 28575.
von Frisch, K. (1914). Der Farbensinn und Formensinn der Biene. *Zool. Jahbr. All. Zool.* **37**, 1-187.
Vorobyev, M. and Osorio, D. (1998). Receptor noise as a determinant of colour thresholds. *Proc. R. Soc. Lond. B* **265**, 351-358.
Vorobyev, M., Brandt, R., Peitsch, D., Laughlin, S. B. and Menzel, R. (2001). Colour thresholds and receptor noise: behaviour and physiology compared. *Vis. Res.* **41**, 639-653.
Wichmann, F. A. and Hill, N. J. (2001). The psychometric function: I. Fitting, sampling, and goodness of fit. *Percept. Psychophys.* **63**, 1293-1313.
Wild, R. (2011). Is visual acuity affected by cone cell signal suppression in blackbar triggerfish, Rhinecanthus *aculeatus*? *Masters Thesis*, University of Auckland.
Wilkins, L., Marshall, N. J., Johnsen, S. and Osorio, D. (2016). Modelling colour constancy in fish: implications for vision and signalling in water. *J. Exp. Biol.* **219**, 1884-1892.
Wright, A. A. (1972). The influence of ultraviolet radiation on the pigeon's color discrimination. *J. Exp. Anal. Behav.* **17**, 325-337.