Evaluation of colour preference in zebrafish for learning and memory

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Abstract

There is growing interest in using zebrafish (*Danio rerio*) as a model of neurodegenerative disorders such as Alzheimer's disease. A zebrafish model of tauopathies has recently been developed and characterised in terms of presence of the pathological hallmarks (i.e. neurofibrillary tangles and cell death). However, it is also necessary to validate these models for function by assessing learning and memory. The majority of tools to assess memory and learning in animal models involve visual stimuli, including colour preference. The colour preference of zebrafish has received little attention. To validate zebrafish as a model for colour-associated-learning and memory, it is necessary to evaluate its natural preferences or any pre-existing biases towards specific colours. In the present study, we have used four different colours (red, yellow, green and blue) to test natural colour preferences of the zebrafish using two procedures: place preference (PP) and T-maze. Results from both experiments indicate a strong aversion towards blue colour relative to all other colours (red, yellow and green) when tested in combinations. No preferences or biases were found amongst reds, yellows and greens in the place preference procedure. However, red and green were equally preferred and both were preferred over yellow by zebrafish in the T-maze procedure. The results from the present study show a strong aversion towards blue colour compared to red, green and yellow, with yellow being less preferred relative to red and green. The findings from this study may underpin any further designing of colour-based learning and memory paradigms or experiments involving aversion, anxiety or fear in the zebrafish.

Keywords

Alzheimer's disease, zebrafish, colour preference, learning and memory, T-maze, Place preference

INTRODUCTION

Transgenic mice are currently the most commonly used *in vivo* models to provide insight into the pathogenesis and for use in drug development for Alzheimer's disease (AD) [1]. However, as the doubt to the relevance of existing transgenic models to the human condition is increasing, alternative models are being sought. One such model is the zebrafish (*Danio rerio*). These small teleost (bony) freshwater fish, originating from rivers of northern India ([2], reviewed in [3]), are attractive for modelling neurodegenerative diseases, such as AD, since they are inexpensive to maintain, can undergo rapid development and can be easily genetically manipulated (for recent review see [4]).

The zebrafish have orthologues or homologues for most human genes including AD related genes such as the presenilins [5-8], amyloid precursor protein [9, 10], apolipoprotein E [11], pen-2 [12, 13] and tau [14].The zebrafish has been used to model a number of neurodegenerative diseases, including Huntington's disease (HD) [15-17]where aggregated polyQ protein has been shown to be toxic to zebrafish embryos and ALS [18, 19]in which the transgenic zebrafish over expresses mutant superoxide dismutase 1(SOD1) and have been shown to have motoneuron loss, muscle atrophy and premature death. A zebrafish model of AD, encapsulating behavioural and pathological hallmarks of the human disease condition has yet to be generated; however, zebrafish expressing human beta amyloid (Aβ) in melanophores (melanocytes) [20] has been reported. A tau transgenic zebrafish model, expressing the human TAU-P301L, has also been recently developed and shown to exhibit accumulation of phosphorylated tau, neurofibrillary tangles and neuronal toxicity [21]. Although this model has been characterised biochemically, behavioural assessments have been limited to assessing escape response to a touch stimulus. This behavioural assay assesses movement deficits and reflects the cell death observed only in the spinal cord of zebrafish embryos. One study developed a zebrafish model for frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17). This model showed neuronal expression of 4 repeat human tau and accumulation of tau in the neuronal body [22].

As more neurodegenerative transgenic models are being developed, there is greater interest in developing behavioural assays to functionally validate these models and also to provide functional assays for high throughput drug evaluation. The behavioural assays of learning and memory can be employed even at the larval stage of zebrafish. For example, habituation of startle reflex has been used to assess the effects of rolipram, memantine and donepezil [23] on non-associative learning in zebrafish larvae. Alternative assays for learning and memory in zebrafish are being trialled. These include T-maze [24-28], plus maze [29, 30], Y-maze [31], light and dark chamber [32, 33], shuttle box active appetitive conditioning [34],place conditioning task [35], appetitive choice discrimination tasks [36], alternation memory task [37], one trial inhibitory avoidance learning task [38] and conditioned place preference testing [25, 28, 39-41]. The majority of these assays are based on colour discrimination and involves visual stimuli (either rewarding or aversive) to be used as cues for learning in zebrafish. Such examples include a one trial inhibitory passive avoidance-learning task where an electric shock has been used to train zebrafish to inhibits swimming into a dark compartment from a white one [38]. In another study, visual stimuli such as plastic sleeves of different colours (red, blue, green and purple colour)have been used to associate with the food reward in the T-maze [27]. One colour has also been used as a cue to train zebrafish to swim to an environmental rich chamber filled with coloured marbles and gravel [24-26, 28, 42]. The plus maze also utilizes a red cue card to predict reward [29, 30].Light and dark chambers have been used to test anxiety, under the assumption that the natural preference of zebrafish for dark environments over bright environments is a predator-avoidance strategy, as in rodents. Place conditioning, as assessed by Eddins et al., [35] involves a three-chambered tank that had a correct and incorrect choice. Restricting swimming to a small space in the corner punished incorrect choices, whilst providing free space for swimming rewarded the correct side choice. In the appetitive choice discrimination task, zebrafish learned to go to the compartment with a light stimulus in order to receive the food reward [36].

It is clear that a number of behavioural evaluation methods have already been developed for zebrafish [27, 30, 37, 43, 44]. However, many of these methods have yet to be validated by factors known to alter learning and memory. A problem may also exist if there are natural preferences for particular visual stimuli (E.g., colour) for behavioural paradigms that rely on visual discrimination, thereby confounding the interpretation of the results. For instance, in a light and dark chamber apparatus, "training" a zebrafish to go towards the dark chamber may reflect a natural preference of zebrafish for a dark environment rather than learning. Thus, without considering the natural preference, learning and memory in zebrafish may be misinterpreted. Similar confounds could arise in an experiment which uses two colours (E.g., red & yellow).

Colour discrimination of rodent models such as rats has already been established. These studies have shown that albino, gray and gray hooded rats can discriminate between red and blue, red and green, red and yellow and blue and yellow colours [45, 46]. The results showed that albino rats prefer green and blue colour while gray and gray hooded rats failed to discriminate between blue and green and green and yellow colours. A recent study has investigated colour discrimination in two species of African mole-rats (Bathyergidae, Rodentia) [47] and showed that these rats avoided box illuminated with blue and green-yellow light. Cage colour has also been shown to influence behaviour of laboratory mice (*mus musculus*) [48]. Here the authors showed that mice preferred white coloured cages over red coloured cages, which have been shown to induce anxiety-like behaviour. These studies in rodents provide significant evidence that colour preference play a significant role in the behavioural characteristics of animals. Indeed, natural colour preference towards a specific colour may influence visual discrimination learning [49], memory and decision making of an animal [50].

The colour preference in zebrafish is yet to be established. Therefore, the rationale of our study is that although many colour based learning and memory methods are being developed, the natural

preference of zebrafish towards or away from particular colours have not yet been investigated, which could be a potential confound to the results of tasks involving colour discriminations.

It is known that zebrafish have colour vision with peak absorbance in ultraviolet (362 nm), violet (415 nm),cyan(480 nm) and yellow(570 nm) [51, 52] which is comparable with that of humans (peak absorbance blue (420 nm), green (536 nm) and yellow (564 nm) [53]. In addition, the behavioural spectra of the fish closely match the sensitivity of their retina cones [54];however, it is not known if the colours that zebrafish can discriminate elicit spontaneous approach or avoidance behaviours. The aim of the present study was to determine the natural colour preference in zebrafish. Gravel and plastic sleeves of four different colours (red, yellow, green and blue) were used to test colour preference of zebrafish in the Place preference (PP) and the T-maze.

MATERIALS AND METHODS

Animals

All experiments were approved by Edith Cowan University Animal Research Ethics Committee and the procedures were conducted in accordance with National Health and Medical Research Council, Australia (NHMRC), Guidelines for the Care and Use of Laboratory Animals. Seventy-two mixed sex (males = 53& females = 19) wild type (AB) zebrafish were used for this study. Animal maintenance conditions were slightly modified from described previously [55]. In brief, zebrafish were housed in an environmentally controlled room with 14:10h light: dark cycle (lights on at 08:30-22:30 h). Red ocean salt was added to the system water at a concentration of 0.3mg/ml (Aquatic Habitats, Florida, USA).Temperature was maintained at28.5°C by an automatic temperature controller. Fish were fed twice daily with live brine shrimp (Vebas Aquariums, O'Connor, Western Australia) in the morning and high protein dry food during the evening (Aquasonic, Australia). Fish were separated in 3-litretanks (12 fish per tank) eight days prior to habituation in the testing apparatus. All the fish were tested at 25 weeks old and were from the F3 generation of an AB strain.

Colour preference assessment

Place Preference (PP).

Fish were tested in a two-chambered place preference (PP) box (Figure 1A)as described previously [26], but with a slight modification (i.e., gravel at the bottom instead of black dots on one side). Briefly, the test apparatus was 23 x 15 x 15cm and was filled with water up to 12cm from bottom. The apparatus was divided into two equal halves with a perforated wall that allows movement albeit impeded. The temperature of the watering the apparatus was maintained at 28^{o}C and the pH of 7.3 was controlled by using sodium bicarbonate (Sigma Aldrich Australia).

As zebrafish are a shoaling fish, all fish were initially habituated in groups in the testing apparatus, and then the size of the shoal was reduced by about half each time the shoal had explored all compartments, until each fished explored all compartments singly. Fish were habituated in the apparatus by placing a barrier with holes through it, which allowed movement, albeit impeded, from one compartment to another. This was done to ensure that the fish learn about the presence of two different compartments in the apparatus. Fish were allowed to explore the apparatus for four minutes. On the following day, one chamber of the apparatus was filled with gravel of one colour, and the other with a different colour. Six different colour combinations of four colours were tested i.e. six groups (red and yellow, red and green, red and blue, yellow and green, yellow and blue, green and blue).Each fish was tested individually (n=12 per group) in the apparatus without any barrier to allow free movement from one side to another. Each fish was placed in the alternative compartment of the place preference box and was allowed to freely swim from one compartment to other. The position and side of entry of each fish were fully counterbalanced by placing them alternatively in each side of the PP box throughout the experiment. This was performed to control for spatial preferences of the zebrafish. The number of entries into each compartment and time spent in each compartment were recorded.

T-maze Coloured Arm Choice Procedure.

A transparent Plexiglas®[poly (methyl methacrylate) (PMMA)] T-maze (Figure 1B) as described by [27] was used. In brief, the dimension of the stem of the maze was 50 × 10 × 10 cm. An area measuring 10 × 10 × 10 cm was closed off at the foot of the stem to form a start box. Each arm of the maze was 20 × 10 × 10 cm. Plexiglas® doors were used to block off the arms of the maze from the stem. The blue, green, red, and yellow plastic sleeves were constructed to fit around three sides of each arm of the T-maze. System water at the temperature of 28^{o}C and pH 7.3 was used to fill the maze to a height of 8 cm. Water temperature was maintained at 28^{o}C throughout the experiment. All of the equipment were custom-designed and built by the Instruments Manufacturing Corporation, Ambala Cantt, India.

Habituation of the zebrafish to the apparatus without coloured sleeves was performed as described above prior to commencement of testing. Testing was done for six consecutive days (one group per day) after their daily feeding in the morning. On the probe trial, each fish was placed in the start box for 30 seconds and the door was opened and then closed following exit of fish from the box. Once the fish entered any of the short arms of the T-maze, another door was closed to prevent the fish from re-entering the long arm. Fish were then observed for 4 minutes. Number of entries into each coloured arm and open area in the middle (colourless compartment, “blank”), the time spent in each area, and first preference of entry was recorded.

Spectral Measurement of Plastic Sleeves and Gravel

To ensure that colours used were emitting at appropriate wavelengths for both colour and similarity to the peak absorbance wavelengths of zebrafish vision, reflectance spectra of different coloured gravel and coloured sleeves were measured using visible and infrared spectrometers.

Plastic sleeves and gravel with four different colours were used for these experiments. Optical properties of these objects were characterized with two different commercially available (visible and

near infrared) spectrometers. The visible spectrometer has optical range of 400-850 nm and the infrared spectrometer an optical range of 850-2100 nm. The experimental setup for measuring the reflectance spectrum is shown in Figure 2. The peak values of the measured spectral reflectance curves for the four plastic sleeves and gravel of different colours are presented in Table 1 and Table 2, respectively. It should be noted, that blue plastic sleeve has very low reflectance with peak value at 643 nm. Note that the light source was outside the sleeves, so relatively low reflectance means relatively high light intensity inside the maze. The resolution for both spectrometers is 0.3 nm. The spectrometers were calibrated before the measurements according to the manufacturer's instructions.

The reflectance spectra of different coloured plastic sleeves and gravel are shown in Fig. 3A & 3B respectively. The peak wavelengths of coloured gravel (in nm) were 443.69 (blue), 515.17 (green), 551.17 (yellow), 615.4 (red) and the peak wavelengths of the coloured plastic sleeves (in nm) were 443.69 (blue), 515.17 (green), 539.45 (yellow), 620 (red).

Statistics

Data were analysed with a two-tailed paired t-test for PP experiment for the preference of one colour over another in each combination using R.2.10 software. Data from experiments using the T-maze were analysed with one-way ANOVA followed by post hoc (Tukey test) using R.2.10 software. The null hypothesis was rejected when $p<0.05$.

RESULTS

The zebrafish were first assessed for colour preference in the PP box. The total numbers of entries in each compartment of PP box were recorded to determine if zebrafish entered and investigated both compartments. The number of entries (Table 3) and time spent in each compartment (Figure 4)were recorded. The average number of entries was similar in both coloured compartments in all combinations, indicating that zebrafish entered and investigated both compartments equally. However,

differences were observed in the time spent in the coloured compartments (Figure 4).The zebrafish spent significantly less time in the blue side in all colour combinations that include red, green or yellow ($p<0.5,0.01,0.001$ respectively). No significant differences were observed in the length of time spent in green, red, or yellow when these colours were tested in 3 different combinations. Overall, these data indicate that relative to red, green and yellow side, the zebrafish avoided the blue side of the PP box.

Zebrafish were also assessed in the T-maze containing coloured plastic sleeves fitted around the three sides of the short arms of the T-maze (Figure 1B).The number of entries (Table 4) and time spent in each compartment (Figure 5) were recorded. The average numbers of entries into the coloured arms of the T-maze were similar for the majority of colour combinations. However, significantly fewer numbers of entries on average into yellow arm (in the red-yellow/ green-yellow combination) were observed. The time spent in the blue arm (in the blue-yellow combination) was significantly less than spent in the yellow arm ($p<0.05$). Similarly in the red-yellow combination, the fish spent significantly less time in the yellow arm. The less time spent in the yellow arm could reflect the fewer entries into this compartment. Interestingly in all colour combinations the fish spent significantly less time in the blue coloured arm and the "blank" compartment, indicating that the fish had similar aversions to "blue and blank". Overall, the results obtained from PP box and T-maze showed that the rank ordering of preferences for colour was Red = Green > Yellow >> Blue.

DISCUSSION

Recently zebrafish have gained popularity as a model for neurobehavioral studies [29, 30, 34, 56, 57] and aging[42, 58-60], due to the species' high fecundity, transparency during development, easy availability for forward and reverse genetic screens and social nature [23, 56]. Zebrafish have the potential to be a good animal model for Alzheimer's disease especially for the behavioural studies associated with the disease as recent evidence has shown that zebrafish can display the learning and memory deficits induced by drugs with similar properties in rodents and humans [23, 26, 57, 61].

Moreover, associative learning with the use of colours discrimination in zebrafish is of interest for behavioural studies, but there have been few studies to date exploring the capabilities of learning and the factors that may influence it in zebrafish, and there is therefore a paucity of information on natural preferences towards particular colours [52]. This could be a major confounding factor in behavioural studies [56, 62].In this study, we investigated the natural colour preference of zebrafish using four different colours with two different apparatus (PP box and T-maze).

The T-maze is considered to be more sensitive than the PP box because the PP box is limited to two choices, (remain in one compartment or swim to the next), where as the T-maze offers three choices after crossing the stem (one colour vs a second colour vs a colourless compartment or “blank”).A choice in a two-colour choice, such as the place preference, could be due to either hedonic properties of one colour or aversive properties of the other colour. For example, two colours could both be aversive, but one less so than the other resulting in a colour “preference”. In the T-maze, there is a neutral reference point, allowing some standard method of determining aversive or hedonic properties of colour. Secondly, the colour is the dominant visual cue in the T-maze, as the sleeve completely encircles the arm of the maze, while in the PP box the coloured gravel placed only on the bottom, is less encompassing, with the other outside visual cues being present.

There are potential confounds to our interpretation in these tests. However, a number of measures were taken to minimise these confounds. For example, spatial preferences in both procedures and external visual cues in the place preference task were controlled for by alternating the sides in which the colour cues were on. Another potential confound is the position of the experimenter. Zebrafish may have a preference for the experimenter, if they associate his/her presence with food, or an aversion if they associate the experimenter with possible predation. This was controlled by counterbalancing the position of the experimenter for both sides by standing equal number of times towards each side. One possible confounding factor could have been the habituation of zebrafish in order to make them aware of

two compartments in the PP apparatus as the test involves the use of colours and does not involve any reward. This was minimized by habituating the zebrafish to the presence of two separated compartments by creating a barrier that allows complete, albeit impeded, movement from one compartment to another. On testing, the barrier was removed allowing the fish to swim freely in both coloured compartments.

Our results show, for the first time, that zebrafish have an aversion to the colour blue, defined as the spectral frequencies454 nm and 643 nm. The zebrafish spent less time in blue compartments, regardless of the other paired colour compartment in both PP and T-maze tests. The spectral properties of the eight different coloured objects (four different coloured gravel and four different coloured plastic sleeves) were within the visible range of optical spectrum (400-750 nm). This indicates that the fish were capable of discriminating between these colours. Colour preference has been shown to play an important role in shoaling and foraging behaviours [63, 64], since fish can have many body coloured patterns and they may select their shoal mates based on these patterns [64]. It is also important to note that behavioural responses in animals may be strain-dependent[1] (i.e. in white and gray hooded rats [45, 46]). In the present study, behavioural testing was performed on the (wild-type AB strain) where we have shown that zebrafish have a preference towards red and an aversion towards blue. However, similar to rodents, colour preferences may be different in different zebrafish strains of which there are a number (e.g. WIK, LFS, BLF), which would require testing prior to conducting any colour based learning and memory experiments. Strain differences in colour preference have yet to be studied in zebrafish and would be an important consideration when establishing behavioural assays in zebrafish.

An explanation for colour preference of zebrafish for red and green over yellow and blue remains to be determined. It could be speculated that the preference for red and greens over blue is due to the natural foraging behaviour of zebrafish as their natural diet is rich in microcrustaceans[65, 66] and carotenoids [67] which are red colour, whilst green is the colour of their natural environment. Evidence for this was provided by Spence & Smith, [52] where zebrafish were given different coloured food and

showed that they prefer food with optical spectrum from 650-700nm. Taken together, all the past studies on the zebrafish vision indicate that the objects used in this study were visible to the zebrafish. It is also important to note that the blue coloured plastic sleeve used in this study had poor reflectance, high transmittance and longer wavelength (643nm) whereas blue gravel had a shorter wavelength (454 nm), higher reflectance, and lower transmittance. Despite these two examples of "blue" covering a range of shades, and brightness, the behaviour was similar. Therefore, zebrafish had an aversion to the coloured objects with specific wavelengths 454nm and 643nm.

Only one other study in the literature has reported on the colour preferences of zebrafish [52].The study presented different coloured plastic strips (red, green, blue white and black)to the fish after feeding with different coloured diet (blue, green, red and white,) with the number of bites recorded. The authors showed that the red plastic strip was the most preferred. This method is based on the foraging behaviour of the fish and was a measure of colour preference for potential food items only. The PP box and the T-maze are established tools for assessing preference, learning and memory in a variety of animal models and have recently been adapted for use with zebrafish[25-27, 32, 33]. For example, one study [27]used T-maze for visual discrimination learning in zebrafish with blue, green, red and white plastic sleeves lined with either horizontal or vertical black stripes to show that zebrafish can be trained for preference to a particular colour. However, whether the zebrafish preferred one colour over another in a combination of colours, which could be a confounding factor for such studies, was not assessed. For example if a fish prefers red colour over blue then training them to associate red colour with food is not actually reflecting the learning rather it is the natural preference of fish towards red colour and it is a major confound in any interpretation of the results. On the other hand, fish probably learn to associate red colours to food faster, and more effectively than blue colours. So, if the aim of a study is to produce fast, high asymptote learning, it may be advantageous to use red coloured cues. Our current study provide strong evidence that zebrafish prefer certain colours (red and green) when tested in

the PP box and T-maze and indicates that either colour (or combination of colours) will be suitable for such learning and memory tests. Since similar results were shown with two different apparatus, it may be that the results are generalizable to other behavioural assay equipment (such as plus maze and Y maze).

Our results show natural preferences for reds and greens over blues, no colour, and yellow. This information is useful in choosing colours for future colour-based learning and memory paradigms. Reds and greens are equally preferred over other colours, and are good choices for appetitive experiments. Red versus green would be a good choice for discriminative stimuli where there is no natural preference for one over the other. Blues are more aversive than the other colours, and might be useful for validating experiments involving aversion, anxiety or fear. As the zebrafish is becoming an increasingly popular *in vivo* tool for neurodegenerative research, there is a requirement for establishing assays that assess memory and learning. Validating colour preference is important to consider when developing further colour-cued learning and memory paradigms in zebrafish.

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Plastic sleeve	Maximum reflectance	Wavelength (nm)
Red	15	624
Green	9	519
Blue	1.5	643
Yellow	21	545

Table 1. Peak values for reflectance measurements taken for red, green, blue and yellow plastic sleeves.

Gravel	Maximum reflectance	Wavelength (nm)
Yellow	41.37	568
Green	15.8	517
Red	32.2	619
Blue	10.3	454

Table 2. Peak values for reflectance measurements taken for yellow, green, red and blue gravel.

Place preference box																
	Blue Green			Blue Red			Blue Yellow			Red Yellow			Green Yellow			Green Red	
	Blue	Green		Blue	Red		Blue	Yellow		Red	Yellow		Green	Yellow		Green	Red
	4	3		14	13		2	1		21	22		22	21		10	9
	10	11		1	2		1	1		7	6		6	7		6	7
	11	10		9	8		1	1		8	9		10	9		4	3
	2	3		7	8		5	6		13	12		14	15		6	7
	11	10		2	1		0	1		10	11		15	14		6	5
	6	7		11	12		7	8		5	4		10	11		13	14
	2	1		7	6		5	4		2	3		25	24		10	9
	7	8		3	4		1	1		8	7		7	8		8	9
	13	12		2	1		5	4		1	2		8	7		12	11
	1	2		4	5		7	8		8	7		1	1		24	25
	6	5		9	8		1	1		9	10		2	1		12	11
	11	12		6	7		15	16		10	9		8	9		3	4
Average	7	7		6	6		4	4		9	9		11	11		10	10
SD	4.16	4.08		4.06	3.91		4.24	4.6		5.18	5.28		7.28	7.01		5.62	5.81

Table 3. The number of entries into each compartment of colour combinations in the place preference apparatus was assessed. The average number of entries (rounded off to the nearest whole number) ± SD was calculated and shows that fish entered both compartment equal numbers of times. The numbers in each row indicate the score in individual trial (n=12).

T-Maze																
	Blue Green			Blue Red			Blue Yellow			Red Yellow**			Green Yellow*			Green Red	
	Blue	Green		Blue	Red		Blue	Yellow		Red	Yellow		Green	Yellow		Green	Red
	1	4		7	8		6	15		4	4		7	1		4	1
	13	10		5	5		7	3		10	0		9	0		8	7
	11	5		5	3		6	10		5	0		5	2		12	10
	9	7		4	6		1	4		12	0		5	5		8	5
	9	10		3	4		2	2		5	6		10	0		10	7
	7	8		7	5		1	1		7	1		4	0		10	12
	4	10		5	3		2	5		6	7		8	2		13	15
	5	13		9	10		2	5		10	0		5	0		9	8
	11	7		13	9		2	1		5	2		21	20		9	7
	8	10		6	8		3	2		2	0		4	9		8	6
	5	10		8	3		4	6		13	10		9	11		4	13
	7	12		4	8		5	10		5	1		12	6		9	7
Average	8	9		6	6		3	5		7	3		8	5		9	8
SD	3.4	2.69		2.74	2.52		2.11	4.31		3.44	3.4		4.77	6.11		2.67	3.81

Table 4. The number of entries in each coloured compartment of colour combinations in the T-Maze. Shaded box represents first preference. The average number of entries (rounded off to the nearest whole number) ±SD was calculated and shown that in the majority of combinations the fish entered equal numbers of times. The numbers in each row indicate the score in individual trial (n=12).* $p<0.05$, ** $p<0.01$.

Figure Legends

Figure 1: Schematic illustrations of the apparatus used. **A**. PP box with the dimensions 23 x 15 x 15cm, with different coloured gravel (red, blue, green, yellow), divided into two equal halves with a perforated wall. **B**. T-maze with different coloured plastic sleeves (red, blue, green, yellow) to fit around the arms of the maze. The stem of the maze was 50 × 10 × 10 cm; start box (10 × 10 × 10 cm) and each arm was 20 × 10 × 10 cm.

Figure 2: The experimental set up for measuring the reflectance spectrum of different coloured objects i.e. plastic sleeves and gravel

Figure 3 A: Reflectance spectra for yellow, green, red and blue gravel used in the place preference experiment and (**B**) red, green, blue and yellow plastic sleeves used for the T-maze. The peak wavelengths of coloured sleeves and gravel are within the spectral vision range of zebrafish.

Figure 4: Total time spent in each compartment with six different colour combinations located within the place preference apparatus. Data represents mean± SEM of n=12 zebrafish. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Figure 5: Total time spent in each compartment of the T-maze with six different colour combinations. Data represents mean± SEM of n=12 zebrafish. * $p<0.05$, *** $p<0.001$.

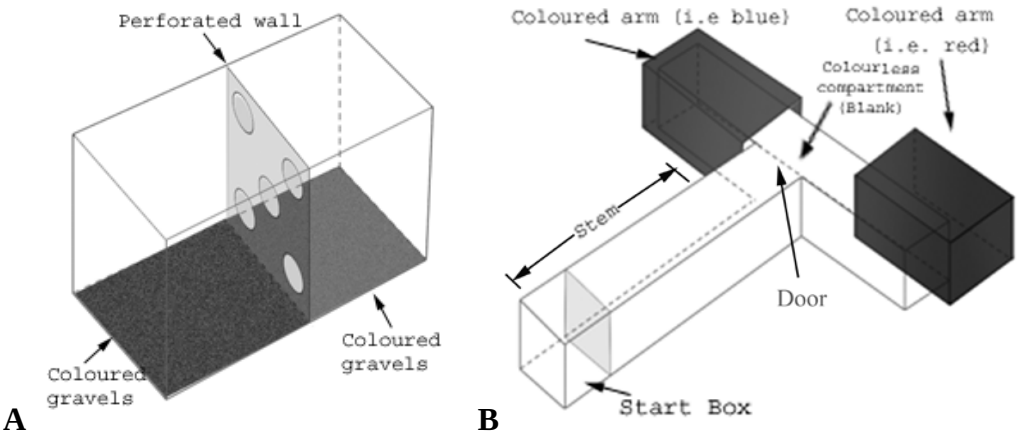


Figure 1

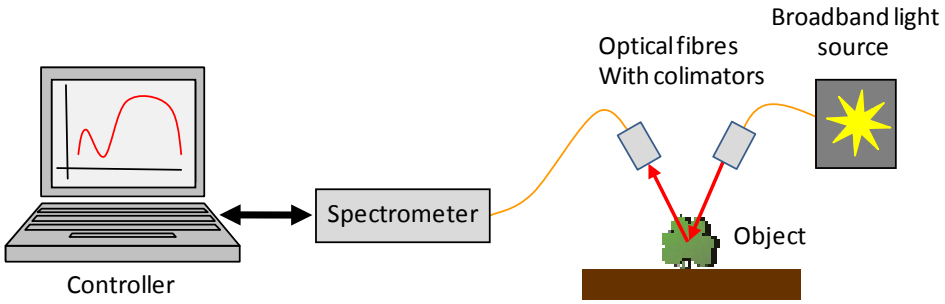
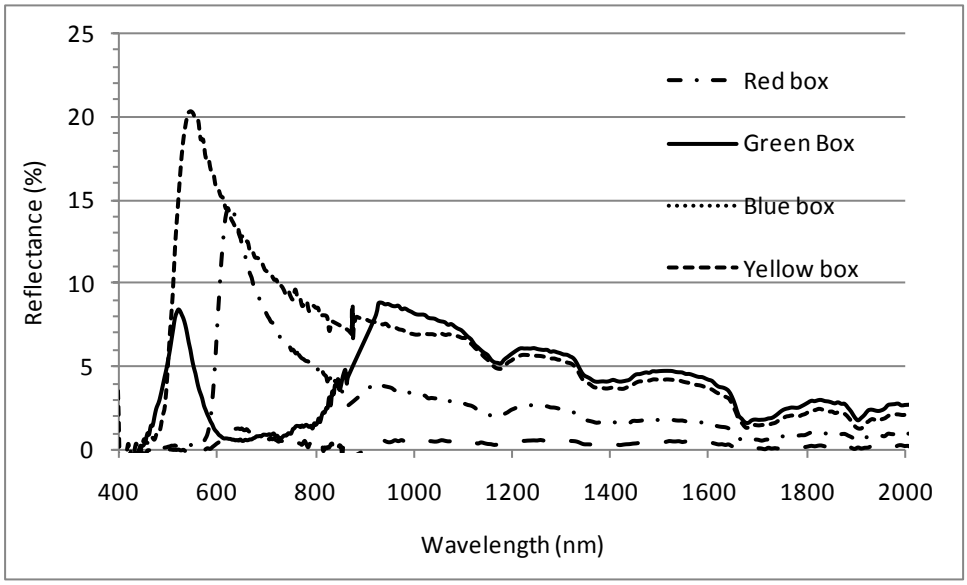
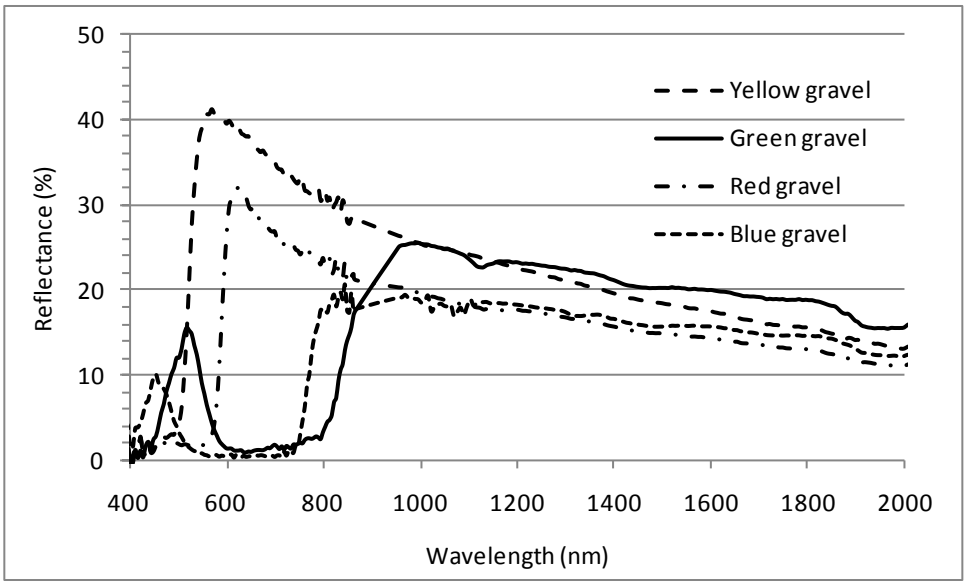


Figure 2





B

Figure 3

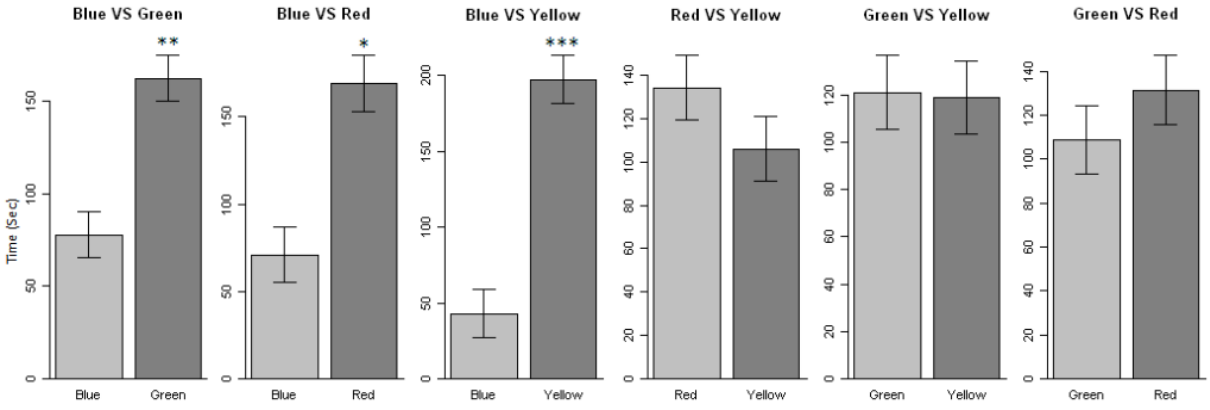


Figure 4

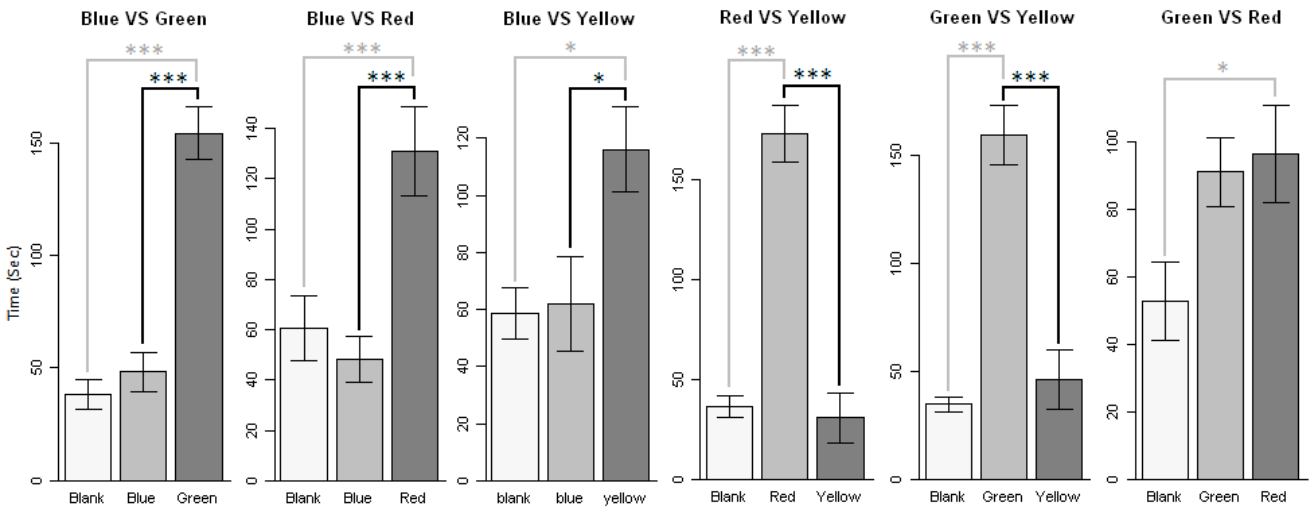

Blue VS Green
Blue VS Red
Blue VS Yellow
Red VS Yellow
Green VS Yellow
Green VS Red
Time (Sec)
***
*
Blank
Blue
Green
Red
blank
blue
yellow
Yellow

Figure 5