Zebrafish embryos and larvae as a complementary model for behavioural research
Ahmad, F.

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Author: Ahmad, Farooq
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Chapter 7

The colour preferences of larval zebrafish: effects of lighting and anxiolytics

Faroq Ahmad and Michael K. Richardson
The colour preference of larval zebrafish

Abstract
The zebrafish (*Danio rerio*) displays a wide range of well-defined behaviours. Colour preference is a behavioural trait in which animals are attracted to, or show aversion towards, particular colours. The preference for a certain colour can be influenced by various environmental factors. Here, we examined the preference of zebrafish larvae for light intensity zones, colours and effect of diazepam on the colour preference. We also tested whether lighting conditions during rearing can influence the colour preference of larval zebrafish using a simple open field test. Larvae of 6 days post fertilization were tested, using an automated behavioural recording system, for their preference and exploration patterns in different light intensity zones in a greyscale field. The results showed that larval zebrafish preferentially explore the lightest environments and avoid dark zones (i.e. all shades of grey). They also show freezing behaviour in the complex environment of the greyscale field. We showed in a previous study that zebrafish larvae preferred orange and green and showed aversion to red, yellow, black and blue zones, as well as freezing behaviour in aversive colours. Here, we pre-treated the zebrafish larvae with the anxiolytic diazepam before exposing them to the variable colour open field. Diazepam abolished the colour preference and freezing behaviour in the colour-enriched open field. Then, to see whether lighting conditions during rearing have an effect on colour preference, the larvae were reared in (i) normal light cycle (14h:10 h light-dark per day); (ii) constant darkness; (iii) constant light. Zebrafish larvae reared in constant light, moved and spent more time in the orange zone and avoided red, blue and black. Orange was also preferred by the zebrafish larvae raised in constant darkness, whereas red was avoided; these larvae also moved more in blue and black zones as compared to larvae raised in constant light. Preference for orange, and aversion to red, were constant findings in all three rearing conditions, suggesting an endogenous preference and aversion, respectively, for these colours. Our results suggest that 6 d zebrafish larvae have a strong set of colour preferences that can be modulated by the anxiolytic diazepam. The preference for orange and aversion to red were constant, regardless the lighting conditions during rearing. By contrast, preference or aversion for black, blue and yellow can be modulated by exposure to a particular light regime during rearing. We conclude that colour preference in the zebrafish larva has both endogenous and plastic components.
Introduction

The zebrafish is a small teleost fish with several advantages as a biomedical research model [415]. It has become increasingly used in various fields, including behavioural studies [58,60,87,416]. Zebrafish embryos and larvae are amenable to real-time visualization of the anatomical changes associated with development. This is because the embryonic tissues and the chorion (an extra-embryonic membrane present for 2-3 days after fertilisation) are initially transparent. Together with short maturation times (2 months) [417] and complex behavioural repertoire, the zebrafish embryo model is useful for many neurobiological research programmes including the tracking of changes to the developing nervous system [418-420].

The larval zebrafish has become a popular pharmacological model for high-throughput screening using automated video-recording systems[46,87,163]. Many aspects of behaviour have been studied in zebrafish including the so-called ‘exploratory-driven anxiety model’ category, which includes tests such as the elevated plus maze, the open field, and the light–dark preference tests [421,422].

Complex behavioural assays have been used to measure more subtle changes in behaviour. These include addiction [423], anxiety [298], aggression [424], learning/memory [300,425-427], locomotion [122], social responses [428] and anti-predatory behaviour [297]. These behavioural repertoires have been employed for studies of drug abuse [429,430], drug development [431], and toxicant exposure [98,197,432,433].

One behavioural parameter that has only been examined in a few studies is colour preference [61,188,434]. However, this is an interesting phenomenon because preference for a specific colour may lead to changes in visual discrimination learning, memory and decision-making [188]. Furthermore, it has been shown that specific colour preference or avoidance can be used to study anxiety-like behaviours in zebrafish [61].

In humans, mood can be influenced by the colour of the surroundings [435,436]. Some studies also report that colours affect human behaviour as indicated by performance on various tasks such as proofreading and problem solving [437,438]. Change of behaviour in rhesus monkeys was also noted in response to different colours [439]. So, these studies suggest that colour can influence behaviour, at least in higher primates.
The fact that many animal species have colour vision indicates that colour discrimination can have survival advantages [440]. In the animal world, colour per se is considered to be an important factor for prey recognition [118], food selection [441-443] and determining the palatability of food [444]. Likewise, in teleosts, colour patterns are used for species recognition, for avoiding visually-hunting diurnal predators and for mating success [445].

There are few reports on the innate colour preferences of fish species. Adult stickelback prefer red or blue over green [446], while adult Nile tilapia have a preference for yellow colour [447]. In the wild, zebrafish inhabit shallow, slow-flowing freshwater [28] with visibility to a depth of approximately 30 cm [28]. They are highly visual animals and have four types of cones and rod photoreceptors which makes them a useful animal to study rod and cone-mediated visual responses [448].

Adult zebrafish can discriminate between colours and have been shown to avoid blue [188]. Much less is known about colour preferences in zebrafish developmental stages. It is also not known whether preference or aversion to certain colours is genetically programmed, or is an epigenetic (acquired) trait; nor is it clear whether factors such as light intensity, abnormal lighting conditions or even pharmacological agents can alter colour preference.

In many vertebrates, visual sensitivity and other retinal phenomena are regulated by a circadian mechanism [449], and zebrafish also show a circadian influence on behaviour [339]. A recent study on zebrafish larvae has shown that abnormal light during development can lead to disrupted development and growth in zebrafish larvae [313]. These larvae adapt to the photic environment by showing changes not only in the abundance of cones, but also in opsin expression in the retina [450,451]. Together, these studies suggest that light may influence the growth, development, colour preferences and behaviour of zebrafish.

Here, we have studied the effect of light intensities on exploration by larval zebrafish. We have shown previously that zebrafish larvae have a significant preference for orange and green, and an aversion to blue, black, red and yellow [61]. To see whether anxiety was a factor in mediating these colour preferences, zebrafish larvae were treated with diazepam, a standard anxiolytic compound [82,83]. To further determine whether colour preferences could be modulated by abnormal lighting conditions during rearing, zebrafish larvae were
raised in three different lighting regimes: (i) 14:10h light-dark cycle; (ii) constant dark (iii) constant light; they were then assessed for their colour preference.

Materials and Methods

Statement of ethics on animal use

All experimental procedures were conducted in accordance with The Netherlands Experiments on Animals Act that serves as the implementation of "Guidelines on the protection of experimental animals" by the Council of Europe (1986), Directive 86/609/EC, and were performed only after a positive recommendation of the Animal Experiments Committee had been issued to the license holder.

Animal husbandry

Male and female adult zebrafish (*Danio rerio*) of AB wild type were purchased from Selecta Aquarium Speciaalzaak (Leiden, The Netherlands) who obtains stock from Europet Bernina International BV (Gemert-Bakel, The Netherlands). Fish were kept at a maximum density of 12 individuals in plastic 7.5 L tanks (1145, Tecniplast, Germany) containing a plastic plant as tank enrichment, in a zebrafish recirculation system (Fleuren & Nooijen, Nederweert, The Netherlands) on a 14h light: 10h dark cycle (lights on at 7:00 AM; lights off at 09:00 PM). Water and air temperature were maintained at 24 °C and 23 °C, respectively. Fish were purchased at the juvenile stage and were allowed to adapt to our facility for at least 2 months before being used as adult breeders. The fish were fed daily with dry food (DuplaRin M, Gelsdorf, Germany) and frozen artemias (Dutch Select Food, Aquadistri BV, The Netherlands).

Zebrafish eggs were obtained by random mating between sexually mature individuals. Briefly, on the day before eggs were required (16h prior to collection), a meshed net allowing eggs to pass through but preventing adult fish from accessing and eating the eggs, was introduced in the home tank of a group of 12 adult fish. Each breeding tank was only used once per month to avoid handling stress and ensure optimal eggs quantity and quality.

The eggs were harvested the next day (30 min after the onset of lights at 07:00) and age was set as 1 day post fertilization (dpf). Five eggs were transferred into each well of a 6-well plate in which each well had a diameter of 34.8 mm and contained 10 ml egg water (0.21 g/l Instant Ocean Sea Salt and 0.0005% (v/v) methylene blue) and incubated in an
isolated room at 28 °C and 50% humidity under a light-dark cycle of 14h:10h (lights on at 07:00 /lights off at 21:00). Note that in order to eliminate further sources of disturbance or stress, the medium was not refreshed except on dpf 2 when the medium was completely replaced by fresh egg water and non-fertilized eggs were removed. Larvae were allowed to develop undisturbed under these conditions until behavioural testing at 6 dpf.

On the day of testing, two larvae were gently transferred by plastic Pasteur pipette (VWR International B.V., The Netherlands) into the colour or control Petri dishes (one larva per Petri dish). At the end of the behavioural test, zebrafish larvae were gently removed from the Petri dish and rapidly euthanized with an overdose of tricaine mesylate (MS-222 or Finquel; Argent Chemical Laboratories, USA) and stored at -20 °C.

**Field types**

**Greyscale field**

The greyscale field (GSF) consisted of a 9.2 cm Petri dish with six radial compartments around a central compartment (Figure 7.1A). The central compartment had no filter, and was small (2cm diameter, which is 5% of the total surface area of the dish) in order to encourage the exploration of the peripheral fields by the larvae. Six greyscale photographic filters (Lee filters, Hampshire, UK) with varying opacity (0-100%) were attached in random sequence to the underside of Petri dish to form a series on non-overlapping radial segments or fields. The 0% segment consisted of a transparent filter. The vertical walls of the Petri dish were also covered with the corresponding greyscale filters. Each compartment was evenly illuminated with white fluorescent light from below (500 lux).

**Standard open field**

The standard open field (SOF) was of the same dimensions as the greyscale field described above, but was left completely transparent with no greyscale filters (Figure 7.1C). Furthermore, the vertical walls of the Petri dish were covered with white duct tape to eliminate potential influence of the surrounding environment on zebrafish larvae exploration.

**Colour-enriched open field**

The colour-enriched open field (CEF) has been described before [61] and like the SOF and GSF have the same dimensions. In contrast to the SOF and GSF, the CEF had one of six different colours (blue, green, red, yellow, orange and black) on the underside of each
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radial compartment (Figure 7.1B). This pattern was achieved by attaching photographic colour filters (Lee filters, Hampshire, UK) in random sequence to the underside of the Petri dish. The vertical walls of the Petri dish were also covered with the corresponding colour filters. The whole apparatus is depicted in Figure 7.2.

Figure 7.1 Experimental fields. (A) Greyscale field (B) Colour enriched field (C) Standard open field. All three fields consisted of a 9.2 cm Petri dish with six radial compartments around a central compartment. The central compartment had no filter, and was small (2 cm diameter, which is 5% of the total surface area of the dish) in order to encourage the exploration of the peripheral fields by the larvae. In the greyscale field, six different filters of varying degree of opacity were used. In the colour-enriched field six different colour filters were used. In each field, the surface area of the central zone was 3.14 cm\(^2\) and the radial zones 10.55 cm\(^2\). In (C), there are no filters at all and so the whole field is homogeneous; the dotted lines are given purely for convenience to indicate ‘virtual’ boundaries used for recording purposes.

**Experiment 1: Effect of light intensity on zebrafish exploration patterns**

It has already been shown that zebrafish larvae avoid dark and prefer light environments [54,452]. However, to check whether different intensities of white light have an effect on exploration patterns, we recorded the locomotor activity of zebrafish larvae in GSF and SOF.
**Experiment 2: Colour preference of zebrafish larvae and pharmacological modulation**

We studied effects of the anxiolytic diazepam (Sigma-Aldrich, Zwijndrecht, The Netherlands) on zebrafish colour preference and exploration patterns. This was done by recording the larval zebrafish activity using two CEFs. The one field was used to study the colour preference of zebrafish larvae as a control while the other was used to see the effect of diazepam on colour preference. Diazepam is commonly employed in pharmacological validation of anxiety models in several species [63,76,421,453]. The diazepam was dissolved in dimethylsulphoxide (DMSO) to give final concentrations in egg water of 2.5 µM diazepam and 0.005% DMSO. The treatment duration was 7 min followed by a rapid wash-out with plain egg water (3 s). The larvae were then immediately transferred to the testing apparatus.

**Experiment 3: Effect of abnormal lighting regimes on colour preference**

To see if abnormal lighting conditions can alter the colour preference of zebrafish larvae, they were exposed to different lighting regimes during rearing (from the onset of fertilization to the day of testing) before testing in CEFs.

**Lighting regimes for rearing**

**Light-dark (LD):** These larvae were reared in a normal light-dark cycle consisting of 14h light: 10h dark cycle (lights on at 07:00, lights off at 21:00). The intensity of the light was 400 lux measured by digital lux meter MASTECH®, MS 6612)

**Constant dark (DD):** These larvae were reared in a room which was continuously dark for the whole period. To further ensure that no light fell on the embryos during this period, the plates were kept wrapped in aluminium foil.

**Constant light (LL):** This lighting regime was achieved by rearing the zebrafish embryos in a room with lights kept permanently switched on until the day of testing.
Figure 7.2: Experimental setup (A) The colour-enriched field (on the left of the platform) and standard open field (on the right of the platform) consists of a plastic Petri dish (9.2 cm in diameter) virtually divided into six equally sized radial compartments. Colour-enriched field is identical to the standard open field except that the bottoms and walls of each of the six radial compartments were coloured using colour photographic filters (yellow, red, green, orange, blue, and black). A circular and transparent zone (2 cm in diameter) is delineated in the centre of both open field apparatuses and serves as a starting location. One standard and one colour-enriched open field are placed on top of the light/infrared platform at a time. Behavioural activity in both open field apparatuses was video-recorded simultaneously using an infrared camera (35 frames/s) located above the experimental setup. The duration of the test is 15 min. Automated video recording relies on both white and infrared lights, which are provided by the specialized light/infrared platform. (B) In addition to the six radial zones, we also included two other zones, namely the inner and outer zones. In both open fields, the inner zone consists of the centre circle into which the larva was pipetted at the start of the experiment (white area, 2 cm in diameter) while the outer zone consists of the remaining area, including all the radial zones surrounding the centre zone (solid grey). Note that thigmotaxis is measured in the outer zone only and is reported both as the percentage of TDM in the outer zone as well as percentage of time spent in the outer zone. In experiment 1, the greyscale field (Figure 7.1B) was used instead of colour-enriched field.

**Experimental procedure**

The detailed experimental procedure has been described elsewhere [61]. Briefly, ZebraLab behavioural system (ViewPoint S.A., Lyon, France) settings were adjusted and the threshold level of the camera was set to accurately record and analyse swimming behaviour.
by using the chronolog function of the ZebraLab software. We next delineated the contour of each of the six radial zones in addition to the outer and inner zones within the Petri dish. This step was necessary to ensure that behavioural activity is not only automatically recorded and analysed for each zone separately but also as a whole regardless of zone delineation. To check whether different light intensities have any effect on zebrafish exploration patterns, we used GSF and SOF (in the case of experiment 1) simultaneously. One zebrafish larva (6 dpf) was randomly chosen and transferred to the central zone of the respective open field apparatus (either the GSF or OSF) using a plastic Pasteur pipette (VWR International B.V., The Netherlands). Automated video recording was carried out using both white and infrared lights, and began immediately on release of the zebrafish larvae into the central zone. The infrared and white lights built in to the platform of the ZebraLab behavioural system (View Point, France) were used for these recordings.

The experimenter was out of view from the experimental setup to eliminate any chances of interference on zebrafish larvae exploration. In this way, the behaviour of each larval zebrafish was tested for 15 min. Social interaction between the zebrafish larvae may play an important role in the exploration of an open field test [399,454]. Therefore, we randomly selected zebrafish larvae for testing in both fields to minimize any effect it may cause on the exploration pattern.

**Behavioural end-points**

The behavioural parameters measured were as follows:

1) Total distance moved (mm); the total distance moved by zebrafish larvae regardless of zone was calculated in order to examine the effect of field type on the locomotor activity of the larva.

2) Latency to leave the centre; the time taken by zebrafish larvae to leave the centre where they were initially placed at the beginning of the experiment was used to examine the tendency of the larvae to explore the outer zones.

3) Centre avoidance: the percentage of TDM in the inner zone was used to determine centre avoidance. Specifically, centre avoidance was calculated as the ratio between TDM in the outer zone and TDM over the whole test arena (the sum of the inner and outer zones). The percentage of TDM in the outer zone was obtained by multiplying this ratio by a factor of 100 as depicted in the formula below:
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Centre avoidance (% TDM inner zone) = \[ \frac{TDM \text{ inner zone}}{(TDM \text{ outer } + \text{ inner zone})} \times 100 \]

This calculation was performed in order to correct for individual differences in locomotor activity as recommended by Bouwknecht and colleagues [422]. Centre avoidance can also be presented as the percentage time spent in the inner zone as shown in the formula below. However the latter calculation must be applied with caution since erroneous conclusions can be obtained if animals do not display sufficient levels of locomotor/exploratory activity [422].

Centre avoidance (% time spent inner zone) = \[ \frac{\text{time spent in inner zone}}{\text{total duration of test}} \times 100 \]

Note that the inner zone consisted of the centre area (2 cm in diameter) while the outer zone consisted of the remaining area surrounding the centre zone. In the present study we measured centre avoidance using both calculation methods shown above.

Zone preference/avoidance: the percentage TDM within each of the 6 radial zones and well as in the starting location (neutral central zone) was used to determine zone preference and central zone preference, respectively. As for centre avoidance, zone preference was calculated as the ratio between TDM in each radial zone and TDM over the whole test apparatus. The same calculation was applied to the central zone. Zone preference/avoidance was also calculated in terms of TTS. The % of TDM and TTS in the each zone was obtained by multiplying this ratio by a factor of 100 as depicted in the formulas below:

\[ \text{Colour zone preference} \% = \left( \frac{TDM \text{ radial zone}}{TDM \text{ whole apparatus}} \right) \times 100 \]

\[ \text{Colour zone preference} \% = \left( \frac{TTS \text{ radial zone}}{TTS \text{ whole apparatus}} \right) \times 100 \]

Furthermore, in order to ascertain that preference for a given zone was not related to the spatial properties of the room, we also performed these calculations for the SOF, which was colourless. We predicted a random pattern of exploration with no specific preference for any of the radial zones (which would correspond to the greys zones in the GSF). Such results would allow us to rule out biases in data interpretation related to spatial properties of the room.
4) Freezing behaviour: the time spent (%) in immobility over the total duration of the test was measured as an index of anxiety-like behaviour. Immobility was defined as the absence of movement for ≥1 s (with the exception of movements required for respiration). We calculated the ratio between duration of freezing behaviour(s) and total duration of the test (i.e. 900 s). The freezing behaviour (%) was obtained by multiplying this ratio by a factor of 100 as depicted in the formulas below:

\[
Freezing \% = \left( \frac{\text{Duration of freezing behaviour(s)}}{\text{total duration of the test}} \right) \times 100
\]

5) Number of entries: frequency of visit to each zone was calculated over the duration of the test which shows the eagerness of larval zebrafish to explore particular area.

Statistical analysis

Statistical analysis and graphs were performed using GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego California USA; www.graphpad.com). All data were arcsine square root transformed prior to statistical test due to its approximative variance-stabilizing property. Student's t-tests were performed to analyse impact of different open fields and drug treatment on general locomotor activity (Figure 7.3, Figure 7.7A,B), latency to begin exploration (Figure 7.6A), and freezing behaviour (Figure 7.6B).

A one-way ANOVA test was performed to analyse zone preference/avoidance in SOF and GSF (Figure 7.4A and B), effect of light and dark rearing conditions on general locomotor activity and freezing behaviour (Figure 7.8A and Figure 7.8B respectively). A Tukey’s post hoc test was used to analyse multiple comparisons. Two-way ANOVA analyses with drug treatment as a between-subjects factor and colour zones (i.e. centre, yellow, red, green, orange, blue, and black) as a within subjects factor were performed to analyse colour preference (Figure 7.7C,D) and also in response to light and dark rearing conditions (Figure 7.9A, B) as well as number of visits per colour zone (Figure 7.7E, F and Figure 7.11), centre avoidance in SOF and GSF (Figure 7.5A-D) and centre avoidance in response to lighting regimes (Figure 7.10A, B). Significant main effects were further decomposed using pair-wise comparisons with a Bonferroni's correction, for multiple comparisons. Data are presented as mean ± SEM, and a probability level of 5% was used as the minimal criterion of significance.
Results

Effect of light intensity on zebrafish exploration (GSF vs SOF)

General locomotor activity

Figure 7.3 shows total activity level in GSF and SOF. Student’s T-test (two-tailed) reveals no significant differences in general locomotor activity of zebrafish larvae between SOF and GSF \([T_{(61)}=1.816, p=0.0742]\).

Zebrafish preferences for specific light intensity zones (in terms of TDM and TTS)

Zebrafish preference for specific greyscale zones was measure in terms of TDM (%) in each of the six radial zones. The zebrafish preference for various greyscale zones is shown in Figure 7.4A. One-way ANOVA for repeated measures revealed that there is no effect of zone \([F(5,198)=0.3141, p=0.9041]\).

TDM (%) for GSF is shown in Figure 7.4B. One-way ANOVA for repeated measures showed a significant main effect of light intensity on zebrafish exploration where they moved more in the 0% zone with no grey filter than either light or dark zones in the GSF \([F(5,165)=23.25, p<0.0001]\).
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Figure 7.4. Zone preference/avoidance measured as TDM (%) per zone. Zebrafish larvae tested in the SOF (panel A) displayed expected patterns of exploratory behaviour. They moved equally in all zones except centre where they moved more. In the GSF (panel B), zebrafish larvae moved significantly more in the centre and 0% zone (neutral zones) while they moved equally in all other zone irrespective of the density of greyscale filters. (C, D) Zone preference/avoidance as measure by the % of TTS per zone in SOF and GSF respectively. The results corroborate the findings in panel A and B for SOF and GSF respectively suggesting that TTS per zone is correlated to TDM. Different letters above bars show statistical significance.

Zebrafish preference for specific greyscale zones was also measured in terms of TTS (%) in each of the six radial zones as well as the central zone (Figure 7.4C). One-way ANOVA for repeated measures revealed that there is no effect of ZONES on TTS in SOF \([F(5,198)=1.135, p=0.343]\). Figure 7.4D shows TTS (%) in GSF. One-way ANOVA for repeated measures showed that zebrafish larvae spent significantly more time in the 0% zone than any other zone in the GSF \([F(5,168)=21.29, p=<0.0001]\).

Centre avoidance (in terms of TDM and TTS)

Two-way ANOVA was performed to see whether zebrafish display centre avoidance in terms of TDM. A significant interaction was found between field types and zones \([F(1,116)=19.55, p=<0.0001]\). The results are depicted in Figure 7.5A. The zebrafish larvae

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moved significantly more in the inner zone of the GSF as compared to inner zone of SOF (p<0.01). When outer zones of both field types were compared, it was found that zebrafish larvae moved significantly more in the outer zone of SOF relative to the inner zone of GSF (p<0.01).

Centre avoidance of zebrafish larvae in terms of TTS was also calculated and is shown in Figure 7.5B. A significant interaction was found between field types and zones \[F_{(1,116)}=15.75, p=<0.0001\]. Specifically, the zebrafish larvae spent significantly more time in the inner zone of the GSF as compared to inner zone of SOF (p<0.01). When outer zones of both field types were compared, it was found that zebrafish larvae spent significantly more time in the outer zone of SOF relative to the inner zone of GSF (p<0.01).

![Figure 7.5](image-url) Figure 7.5. Patterns of zone preference/avoidance. (A) Zebrafish centre avoidance was calculated as % TDM. Zebrafish larvae moved more in the inner zone of GSF as compared to inner zone of SOF. In case of outer zones, zebrafish larvae moved significantly less in the outer zone of GSF than that of SOF. (B) Centre avoidance of zebrafish larvae was also calculated in terms of % of TTS in SOF and GSF. The results of % TTS in SOF and GSF largely corroborated with panel A.

Latency to leave the centre

Figure 7.6A shows the latency to leave the central zone. There was a marked increase in the latency to leave the central zone in the GSF. However, in SOF, zebrafish larvae left the centre soon after releasing them and spent little time in the centre \[T_{(62)}=5.416, p<0.0001\].

Freezing behaviour

The time spent in relative immobility for the duration of the experiment was extracted from the data and is shown in Figure 7.6B. A student’s T-test showed that there was no
significant difference in time spent in immobility between GSF and SOF \[T_{(62)}=4.405, p<0.001\].

![Graph](image)

Figure 7.6. Patterns of anxiety-like behaviours. (A) Analysis of the latency to leave the centre zone to explore any other radial zone is significantly higher in the GSF than in SOF. (B) Expression of freezing behaviour which was calculated as time spent in relative immobility shows that zebrafish larvae in GSF spent significantly more time in relative immobility than in SOF.

**Colour preference of zebrafish larvae in CEF and its pharmacological modulation**

We have previously reported the colour preference of zebrafish larvae [61]. Here, we looked at the effect of diazepam on this preference.

**Effect of diazepam on general locomotor activity**

TDM (%) is shown in Figure 7.7A. Student’s t-test showed no significant differences in general locomotor activity of diazepam exposed and control zebrafish larvae \[T_{(46)}=1.573, p=0.1226\].

**Effect of diazepam on freezing behaviour**

Figure 7.7B shows the freezing behaviour of the zebrafish for diazepam-treated and control groups. The larvae treated with diazepam showed significantly less time spent in immobility as compared to control zebrafish larvae. \[T_{(46)}=2.444, p=0.0184\].
Effect of diazepam on colour preference (in terms of % TDM and TTS)

Colour preference was assessed by measuring the TDM (%) in each of the six radial zones as well as in the central colourless zone for control and diazepam treated larvae. Figure 7.7C shows effect of diazepam on the colour/zone preference. A two-way ANOVA analysis reveals a significant interaction between colour zones and treatment \[ F(6, 276) = 4.804, p<0.0001 \]. A post hoc Bonferroni test was used to further decompose the interactions and correct for multiple comparisons. The results show a significant difference in terms of % TDM per zone between control and diazepam treated larvae. Specifically, control larvae moved significantly less in red, yellow, black and blue (p<0.05) than did treated larvae.

Colour/zone preference was also assessed by measuring the TTS (%) in each of the 6 radial zones, and the central zone, of the CEF (Figure 7.7D). A two-way ANOVA analysis reveals a significant interaction between colour zones and treatment \[ F(6,276) = 5.189, p<0.0001 \]. A post hoc Bonferroni test was used to further decompose the interactions and correct for multiple comparisons. The results showed significant differences in terms of % TTS per zone between control and treated zebrafish larvae. Specifically, control larvae spent significantly less time in the red, yellow, black and blue zone (p<0.05) as compared to diazepam treated larvae.

Number of entries per zone

The frequency of visits per zone is presented in Figure 7.7E. A two-way ANOVA analysis reveals no significant interaction between colour zones and treatment \[ F(6,276) = 2.017, p = 0.2329 \]. Bonferroni post hoc analysis indicates that the number of entries to the zones were not significantly different between control and treated larvae (p>0.05).

Time spent per entry

The time spent in each zone per visit is shown in Figure 7.7F. A two-way ANOVA analysis reveals significant interaction between colour zones and treatment \[ F(6,276) = 3.654, p = 0.0017 \]. Bonferroni post hoc analysis indicates that the time spent per visit by control larvae in colour zones were significantly different from diazepam treated larvae. Specifically, control zebrafish larvae spent less time per visit in red, yellow, black and blue zones than did treated larvae.
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A. Total activity level

B. Freezing behaviour

C. Colour preference (% TDM)

D. Colour preference (% TTS)

E. Number of entries

F. Time spent per entry

Figure 7.7. Effect of diazepam on colour preference. (A) The total activity level of control and treated zebrafish larvae was not significantly different (p>0.05). (B) The zebrafish larvae treated with diazepam spent significantly less time in relative immobility (p=0.0219). (C) The zebrafish larvae avoided red, yellow, black and blue colours, however, this avoidance was abolished with diazepam treatment. (D) The colour preference was also calculated in terms of total time spent. The results corroborated the results with panel C. The control larvae preferred orange, green and central zone and avoided red, yellow, black and blue. However, the treated larvae did not show any significant preference or avoidance. (E) The number of visit per zone showed that zebrafish larvae visited each zone and no significant difference was found. However, when time spent per entry was calculated (F) it showed that control zebrafish larvae spent significantly less time per entry in red, yellow, black and blue colours while when treated with diazepam, larvae visited all colours equally.
Effect of lighting conditions during rearing on the subsequent colour preferences of zebrafish larvae (CEF vs SOF)

General locomotor activity
The general locomotor activity of zebrafish larvae reared in either constant light (LL), constant dark (DD) and normal light-dark cycle (LD) is shown in Figure 7.8A. One-way ANOVA for repeated measures reveals a significant influence of environmental complexity on general locomotor activity \( F(2,71) = 5.775, p = 0.0048 \). Specifically, the total activity level was significantly higher in the zebrafish larvae raised in normal light-dark cycle (LD) than the larvae raised in LL or DD.

Freezing behaviour
The time spent in relative immobility (freezing behaviour) is shown in Figure 7.8B and analysed using a One-way ANOVA for repeated measure. LL and DD zebrafish larvae remained in a state of relative immobility significantly more than the LD larvae \( F(2,71) = 6.573, p = 0.0024 \).

Colour preference (in terms of % TDM and TTS)
Colour/zone preference was assessed by measuring the TDM (%) in each of the 6 radial zones as well as in the central zone (Figure 7.9A). A two-way ANOVA analysis reveals a significant interaction between colour zones and types of open field \( F_{(12, 702)} = 7.358, p \).
p < 0.0001]. A post hoc Bonferroni test was used to further decompose the interactions and correct for multiple comparisons. The results show a significant difference in terms of % TDM per zone between all lighting regimes. The DD larvae moved significantly more in the black (p < 0.05) and blue zones (p < 0.001) as compared to LD and LL larvae. The LL larvae moved significantly more in the orange (p < 0.05) and yellow zones (p < 0.05) as compared to LD and DD larvae.

Colour/zone preference was also assessed by measuring the TTS (%) in each of the 6 radial zones as well as in the central zone (Figure 7.9B). A two-way ANOVA analysis reveals a significant interaction between colour zones and types of open field [F(12,702) = 6.041, p < 0.0001]. A post hoc Bonferroni test was used to further decompose the interactions and correct for multiple comparisons. The results show a significant difference in terms of % TTS per zone between LD larvae compared to LL or DD larvae. Specifically, DD larvae moved significantly more in the black (p < 0.05) and blue zones (p < 0.0001) as compared to LD and LL larvae.

Centre avoidance (TDM and TTS)

The results of centre avoidance in terms of TDM are shown in Figure 7.10A. Two-way ANOVA analysis was performed which showed significant interaction between lighting conditions and zones [F(2,158) = 21.13, p < 0.0001]. It was found that LD zebrafish larvae moved significantly more in the inner zone than LL and DD zebrafish larvae which moved significantly more in the outer zone.

Centre avoidance in terms of TTS is shown in Figure 7.10B. Two-way ANOVA analysis revealed a significant interaction between lighting conditions and zone [F(2,158) = 149.9, p < 0.0001]. A post hoc Bonferroni test was used for multiple comparisons. Specifically, LL and DD zebrafish larvae spent significantly more time in the outer zone than LD zebrafish larvae.
The colour preference of larval zebrafish

Figure 7.9. Colour preference/avoidance in response to different light regimes in terms of % TDM (panel A) and in terms of % TTS (panel B). The zebrafish larvae were continuously exposed to different light regime from the onset of fertilization until the day of testing (6 dpf). The zebrafish larvae reared in normal light dark cycle preferred the orange and green colour while avoided the red, yellow, black and blue colour both in terms of % TDM and % TTS. The zebrafish larvae reared in constant dark, moved and spent more time in the blue and black and avoided green, red and yellow. The zebrafish larvae reared in constant light preferred orange, green and yellow while avoided red, black and blue as compared to larvae reared in normal light-dark cycle.
Figure 7.10. Patterns of preference/avoidance. Centre avoidance was displayed by zebrafish larvae in terms of % TDM (A) and % TTS (panel B). The zebrafish larvae raised in either constant light or constant dark moved significantly less in the inner zone than zebrafish larvae raised in normal light-dark cycle. In the outer zone, zebrafish larvae raised in normal light-dark cycle, moved significantly less than zebrafish larvae raised in constant light or constant dark. The % TTS results corroborate the finding in panel A.

Number of entries per zone

The frequency of visits per zone is presented in Figure 7.11. A two-way ANOVA analysis reveals a significant interaction between colour zones and lighting conditions \(F_{(12,546)} = 3.265, p < 0.001\). Bonferroni post hoc analysis indicates that LL zebrafish larvae visited green, black and blue significantly less than either LD or DD larvae. The number of entries in orange was significantly higher in DD larvae than LD and LL larvae \(p<0.05\).
The colour preference of larval zebrafish

Figure 7.11. (A) Number of entries. Analysis of the frequency of visits in each of the colour zones shows that zebrafish larvae reared in normal light-dark cycle visited centre, red, black and blue zones significantly less than orange and green (p<0.05). The zebrafish larvae reared in light made fewer entries in red, green, black and blue zones (p<0.05). The zebrafish larvae reared in constant dark, made significantly more number of entries in orange (p<0.05).

Discussion

We have already developed a novel standard open field test for zebrafish larvae [61]. Here we aimed to provide a variant of the open field using different degrees of greyscale light intensity zones instead of different colours. We used light and dark greyscale filters with varying degrees of darkness and examined the pattern of exploration. Then, we validated our previously-developed colour preference test to see that anxiety-like behaviour, as shown by freezing and centre avoidance, can be modulated with the anxiolytic compound diazepam. Further, we showed that light conditions during rearing lead to differences in the subsequent zebrafish colour preferences.

Exploratory behaviours in the SOF and GSF

One of the goals of this study was to assess the impact of environmental complexity on patterns of exploratory behaviours displayed by animals in the novel fields. We have previously shown that zebrafish larvae display dark-avoidance and thigmotaxic-like behaviour. Here we tested different shades of dark in one field to see if degree of opacity has any effect on the exploratory behaviour and preference for certain zones. We observed that zebrafish showed a response to a novel field similar to that shown by adult zebrafish...
The colour preference of larval zebrafish

[80] and rodents [215]. Specifically, zebrafish larvae explored the novel SOF and showed a relative avoidance of the central zone. However, zebrafish larvae preferred the light (0%) area of the GSF. This is consistent with other reports on larval zebrafish light/dark preferences [49,452,455] and contrasts with the strong preference for the dark compartment shown by adult zebrafish [85].

We found zebrafish larvae moved and spent more time in the central zone (even though it constitutes only 5% of the total area). This again is in contrast to adult zebrafish which display thigmotaxis [32,208,219]. However, in the current study, centre exploration (≥20%) may be attributed to the placement of the zebrafish in the centre zone at the beginning of the experiment, which might have resulted in freezing, and a biasing of subsequent movement towards the centre zone.

Zebrafish larvae moved more in the central zone of GSF (37%) as compared to central zone of SOF (25%). In terms of TTS, zebrafish larvae spent more time in the central zone of GSF (38%) than in the central zone of SOF (28%). This might be explained on the basis of complexity of the novel environment [61]. Thus, the GSF consisted of different shades of greyscale filters and may therefore represent a complex environment that the zebrafish larvae were reluctant to explore (as biasing their movement to the centre). This could explain why we did not see the thigmotaxis-like behaviour reported in adult zebrafish [32,80,219] and rats [215] placed in novel environments. The same inference can also be drawn from the latency of the zebrafish larvae to leave the centre in both fields. Zebrafish larvae left the centre immediately after forced placing in the SOF; however, there was a greater latency to leave the centre in the case of GSF. One explanation for this could be the complexity of the environment and a longer time taken by the larvae in assessing it before they started to explore.

The exploratory behaviour of zebrafish larvae in the GSF suggests preference and/or avoidance for certain zone is not dependent on the light intensity. The zebrafish larvae avoided the all the greyscale zones and preferred to explore in the light zone either with a filter or without a filter. This result formed the base of our next experiment in which zebrafish colour preference and avoidance patterns were observed.

Zebrafish colour preference and diazepam treatment

We have already reported an open field model for larval zebrafish colour preference and for anxiety-like behaviour displayed in a complex environment [61]. Here, we report that the
anxiolytic diazepam can abolish the anxiety-like behaviour (freezing). In our previous study [61], untreated zebrafish preferred orange and green while avoiding red, yellow, black and blue zones, a finding confirmed here.

A preference and aversion to certain colours is also indicated by the behaviour of zebrafish larvae in a particular colour zone. Less locomotor activity, and less time spent, in aversive colours suggest an effect of colours on some cognitive response. In the current study, red, yellow, black and blue were least preferred and gave rise to anxiety-like behaviour (freezing) in the larvae.

The aversion to red has been reported in many species including rodents [456], rhesus monkeys [457], domestic chickens [458] and adult zebrafish [434]. It has also been argued that colours such as red and yellow serve as effective warning signals [459]. This suggests that an aversion to specific colours in the current study might be a response to a perceived warning stimulus. In humans, red is also regarded as a distracting colour [438] that evokes a negative mood more than do other colours [437,460]. The preference or avoidance of certain colours might be explained by the moods which these colours evoke. Extrapolating these results to the zebrafish, it could be envisaged that aversive colours produce an anxiety-like state, while preferred ones either lower, or do not change, the level of anxiety.

To test whether an anxiolytic is able to change the colour preference, zebrafish larvae were treated with diazepam. This was used because it has been shown that it reduces anxiety-like behaviour in zebrafish larvae [54,208]. It has also been proved effective in adult zebrafish where it decreased the white avoidance [67]. In the case of zebrafish larvae in an open field test, larvae treated with diazepam and other anxiolytics spent significantly more time in the dark zone than the control larvae; some anxiogenics had the opposite effect [54]. Similarly, in the current study, the aversion towards certain coloured zones was significantly reduced by diazepam treatment, and the larvae moved and spent time equally in all zones irrespective of the colour. Hence, our findings are in accordance with our previous study showing that zebrafish larvae show specific preference/aversion of certain colours and additionally show that these preferences and aversions can be modulated by diazepam treatment.
Effect of abnormal lighting conditions during rearing on colour preference/aversion

General patterns of avoidance behaviour in zebrafish have been studied in detail [125,461]; they can be induced by such factors as size and shape of a potential predator, and its colour patterns, which can induce fear and hence avoidance behaviour [462]. In view of these studies, it is interesting to know the colour preference of zebrafish. Adult zebrafish have been showed aversion to blue while red and green were preferred over yellow [188,434]. Young zebrafish larvae show preference for orange and green and aversion to blue, black, red and yellow [61]. Knowledge of colour preference may help in the studies of learning and memory [425,434] or aversion, anxiety or fear [61]. But it is essential to know about the preference of particular species as it can change in response to environmental factors such as light in the present study.

Several environmental factors such as light play an important role in the growth and development of zebrafish larvae [313]. In the present study, the preference/aversion of zebrafish larvae changed in response to the rearing environment. We have already reported the innate colour preference of larval zebrafish in which zebrafish showed a preference for orange and green while red, yellow, black and blue were avoided [61]. In the current study, zebrafish larvae reared in constant light avoided red, black and blue while preferred orange in terms of TTS and TDM. The zebrafish larvae reared in constant dark moved, and spent less time, in red, and preferred orange over other colours; their aversion to black and blue was lost. Irrespective of the raising environment, preference for orange and aversion to red was a constant finding, suggesting an innate preference and aversion for these colours respectively. Preference of orange and green can be attributed to foraging habits [28,463] as well as the biological niche of aquatic species [28]. However more studies are required to clarify these issues.

In the present study, the total activity level was decreased for the larvae raised either in constant dark or in constant light, suggesting that the lighting regime during rearing can affect the subsequent basal activity levels of zebrafish larvae. Freezing behaviour was also modulated by abnormal lighting regimes during rearing suggesting. These results conform with studies on rodents which also showed decrease in locomotor activity after exposure to either constant light or constant dark [464].
Conclusion

We have shown that zebrafish larvae prefer light environment over dark and display anxiety-like behaviour in the complex environment. The larvae prefer orange and green over red, yellow, black and blue. After exposure to diazepam, the colour preference and aversion was lost as was the anxiety-like behaviour of freezing. We further show that light exposure during rearing can modulate colour preference and behaviour of the zebrafish larvae. The zebrafish reared in constant light moved more in the green and orange zones while zebrafish reared in constant dark moved more in the black and blue zones. However, regardless of lighting regime, the zebrafish larvae showed a strong preference for orange and aversion to red indicating that zebrafish larvae have a particular genetic predisposition for the development of colour preference and avoidance.