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# Success of the receptor noise model in predicting colour discrimination in guppies depends upon the colours tested

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ABSTRACT

Accurate knowledge of species colour discrimination is fundamental to explain colour based behaviours and the evolution of colour patterns. We tested how the receptor noise limited model, widely used in behavioural ecology, matched actual colour discrimination thresholds obtained using behavioural tests. Guppies *(Poecilia reticulata*) were first trained to push a target coloured disk placed among eight grey disks of various luminances on a grey plate. Guppies were then tested to find target disks, which varied in colour contrast from the plate. The target disks followed a gradient going from high contrast to inconspicuous against the grey background. We plotted the percentage of correct choices of each colour in the gradient against the model prediction and determined the discrimination thresholds using the inflection point of the fitted sigmoid curve. We performed the experiment on six colour gradients: red, orange, yellow, green, blue and purple. Four colour gradients: red, orange, green and blue, showed a discrimination threshold that matched the model predictions. However, deviations of the model for the yellow and purple gradients suggest that ecological relevance of some colours could affect decision-making in behavioural tests and that we can no longer assume that the rules for colour discrimination are independent of colours.

## 1. Introduction

The information provided by colour signals is used in a wide range of behaviours that affect reproduction and survival including mate choice, foraging and predator-prey interactions (Caine & Mundy, 2000; Candolin, 2003; Couldridge & Alexander, 2002; Endler, 1978; Prudic, Skemp, & Papaj, 2007; Roulin & Bize, 2007). A fundamental step in understanding colour-based behaviours is to determine how colours are perceived (Kelber, Vorobyev, & Osorio, 2003). A number of mathematical models have been developed with the aim to predict the ability of a species to discriminate colours, based on what happens in the retina (De Ibarra & Menzel, 2014; Kemp et al., 2015). We can use visual models to predict visual abilities using known photoreceptor sensitivities and relative abundances that are available in many species (Cronin, Johnsen, Marshall, & Warrant, 2014). However, to determine actual colour perception, which includes processing in the brain as well as in the retina, and to validate predictions from the visual models, it is necessary to perform carefully designed behavioural experiments. These experiments evaluate the ability of individuals to detect or discriminate among colours (Kemp et al., 2015). Currently, behavioural colour discrimination thresholds are only available in a few species (Champ, Vorobyev, & Marshall, 2016; Dyer, Spaethe, & Prack, 2008; Fleishman et al., 2016; Lind, Chavez, & Kelber, 2014; Olsson, Lind, & Kelber, 2015; Vorobyev & Osorio, 1998; Vorobyev, Brandt, Peitsch, Laughlin, & Menzel, 2001).

The receptor noise limited model (RNL, Vorobyev & Osorio, 1998) is commonly used to predict colour discrimination. It assumes that the noise in the photoreceptors is the main limitation to colour discrimination in a given species (Vorobyev & Osorio, 1998). The RNL model estimates $\Delta S$: the sensed distance between stimuli. $\Delta S$ is thus a measure of the likelihood of colour discrimination between two stimuli; when $\Delta S = 1$, two visual stimuli are presumed to be just noticeably different on average (JND, Vorobyev & Osorio, 1998; Endler & Mielke, 2005; Kemp et al., 2015). Above this threshold ($\Delta S > 1$), the viewer is theoretically able to discriminate between the two stimuli. Below this threshold ($\Delta S < 1$) the viewer is unlikely to discriminate between them. The quantity $\Delta S$ is a statistical statement about discrimination and is in fact the Mahalanobis distance between two multivariate signals or measures (Clark, Santer, & Brebner, 2017). This is commonly used in multivariate statistics and signal detection theory, and as such, is a statement about what will be true on average over time and not necessarily true in any one instant. The RNL model has been validated using behavioural experiments in a few species including, humans, honeybees, several bird, lizard and reef fish species (Champ et al., 2016;

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Dyer et al., 2008; Fleishman et al., 2016; Lind et al., 2014; Olsson et al., 2015; Vorobyev & Osorio, 1998; Vorobyev et al., 2001). However, the RNL model has not yet been validated using behavioural experiments in freshwater fish. Given the importance of freshwater fish in colour based evolutionary experiments (Cole & Endler, 2015; Couldridge & Alexander, 2002; Endler, 1991; Fuller, 2002; Kemp, Reznick, & Grether, 2008; Kranz, Cole, Singh, & Endler, 2018; Maan & Sefc, 2013; Seehausen & Schluter, 2004), this is an important gap in our knowledge and the purpose of this paper.

The ecology and evolution of colouration in nature will ultimately be driven by the behavioural response of individual viewers (Endler, Westcott, Madden, & Robson, 2005; Guilford & Dawkins, 1991). Guppies (*Poecilia reticulata*) provide an excellent model to investigate colour perception and validate visual models. They inhabit freshwater streams and show very strong chromatic sexual dimorphism (Houde, 1997). Males display highly polymorphic colour patterns, which are an indication of a male's quality (Houde & Torio, 1992; Pitcher & Evans, 2001) and are assessed by females during mate choice (Cole & Endler, 2015). Guppies also use colour vision for foraging on algae, rainforest fruits, macroinvertebrates and insect larvae (Houde, 1997; Zandonà et al., 2011). To understand the evolution of male colour patterns and colour based behaviours in guppies, it is essential to determine how they perceive colours. Multiple studies have yielded diverse knowledge of the guppy visual system such as spectral sensitivity, arrangement and relative abundance of photoreceptor classes (Long, 1993; reviewed in Sandkam, Dalton, Breden, & Carleton, 2018).

In order to test the ability of the RNL model to predict discrimination, we compared the threshold of colour discrimination derived from behavioural tests with model predictions. We also investigated the possibility colour-dependent variation in model fit. In order to do this we used coloured stimuli in sets of six colour gradients, each ranging from a "pure" (highly chromatic) colour to grey. Each gradient occupied a different area in guppy colour space (Fig. 1). Fish were trained to detect a coloured target stimulus on a grey background among other grey stimuli. The latency before first push was recorded and we predicted that the smaller the $\Delta S$ against the background, the higher the latency. The assumptions of the RNL model are validated if the colour discrimination threshold occurs where the calculated $\Delta S$ between the two stimuli is approximately 1.

## 2. Material and methods

### 2.1. Fish husbandry

We used guppies from a laboratory population descending from a wild caught guppy population, caught in late 2010 at Alligator Creek, Bowling Green Bay National park, Queensland (19°26.79′S 146°58.65′E). To control for age, three replicates each of 80 juvenile (approximately one-month-old with a ~1:1 sex ratio) were placed in 196 L glass tanks seven months prior the beginning of the experiment. The tanks were illuminated by high-frequency fluorescent lamps following a 12 h light-dark cycle and the fish were fed once a day with flake food or brine shrimp.

### 2.2. Experimental setup

We performed the experiment with female guppies because the use of colour by females in sexual selection leads to the evolution of colour pattern in this sexually dimorphic species (Cole & Endler, 2015). Moreover, Lucon-Xiccato and Bisazza (2014) showed that female guppies were easier to retrain than male. We selected six females, all approximately eight months old and isolated them in 20-litre experimental tanks (22 × 35.5 × 26 cm). Each tank had an opaque partition with a sliding door, which allowed females to swim through when lifted. This partition created two equally sized compartments, the home compartment and the experimental compartment (Fig. S1a). A mirror (12 * 12 cm, covering 75% of the left side wall of the home compartment) was placed in the home tank to create the illusion of a companion fish thus avoiding social isolation for the focal female (Agrillo, Miletto Petrazzini, Piffer, Dadda, & Bisazza, 2012). Each tank was illuminated by an Exo-terra Sunray 50 W reptile light, which closely mimicked natural sunlight, including UV (Fig. S1b). We measured the irradiance (Fig. S1b) using a cosine-corrected receptor and an ocean optics USB2000+ spectrophotometer calibrated for photon flux (μmol photons $m^{-1}$ $sec^{-1}$ $nm^{-1}$) with a Li-Cor LI-1800-02 optical radiation calibrator (standard lamp); for details see Endler (1990). The tanks contained one cm of white gravel and 14 cm of water and were maintained at 22 ± 2 °C.

To minimise any human interference on the fish behaviour, a black curtain (1.2 m long and 3.5 m wide) was placed in front of the experimental setup. The experimenters were able to open the sliding door between the two chambers from outside the experimental area using a nylon thread connected to the partition (See Supplementary Fig. S1 for an overview of the experimental set up). The trials were recorded with Panasonic HC-V100 and JVC HD Everio cameras facing down from above the tanks.

### 2.3. Stimuli

To determine colour discrimination in guppies, the experiment

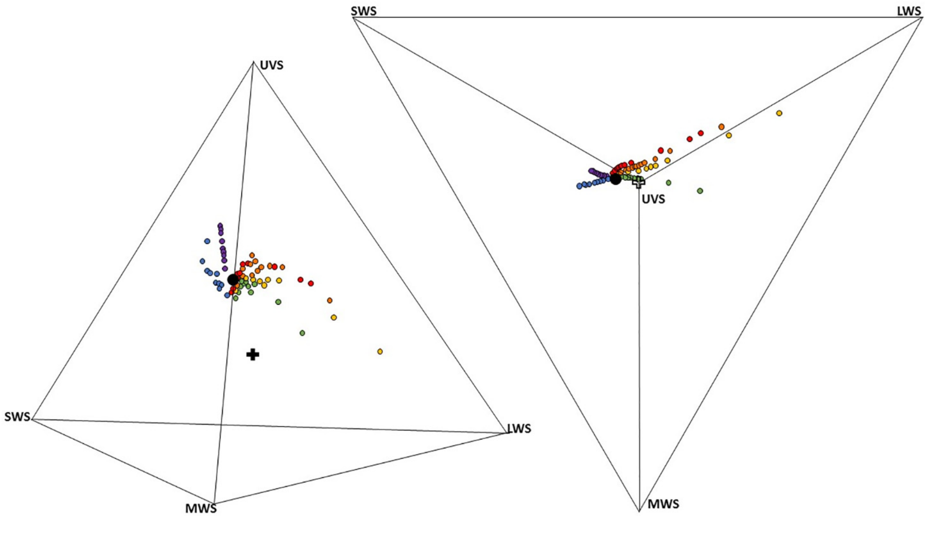

**Fig. 1.** Stimuli generated for the six colour gradients in the tetrahedron of guppy colour vision. Front view, left. Top view, right. The Von Kries correction was applied for all cone captures. The large black dot represents the grey background and the black cross represents the achromatic point for the guppy visual system under the light conditions of the experiment. See Supplementary Fig. S4 for an enlargement of the stimuli.

evaluated the ability of each fish to identify a target coloured stimulus disk among grey disk stimuli on a grey acrylic background plate. The plates and disks were designed using Adobe illustrator CS6 (version 16.0.3) and manufactured with a Trotec laser cutter (Trotec Laser engraver, Trotec Laser Pty Ltd, NSW 2557, Australia). The plates measured 100 × 100 × 6 mm and had 25 evenly spaced holes of 5 mm diameter and 2–3 mm depth. The disks were 10 mm diameter by 3 mm. All plates and disks were painted with an acrylic, non-toxic paint (acrylic paint, Art culture™).

For the target stimuli, we created six colour gradients: red, orange, yellow, green, blue and purple, covering different areas in guppy colour space (Fig. 1). Each gradient contained between 9 and 12 disks varying from a very chromatic (saturated) colour, which was highly conspicuous against the background, to a colour which was very similar to the background and hence inconspicuous. To create the six colour gradients we mixed the original paints (“Warm Red”, “Orange”, “Warm Yellow”, “Green Light”, “Warm Blue” acrylic paint, Art Culture™) in various proportions with the background grey paint (“Grey” acrylic paint, Art Culture™). For the purple gradient, we used a mix between “Warm Red” and “Warm Blue” as the starting colour (See Supplementary Fig. S2 for the reflectance spectra of all stimuli). The most conspicuous colour was the pure (not mixed with grey) coloured paint. To make the gradient we added a drop of the grey background paint to this coloured paint to create the second stimulus in the gradient, and then a second drop of grey to create the third stimulus and continued to add drops until a full gradient was obtained. This produced disks that ranged from the “pure” colour to the grey of the grey plate (Fig. 1 shows the colour gradients according to the guppies’ visual system; Supplementary Fig. S2 shows the reflectance spectra of all gradient stimuli). The orange gradient contained 12 contrasts of stimuli, the red and green 11 contrasts, the blue 10 contrasts, the yellow and purple 9 contrasts. Numbers varied as a result of paint pigment limitations. For each colour gradient, we created three replicate stimuli of the same colour contrast, which were used randomly during trials.

The plates were painted with the grey background paint (“Grey” acrylic paint, Art Culture™). For each trial, we used one coloured target disk and eight grey distractor disks. The distractor disks were used as controls to ensure that the fish were identifying the target disks because of their colour rather than their luminance or position. We generated a gradient of distractor disk with 22 grey steps, created by mixing white and black paint (“White” and “Black” acrylic paint, Art Culture™) in white:black ratios varying from 1:10 to 10:1. Another eight distractor disks were painted with the same colour as the grey background plate. For each trial, we selected eight distractor disks randomly out of the 30 distractor disks.

We used the receptor noise limited model to measure $\Delta S$ between each target stimulus and the grey background and to predict the colour discrimination threshold (JND) as $\Delta S = 1$. We used the formulae in Vorobyev and Osorio (1998) and Kelber et al. (2003). $\Delta S$ depends on the difference in receptor signals between two stimuli ($\Delta q_i$) and the standard deviation of the noise ($e_i$) in the four photoreceptor classes ($i = 1,2,3,4$ for the UVS, SWS, MWS and LWS cone classes; with peak sensitivities to UV, small, medium and long wavelengths, respectively). The retina of the guppy forms a regular mosaic and this yields photoreceptor abundances ratios of 1:1:2:2 for UVS, SWS, MWS and LWS respectively (Laver & Taylor, 2011; Long, 1993). We conservatively used 0.2 as the standard deviation of the noise in a single receptor cell type $i$ (i.e., numerator of the Weber fraction - “$\upsilon_i$” in Vorobyev & Osorio, 1998, “$\sigma_i$” in Olsson et al., 2015) to calculate receptor noise. Some other published studies used 0.1 and even 0.05 but that yields larger and possibly unrealistic values of $\Delta S$ in the guppy. We used the ambient light irradiance, spectral reflectance of the grey background and of each coloured disk, and the guppy cone spectral sensitivities to calculate $\Delta S$ between each disk and the grey background (See Fig. S3 for the spectral sensitivity curves of each opsin used in the model). We used photoreceptor spectral sensitivities based upon both microspectrophotometry (MSP) and opsin expression data (reviewed in Kawamura et al., 2016, Sandkam et al., 2018, Kranz et al., 2018). We used these peak cone sensitivities ($\lambda_{max}$) in our model: SWS1 (UVS, $\lambda_{max} = 359$ nm, Watson et al., 2011), SWS2b (SWS, $\lambda_{max} = 408$ nm, Archer & Lythgoe, 1990), Rh2-2 (MWS, $\lambda_{max} = 465$ nm, Watson et al., 2011) and LWS3 (LWS, $\lambda_{max} = 560$ nm, Watson et al., 2011). We constructed the curves using these $\lambda_{max}$ in the Govardovskii, Fhyrquist, Reuter, Kuzmin, and Donner (2000) A1 template and the guppy ocular media transmission spectrum provided by Ron Douglas. Supplementary material Section S1 provides the MATLAB functions for the template (both A1 and A2, although only A1 is relevant for guppies), the ocular media transmission data, and a sample MATLAB script which combines them into a guppy eye model.

### 2.4. Training

The training was designed to teach the fish to dislodge a disk in order to receive a food reward (brine shrimp) hidden under it. To perform the training and reinforcement trials we placed a drop of brine shrimp nauplii in water in one random hole of the plate and immediately placed it in the freezer for at least 10 h. The freezing process allowed the food to stay in the plate hole during the trial, rather than floating free. The variation in water temperature due to the frozen plate did not exceed 2 °C and we did not observe any modification of the fish behaviour or any sign of stress.

We followed a slightly modified version of the guppy training protocol used by Bisazza, Agrillo, and Lucon-Xiccato (2014). The training had 16 stages (see Supplementary Table S1 for details). During the first stage of the training, only the target coloured disk and two grey disks (of different luminance) were placed on the plate with the target disk covering only 10% of the hole containing the food. To obtain the food, the fish simply had to approach and access the hole partially covered by the target disk. We defined success as when the fish oriented towards the food and started eating within 10 s after entering the experimental compartment. Once the fish had learnt how to obtain the food, we gradually increased the amount by which the disk covered the hole for the next 10 training stages (10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100%) so that the fish eventually learned to push and dislodge the disk to get the food reward (Online Supplementary Video). The time taken to obtain the food reward increased naturally with each of the first 10 training stages. A fish generally spent more than two minutes before dislodging a disk with 100% of the hole covered. We left the fish in the experimental compartment until they removed the coloured disk and ate the food under it. However, if after 10 min the fish did not dislodge the disk (either because she did not push or because she did not push enough), we showed the fish the correct disk and helped her to dislodge it, by gently pushing the disk with a thin metal rod to uncover the food.

Once a fish was able to choose and dislodge the target disk in at least three consecutive trials to get the food, we increased the number of distractor disks gradually (chosen randomly among the group of 30 distractor disks), until eight distractor disks and the target coloured disk, were displayed on the plate in any one trial (Supplementary Table S1). We estimated that a female was fully trained when she dislodged 100% of the target disks over two consecutive training sessions (six trials). During any trial, if the first push was on a grey disk, the trial was counted as a failure, even if the target disk was dislodged later on the same trial. The training required one and a half months for all colour gradients except for the purple gradient, which required two months.

We performed six training trials every day, three between 10 am and 12 noon and three between 3:30 and 5:30 pm. The duration of the training sessions was as short as possible in order to minimise any possible effects of diurnal variation in activity (Reeve et al., 2014), which may influence the female response to the behavioural trails.

### 2.5. Experimental and reinforcement trials

To avoid any olfactory bias no food was placed under the target disk during all experimental trials. To maintain consistency, we used a frozen plate in all trials regardless of whether or not the plate contained food.

A trial was set up with the frozen plate being placed in the centre of the tank's experimental compartment with the eight background grey disks and the target disk arranged randomly over the holes. The experimenter turned the camera on, closed the curtain and opened the sliding door. Once the fish entered the experimental compartment (the curtain was translucent enough for viewing), a timer was put on for two min. After the two min of trials, the experimenter turned off the camera, opened the sliding door again and waved the hand in front and on the side of the experimental compartment, encouraging the fish to go back to its home compartment. We then closed the sliding door and removed the plate and disks.

During the experiment, fish performed eight trials per day, four during the morning session and four during the afternoon session, at the same times as training. Four different stimulus sets (i.e. plate and disks) were placed consecutively in the tank during one experimental session. The first fish to perform the experiment was chosen randomly every day. During each experimental session, three experimental trials and one reinforcement trial were run.

Because tests were not accompanied by a food reward, it was necessary to run a rewarded reinforcement trial during each session to maintain high fish motivation. The reinforcement trial was randomly run first, second, third or last during the session. Reinforcement trials were run the same way as experimental trials but food was placed under the target disk. All reinforcement trials were run with the most conspicuous colour of each colour gradient as the target disk (the one with the highest contrast against background).

Females performed the experiment on three colour gradients, run in Latin Square order (Table 1). All fish performed 10 trials for each colour contrast of a given colour gradient. Thus, each colour gradient was tested on three different fish. A total of 30 trials were run for each colour contrast of each gradient. The very long training time prevented a larger sample size or more gradients tested.

### 2.6. Data collection

The videos were viewed for analysis with VLC media player (version 3.0.4). We recorded the time of entry in the experimental compartment, the fish choice (first disk push is the target disk = correct choice, first disk push is one of the grey disk, or no push during the entire trial = incorrect choice), the time of first push (Latency). We also recorded if the coloured disk was dislodged during the two minute trials. The target disk was dislodged in 11.2% of the experimental trials, and in those cases the fish did not find a food reward under the disk and this could have lead to a motivation drop. However, a fish that dislodged a disk and did not push any disk in the following trials occurred only 0.01% of the trials, leading to the conclusion that dislodging the disk did not decrease fish motivation. For the reinforcement trials, we recorded the time when the coloured disk was dislodged.

**Table 1**
Colour gradient order experienced by each fish.

| Fish | F1 | F2 | F3 | F4 | F5 | F6 |
|---|---|---|---|---|---|---|
| 1st colour gradient | Purple | Orange | Blue | Red | Green | Yellow |
| 2nd colour gradient | Orange | Blue | Red | Green | Yellow | Purple |
| 3rd colour gradient | Red | Green | Yellow | Purple | Blue | Orange |

### 2.7. Statistical analysis

All statistical analyses were performed in *R* (R Core team, 2013) with *R Studio* (R Studio 2016, Version 0.99.8). Normality and homogeneity of the residuals were verified for each model before further analysis.

The reinforcement trials were not included in the statistical analysis because they were always run with the most conspicuous target disk and food placed under this disk. The overall percentage of correct choices (first disk push is the target disk) obtained by the fish during reinforcement trial were 100%, 98%, 96%, 94%, 88% and 62% respectively for the yellow, red, orange, green, blue and purple gradients.

#### 2.7.1. Discrimination threshold for each of the colour categories

To determine the discrimination threshold for the colour gradients we used the inflection point (i.e. point of maximum slope) of a sigmoid curve fitted to the data. A sigmoid curve is the best way to estimate the location of a state change (here change in perception) which occurs in psychometric functions.

For each fish, the percentage of correct choices for each colour stimulus of a gradient was plotted against the estimated $\Delta S$ of this stimulus against the background. We fitted a sigmoid curve to the data using MATLAB (2017a version 9.2.0). The sigmoid curve followed the non-linear regression model: $Y \sim b1/(1 + \exp(b2 - b3 * X))$ where $b1$ is the asymptote of the curve, $b2$ is the inflection times the slope and $b3$ is the slope of the curve. We used sigmoid curves for all colours except for two purple gradients (performed by the fish F3 and F5, see supplementary material S4) where the thresholds were determined by the inflection point of a polynomial function. To obtain a single threshold value for each colour gradient, we plotted the percentage of correct choice of the three fish performing on a given colour stimulus, against the estimated $\Delta S$ of this stimulus and fitted a sigmoid curve. We determined the threshold using the inflection point of this sigmoid curve. The thresholds obtained for each fish were used in the subsequent analysis.

To test the validity of sigmoid threshold determination we compared the sigmoid thresholds with a probability method used in other species (Lind, 2016; Olsson et al., 2015). The second method uses a binomial test and evaluates the minimum number of correct choices necessary to be significantly greater than random choice. This method gave us a threshold of colour discrimination at 40% of correct choices for a colour gradient when a single fish was performing (probability of success on a single trial = 0.11, number of trials per fish per colour contrast = 10, P = 0.02, one-tailed binomial test). Note that random performance is not 50% because this is a choice of one out of nine disks, not two.

#### 2.7.2. Differences in thresholds among colour categories

Once the behavioural thresholds of colour discrimination were determined for each fish, we tested for differences in threshold among colour gradients. We performed a mixed linear model (function lmer in package *lme4*, Bates, Maechler, Bolker, & Walker, 2014) with the discrimination threshold $\Delta S$ as the response variable and colour gradient name as the fixed effect (explanatory variable). We included fish identity as a random factor. We ran a post hoc analysis to control for multiple comparisons (across colour gradients) using the glht function and Bonferroni adjustment (R package *multcomp*, Hothorn, Bretz, & Hothorn, 2017).

#### 2.7.3. Differences in plateau of maximum among colour categories

For each fish tested on each colour gradient, we extracted the Y max (maximum percentage of correct choice) from the fitted sigmoid curve. This value represents the plateau of maximum percentage of correct choice for each fish for that colour gradient. We evaluated the potential effect of the colour gradient tested on the plateau because the ecological relevance of some colours could potentially influence the height of

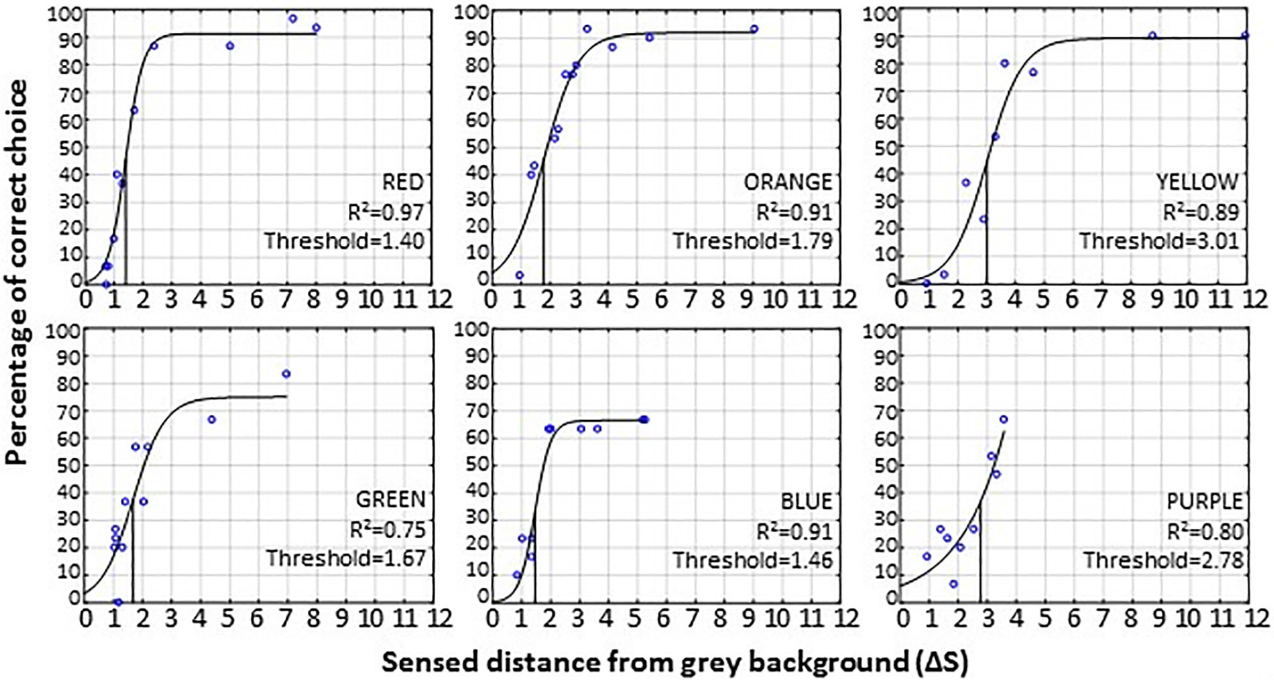

**Fig. 2.** Estimation of the discrimination thresholds between targets and grey background for the six colour gradients. Thresholds were estimated as the point of inflection of the sigmoid curves. Each percentage of correct choice within a gradient is the mean of the correct choice from three fish (n = 30 trials). Note that, although these graphs show means, we used the individual thresholds obtained for each fish (Supplementary Fig. S5, Table 2) for the statistical analysis.

the plateau if, for example, innate rejection or neophobia towards a particular colour prevents individuals from obtaining a high score for that colour. The difference in plateau between pairs of colour categories was tested with a generalised linear mixed model and post-hoc with Bonferroni adjustment, similarly to our tests for differences in threshold among colour categories, with plateau instead of threshold as the response variable.

#### 2.7.4. Differences in fish response (i.e., psychometric function) among colour categories

We ran a generalised linear mixed model (function glmer in package *lme4*, Bates et al., 2014) to test if the colour gradient had an influence on the relationship between $\Delta S$ and the percentage of correct choice. In other words, does the colour influence the way fish responded to the test and thus the shape of the psychometric function? The binomial variable "fish choice" (correct vs incorrect) was the response variable, $\Delta S$, the colour gradient name and the first order interaction between those two variables were the fixed factors. Fish ID was added as random factor. The model followed a binomial distribution with logit link. We performed six models, each with a different colour as the reference variable allowing us to compare colours pairwise. We corrected p-values for multiple comparisons using the Bonferroni method (p-adjust, R package *Stats*).

#### 2.7.5. Latency before first disk pushed

To determine reaction time, which may vary with the difficulty of the trials, we measured the latency before first push in each trial. To test if $\Delta S$ from the background had an effect on the latency of the fish response (first disk pushed; the lower the $\Delta S$ the more difficult a trial was expected to be), we ran a linear mixed model (lmer in R package *lme4*, Bates et al., 2014). To satisfy the assumptions of the linear mixed model, we conducted a Tukey's Ladder of Powers transformation on the latency data before analysis. This transformation produced a normally distributed latency variable and led to normal residuals in the linear mixed model (transformTukey, package *rcompanion*, Mangiafico, 2018). The Latency$^{0.05}$ was the transformed response variable, and fixed factors were $\Delta S$, colour categories and the first order interaction between these two variables. Fish ID was added as random factor. We constructed six models to obtain the effect of the colour gradient on the fish responses to the test (in each model a different colour was tested). We ran a P-value adjustment test using the Bonferroni method (p-adjust, R package *Stats*) to control for multiple comparisons.

### 2.8. Ethical note

The methods adhered to the ASAB/ABS Guidelines for the Use of Animals in Research and were carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). All movement generated to place and remove the experimental plate and disk in the aquarium were performed with highest care to not disturb the fish. During the experiment, the water quality (pH and KH) was controlled fortnightly to insured the best husbandry condition for the fish. This experiment was conducted under Deakin University's Animal Ethics Committee approval number G11-2015.

## 3. Results

### 3.1. Discrimination threshold for each of the colour categories

The two methods used to determine the discrimination thresholds: inflection point and binomial test, yielded results which were strongly correlated ($r = 0.98$, $CI_{95\%}$ [0.94; 0.99], $p < 0.001$, see supplementary Fig. S6). Consequently, we used the inflection point method for subsequent analysis.

Two of the colour gradients: red and blue, showed a threshold between 1.4 and 1.5 $\Delta S$. Orange and green colour gradients showed a threshold between 1.5 and 2 $\Delta S$. The purple and yellow gradients had the highest thresholds, over 2.75 $\Delta S$ (Fig. 2). Sigmoid curves and threshold determinations for each fish are presented in Supplementary Fig. S5 and Table 2 gives the values of individual thresholds. Supplementary Table S2 gives the threshold obtained with the binomial test method (40% of correct choice).

**Table 2**
Discrimination thresholds for the six colour categories (using the inflection method) for each fish individually (Individual Threshold Curves on Supplementary material S4). 'Order' (from 1 to 3) indicates whether the fish have performed the test on a colour gradient first, second or last during the experiment.

| Colour category | fish | Order | Individual Threshold |
|---|---|---|---|
| Red | F4 | 1 | 1.18 |
| | F3 | 2 | 1.37 |
| | F1 | 3 | 1.64 |
| Orange | F2 | 1 | 2.05 |
| | F1 | 2 | 1.87 |
| | F6 | 3 | 1.38 |
| Yellow | F6 | 1 | 3.26 |
| | F5 | 2 | 2.76 |
| | F3 | 3 | 3.03 |
| Green | F5 | 1 | 1.59 |
| | F4 | 2 | 1.81 |
| | F2 | 3 | 1.42 |
| Blue | F3 | 1 | 1.55 |
| | F2 | 2 | 1.36 |
| | F5 | 3 | 1.87 |
| Purple | F1 | 1 | 3.01 |
| | F6 | 2 | 2.31 |
| | F4 | 3 | 3.66 |

### 3.2. *Differences in thresholds among colour categories*

The purple and yellow colour gradients had a significantly higher threshold of detection than the blue, green, orange and red colour gradient (Fig. 3, Table 3). We did not find any significant differences between the purple and the yellow gradient results or among the blue, green, orange and red gradients (Fig. 3, Table 3).

### 3.3. *Differences in plateau of maximum among colour categories*

We did not find any significant differences in the plateau among the six colour gradients (all $P > 0.24$, Supplementary Table S3, See Fig. S5 for the variation among individual plateaus); there was no evidence that the maximum percentage of correct choices varies among colours.

### 3.4. *Differences in fish response (i.e., psychometric function) among colour categories*

We did not find any significant differences among the six colour gradients in the shape of the psychometric function (i.e., form of the sigmoid curve, indicating the way that fish responded to the test); all $P > 0.56$, (Supplementary Table S4). Only $\Delta S$ had a significant effect on the percentage of correct choices in each model ($P < 0.001$).

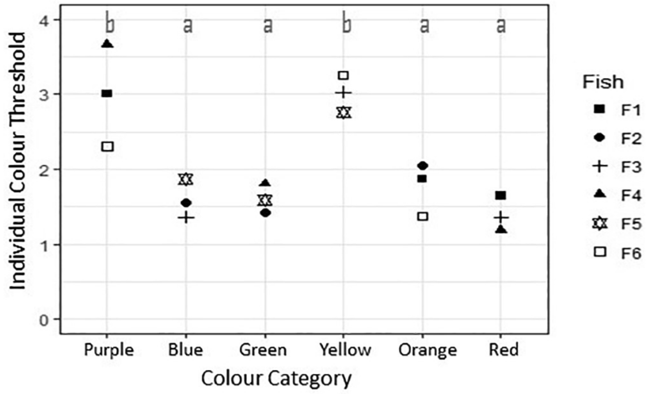

**Fig. 3.** Individual discrimination threshold for each of the six colour gradients using the point of inflection of the sigmoid curve. Different letters denote statistical differences.

**Table 3**
Tests for differences in thresholds between pairs of colours for the six colour gradients. P-values in bold are significant after Bonferroni adjustment for multiple comparisons.

| Colour Gradient pairwise comparison | β | SE | Z | P |
|---|---|---|---|---|
| Green-Blue | 0.01 | 0.30 | 0.04 | 1.00 |
| Orange-Blue | 0.17 | 0.30 | 0.58 | 1.00 |
| Red-Blue | −0.20 | 0.30 | −0.67 | 1.00 |
| Purple-Blue | 1.40 | 0.30 | 4.70 | **< 0.001** |
| Yellow-Blue | 1.42 | 0.30 | 4.77 | **< 0.001** |
| Orange-Green | 0.16 | 0.30 | 0.54 | 1.00 |
| Red-Green | −0.21 | 0.30 | −0.71 | 1.00 |
| Purple-Green | 1.39 | 0.30 | 4.66 | **< 0.001** |
| Yellow-Green | 1.41 | 0.30 | 4.74 | **< 0.001** |
| Red-Orange | −0.37 | 0.30 | −1.24 | 1.00 |
| Purple-Orange | 1.23 | 0.30 | 4.12 | **< 0.001** |
| Yellow-Orange | 1.25 | 0.30 | 4.20 | **< 0.001** |
| Purple-Red | 1.60 | 0.30 | 5.36 | **< 0.001** |
| Yellow-Red | 1.62 | 0.30 | 5.44 | **< 0.001** |
| Yellow-Purple | 0.02 | 0.30 | 0.08 | 1.00 |
| Random Effects | Variance | SD | n | |
| Fish | $1.71 \times 10^{-22}$ | $1.20 \times 10^{-11}$ | 6 | |
| Residuals | 0.13 | 0.36 | | |

### 3.5. *Latency before first disk pushed*

The blue, green, yellow and red gradients showed a significantly longer latency than the purple gradient (Table 4, Fig. 4).

$\Delta S$ successfully predicted the latency before first push for the red and yellow colour gradients. Fish were significantly faster to make a decision on which disk to push for larger $\Delta S$ ($p < 0.001$, Table 4, Fig. 4, Supplementary Table S5). The interaction between latency and $\Delta S$ was significantly different between the red and the green gradient. The red gradient showed a significant positive relationship between latency and $\Delta S$, the green did not; and the two linear fit were significantly different (Table 4).

## 4. Discussion

We performed a behavioural colour discrimination experiment over

**Table 4**
Significant values of the linear mixed model testing if $\Delta S$ between the background and the target stimuli had an effect on the latency of the fish response. This table shows only the significant variables of the model (when $p < 0.05$) after Bonferroni correction for multiple comparisons. For results of the full model, see Supplementary Table S5.

| Intercept colour | Variable | β | SE | DF | t | P |
|---|---|---|---|---|---|---|
| Blue | (Intercept) | 1.19 | 0.01 | 28 | 139.89 | < 0.001 |
| Green | (Intercept) | 1.19 | 0.01 | 19 | 154.10 | < 0.001 |
| Green | $\Delta S$:Red | −0.01 | 0.00 | 1433 | −3.80 | 0.011 |
| Orange | (Intercept) | 1.17 | 0.01 | 19 | 150.79 | < 0.001 |
| Orange | Red | 0.04 | 0.01 | 1415 | 4.49 | 0.001 |
| Orange | Yellow | 0.05 | 0.01 | 1430 | 6.14 | < 0.001 |
| Red | (Intercept) | 1.20 | 0.01 | 17 | 160.17 | < 0.001 |
| Red | $\Delta S$ | −0.01 | 0.00 | 1433 | −7.38 | < 0.001 |
| Purple | (Intercept) | 1.15 | 0.01 | 63 | 110.02 | < 0.001 |
| Purple | Yellow | 0.07 | 0.01 | 1436 | 5.84 | < 0.001 |
| Purple | Blue | 0.04 | 0.01 | 1426 | 3.47 | 0.039 |
| Purple | Green | 0.04 | 0.01 | 1436 | 3.44 | 0.044 |
| Purple | Red | 0.05 | 0.01 | 1437 | 4.56 | < 0.001 |
| Yellow | (Intercept) | 1.22 | 0.01 | 27 | 144.27 | < 0.001 |
| Yellow | $\Delta S$ | −0.01 | 0.00 | 1432 | −6.39 | < 0.001 |

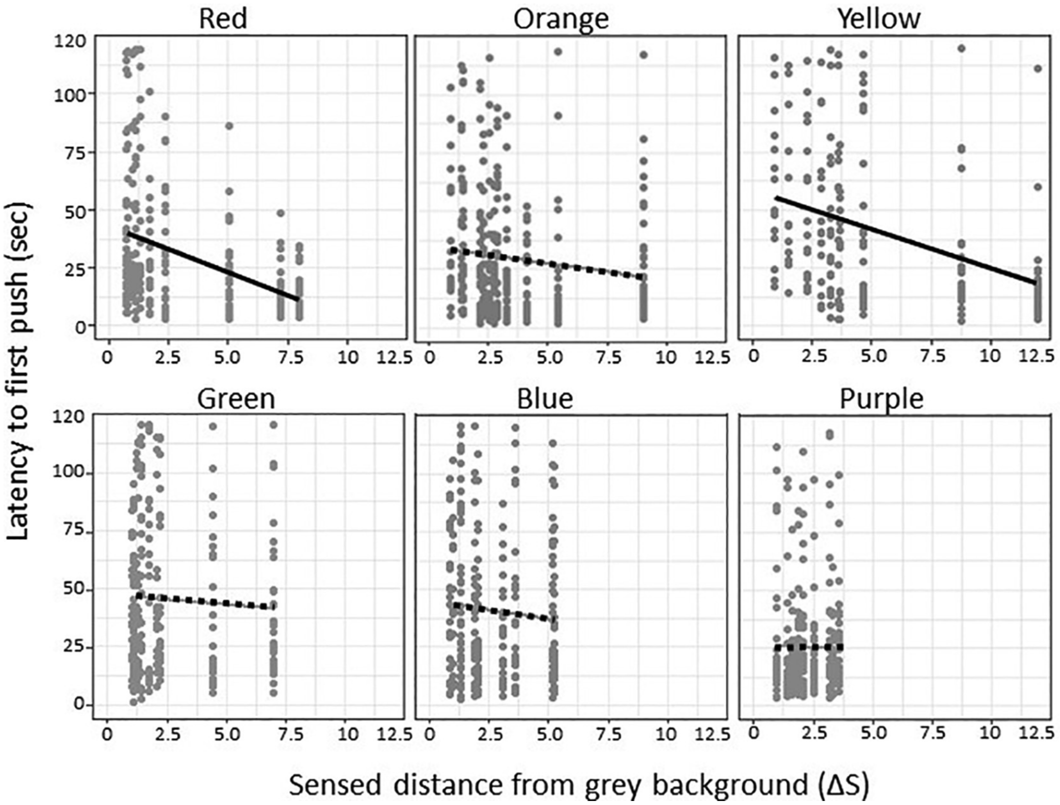

**Fig. 4.** Effect of $\Delta S$ between the target stimulus and the grey background on the latency before first push for the six colour gradients. Solid lines indicate that the interaction was significant.

six colour gradients to evaluate whether the predictions made by the RNL model matched the behavioural threshold of discrimination and whether the quality of the fit varied with colour. Three fish were tested per gradient and thus 30 trials were run per treatment level.

### *4.1. Differences in thresholds among colour gradients*

The threshold predicted by the RNL model was close to the actual discrimination threshold obtained via the behavioural assessment for four colour gradients: red, orange, green and blue. The behavioural thresholds ranged from 1.40 $\Delta S$ to 1.79 $\Delta S$ and were all significantly less than 2 $\Delta S$ for those four colour gradients. At 1 $\Delta S$ two stimuli should be just noticeably different, while at 2 $\Delta S$ the stimuli should be clearly distinguishable. Moreover, the threshold values for those four colour gradients were both homogeneous (i.e. no significant difference among them) and similar to the behavioural threshold of discrimination obtained from another studies on a reef fish (i.e., triggerfish, Champ et al., 2016). This result indicates that the RNL model is a valuable way of estimating the ability of a fish to distinguish at least some colours. Thus it implies that we could use the RNL model to explain colour based behaviour for colours such as red, orange, green or blue area in guppy colour space.

However, two of the colour gradients: purple and yellow showed higher thresholds than predicted by the model (2.78 and 3.01 $\Delta S$ respectively). Several possible reasons that might account for these higher behavioural thresholds.

First, the RNL model assumes that the standard deviation of the noise (numerator of the Weber fraction or $\upsilon$) is constant across all photoreceptor types (see equation 7. in Vorobyev & Osorio, 1998). Because we do not have direct measures of the noise associated with each photoreceptor type, studies using the RNL model have to estimate the standard deviation (SD) of the noise. We chose a value of $\upsilon = 0.2$ to be conservative. Using this value, the guppy behavioural threshold is, for most of the colours tested, close to the prediction made by the RNL model. However, the smaller $\upsilon$, the larger $\Delta S$ becomes (equation 5. in Vorobyev & Osorio, 1998); the lower the noise, the easier to discriminate two stimuli with a given contrast. This would lead to a shift in the sigmoid discrimination function towards higher $\Delta S$ and ultimately to a higher $\Delta S$ threshold as well as a greater mismatch with RNL prediction. Thus, the RNL model predicts quite accurately guppy colour discrimination on a behavioural level, assuming $\upsilon$ is equal to or slightly above 0.2.

In other studies, the values chosen for SD photoreceptor noise ($\upsilon$) range from 0.05 in the triggerfish (trichromatic species, Champ et al., 2016) to 0.21 in budgerigars and 0.2 chicken (tetrachromatic species, Lind et al., 2014; Olsson et al., 2015). In budgerigars, different $\upsilon$ were tested and 0.21 yielded the best predictions in their study (Lind et al., 2014). The actual photoreceptor noise can be measured using electrophysiological techniques but this has only been done with bees so far. Moreover, $\upsilon$ can vary depending on the intensity of the adapting light. For example, $\upsilon$ varies from 1.5 to 3.5 in bee MWS photoreceptors for intensities of $-1$ and $-5$ $\log_{10}$ intensity units respectively (Vorobyev et al., 2001). Variation in photoreceptor noise highlights the complexity of developing a model taking into consideration varying and accurate parameters. Of course, it would be even more complex if we were to account for fluctuating light conditions as occurs in the wild. The accuracy of the model could be improved by taking into account different noise parameters for the different photoreceptor cell types. Colour processing is actually dependent on how much a colour stimulates each of the photoreceptors which have different sensitivities, noise levels, and levels of chromatic adaptation due to the local microenvironment. Unfortunately, this information is not available in most species, which highlights the need to calibrate the model using behavioural thresholds for any species being studied.

A second reason for the mismatch for some colours is that behavioural responses could deviate from model predictions because the

RNL model does not consider colour processing in the brain, which will ultimately influence colour perception. Colours occupying different areas in colour space could be processed under different pathways both in the retina and brain. Even in the retina, the initial opponent processing may involve more than two cone classes. The interaction between sensing and memory could also increase the thresholds for some colours. Thresholds to approach purple or yellow which are higher than that predicted by the RNL may therefore result from processing in the brain and so act in addition to the limits estimated by the RNL.

Third, prior experience could alter the behavioural thresholds. Purple is rarely encountered in natural guppy streams, and never in our laboratory populations, and therefore it is possible that unfamiliarity with purple colour (neophobia) played a role in producing a higher discrimination threshold for this colour gradient, a classical effect of neophobia (Marples & Kelly, 2001). This is supported by the fact that the purple gradient was the gradient requiring the longest training time. Bright yellow (often associated with black) is used as an aposematic signal and individuals of many different species either learn to avoid yellow or have innate avoidance of yellow (Mappes & Alatalo, 1997; Mappes, Marples, & Endler, 2005; Meinwald et al., 1998; Smith, 1975, 1977; Wee & Monteiro, 2017). Aposematism and inherent avoidance of yellow would explain the high percentage of correct choices for the most contrasted stimuli of the yellow gradient; recognising yellow would be important if there were costs to eating yellow food. Aversion would explain the higher threshold for yellow. Given that we trained for a yellow stimulus but yellow may be inherently aversive, as yellow saturation decreases the balance between being rewarded for yellow and the aversive association with yellow would shift towards aversion as the stimulus became more different from the trained yellow. Aversion generalisation is limited (Ham, Ihalainen, Lindström, & Mappes, 2006). Therefore, guppies would avoid the low contrast yellows more than the high contrast yellow, increasing the threshold above that predicted purely from the RNL model.

Although yellow is found on the caudal fin of some guppies it is rare in natural and our laboratory populations. Females could be attracted to males with yellow but only high saturation yellows. However, no studies have tested the effect of the yellow saturation on guppy female preferences.

Finally, the differences in threshold among colour gradients could be explained by the ecological relevance of having acute discrimination for some colours. Discrimination of the red target stimuli on the grey background led to the smallest threshold, and a latency that increase when the difficulty of the trial decrease. This is particularly interesting as red has an ecological relevance for the guppies. For example, the orange patch, which has been shown to be indicative of male quality and attractiveness in some populations (Endler & Houde, 1995; Houde & Torio, 1992; Houde, 1997; Kodric-Brown, 1989; Kolluru et al., 2006) is composed of both carotenoids and a red pteridine pigment (Grether, Cummings, & Hudon, 2005). Moreover, males use more pteridine pigments in their orange patch when the carotenoids availability is higher in their environment (Grether et al., 2005). Females prefer males with the appropriate orange hue, which is directly related to the ratio of pigment that affect male attractiveness (Deere, Grether, Sun, & Sinsheimer, 2012). Thus, fine discrimination abilities among different red chromas may be beneficial for females because male colour patch saturation is linked to quality and will lead to higher quality offspring.

### 4.2. *Plateaus and fish responses among colour categories*

All behavioural data, except for the purple gradient, fit a sigmoid curve with a steep slope and a plateau of discrimination. The absence of a plateau for the purple gradient could be due to physical pigment limitations of the purple colour stimuli; we were not able to create a contrast between the target stimuli and the grey background higher than $\Delta S = 3.56$. This is very small compared to those from the other gradients with maxima at 8, 9, 11.9, 6.9, 5.3 $\Delta S$ for the red, orange yellow, green and blue gradients respectively. This limited the range of $\Delta S$ that we could test. As a result, the purple gradient only contained colours that were relatively difficult to distinguish compared to the other colour gradients. If the rewarded stimulus was difficult for guppies to detect, then a loss of motivation could have occurred for this gradient because the fish were not rewarded as often as for the other gradients. This may have prevented the fish from reaching a plateau in which the fish consistently obtained a high percentage of correct choices. It may also have resulted in a poor threshold estimate because the responses did not have a sigmoid shape.

Noticeably, the similar plateaus obtained for all colour gradients, except purple, indicate that larger chromatic distances do not lead to improvement in discrimination. This result supports previous studies in chickens and triggerfish (Champ et al., 2016; Olsson et al., 2015). It also reinforces the purpose of the RNL which is to give predictions of discrimination for adjacent colours in the animal's colour space but not colours located far apart (Vorobyev & Osorio, 1998). Moreover, no differences were found either in the plateau or in the shape of the sigmoid curve among colour gradients, which indicates that guppies have similar behavioural patterns of discrimination for all colour gradients, indicating similar kinds of reactions towards different colours.

### 4.3. *Latency before first disk pushed*

For the yellow and red gradients, the latency before first push increased when the task became difficult (decreased $\Delta S$). One possibility could be that guppies actively increase their search for the target disk when the red and yellow target disks become less contrasted to the background. Alternatively, guppies detected the less contrasted colours as rapidly as the more contrasted colours, but showed lower tendency to push the disk, leading to increase of the latency. Both possibilities indicate that guppies paid particular attention to those two colour gradients, indicating that they could be ecologically relevant.

Finally, the latency before first push was significantly lower for the purple gradient than most of the other colour gradients (Red, Yellow, Green, Blue). Also, the training time for the purple stimuli was the longest (see material and methods). Moreover, for the same $\Delta S$ among the different colour gradients, and for $\Delta S$ higher than three, the fish showed more incorrect choices when purple stimuli where presented, indicating that purple might either be generally avoided or not as easily discriminable. If the guppies did not detect the target stimulus, they quickly and carelessly pushed disks leading to lowest latency, and lower percentage of correct choice for the purple gradient stimuli.

### 4.4. *Conclusion*

Our results show that guppy behavioural thresholds of colour discrimination were close to the predictions made by the RNL model, assuming that the SD of the noise in a photoreceptor is superior or equal to 0.2. Thus, together with other results on birds, bees, reef fish and humans (also bringing convincing threshold), it seems that the RNL model is a good matrix to understand species colour discrimination. However, deviations of the model for two of our six colour gradients suggest that either the effects of innate behaviours (e.g., neophobia or aposematism), the effect of differential stimulation of the photoreceptor by the colour pattern, or other events in the brain, could modify behaviour to be different from that predicted from the RNL model. Thus, this study emphasizes that the ecological, experience or fitness-related relevance of some colours could affect decision-making in a behavioural context. Therefore, we conclude that the processes of colour discrimination can be highly dependent on which colours are being compared.

In future studies, we recommend using the largest sample size possible that allow full training of all individuals. The number of colour stimuli inside a gradient could be eventually decreased but the number of stimuli around the expected threshold should be as large as possible for accurate threshold location. Differences between sexes could also be investigated because differences in colour-linked behaviours and ecological requirements could affect discrimination thresholds. If the paint pigments used in experiments allows it, we recommend training all individuals with stimuli having the same $\Delta S$ from the background for all colour gradient tested. These refinements will be necessary to ensure that we detect differences in behaviour which are related to the colours of the stimuli.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.visres.2019.04.002.

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