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Zebrafish embryos and larvae as a complementary model for behavioural research

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Exploratory behaviour in the open field test adapted for larval zebrafish: impact of environmental complexity

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Abstract

This study aimed to develop and characterize a novel (standard) open field test adapted for larval zebrafish. We also developed and characterized a variant of the same assay consisting of a colour-enriched open field; this was used to assess the impact of environmental complexity on patterns of exploratory behaviours as well to determine natural colour preference/avoidance. We report the following main findings: 1) zebrafish larvae display characteristic patterns of exploratory behaviours in the standard open field, such as thigmotaxis; 2) environmental complexity (i.e. presence of colours) differentially affects patterns of exploratory behaviours and greatly attenuates natural zone preference; 3) larvae displayed the ability to discriminate colours. As reported previously in adult zebrafish, larvae showed avoidance towards blue and black; however, in contrast to the reported adult behaviour, larvae displayed avoidance towards red. Avoidance towards yellow and preference for green and orange are shown for the first time, 4) compared to standard open field tests, exposure to the colour-enriched open field resulted in an enhanced expression of anxiety-like behaviours. To conclude, we not only developed and adapted a traditional rodent behavioural assay that serves as a gold standard in preclinical drug screening, but we also provide a version of the same test that affords the possibility to investigate the impact of environmental stress on behaviour in larval zebrafish while representing the first test for assessment of natural colour preference/avoidance in larval zebrafish. In the future, these assays will improve preclinical drug screening methodologies towards the goal to uncover novel drugs.

Introduction

An important consideration related to the rising popularity of zebrafish as a novel pharmacological model for high-throughput screening is the demand for validated behavioural assays customized for zebrafish larvae and compatible with the use of automated video-recording systems [87,362-367]. One important category of assays is 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 [368,369]. These assays are traditionally used as behaviour-based screening methodologies for validation of the pharmacological effects of neuroactive compounds, and are recognized as gold standards in preclinical (rodent-based) research [368,370-373]. We recently adapted the open field test for adult zebrafish [173], as well as the light-dark preference test for adult [173] and larval zebrafish [374]. In the current study, we want to extend this repertoire of assays to include and adapt the open field test for zebrafish larvae.

The open field test is well suited for zebrafish larvae since it is a relatively simple, painless, and unconditioned test that can readily assess spontaneous/natural tendency of an animal to explore or avoid a novel environment depending on the degree of aversiveness [369]. The open field test provides measures of reactivity to a novel large space in which an isolated individual is confronted with the dilemma of exploring the novel environment (to find food, escape routes, and mating partners) and the fear of a large unknown space [369,372]. Novel environments, such as those experienced in the open field test, have aversive properties that alter exploratory behaviour and promote thigmotaxis in rodents [369].

Interestingly, features of the environment such as illumination (bright/open zone vs. dark/protected zone), colours, and its topography (periphery vs centre) serve as cues helping an individual assess the aversiveness or safety of a given environment [369,371,375-379]. In the current study, we not only investigated whether zebrafish larvae display characteristic exploratory patterns usually observed in this test, but also how patterns of exploratory behaviours could be influenced by more complex environment comprising a choice of colours. Colour-enriched environments are ecologically relevant contexts for zebrafish, which possesses the ability to view and discriminate colours [172,377,379-385]. In fact, zebrafish is an important model of the visual system [386-389]. This is further supported by the demonstration that zebrafish possess a photopigment spectral sensitivity with peak absorbance in ultraviolet (362nm), blue (415nm), green

(480nm) and red (570nm) [379,390] as well as all the specialized neural cells necessary for colour vision in the retina of larval zebrafish [172,383,385,391].

In addition to their ability for colour discrimination, adult zebrafish are also capable of conditioned learning that relies on colour as a conditioning cue. For instance, adult zebrafish were successfully trained to forage for food of a particular colour that corresponded to the type (colour) of food to which they were exposed in early life [379]. Furthermore, and of particular interest for the current study, apparent innate preference and avoidance for red and blue, respectively, have been observed in adult zebrafish [377,384]. Taken together, these findings suggest that colour discrimination, colour condition learning and/or colour preference/aversion could be used proficiently for development of novel assays in zebrafish, such as a novel colour-enriched open field test [378,380].

Goal of the Study

The goal of this study is to develop a novel standard open field test as well as a variant version of the same test comprising greater environmental complexity such as an array of six different colours (i.e. colour-enriched open field test). We aim to provide a detailed description of the behavioural repertoire displayed by larval zebrafish when confronted with novel environments that differ in complexity such as the absence (i.e. standard open field test) or presence of colours (colour-enriched open field test). Specifically, the following questions were addressed: 1) Do zebrafish larvae display characteristic exploratory behaviours commonly encountered in the 'standard' open field such as centre avoidance and habituation of locomotor activity? 2) What is the influence of environmental complexity (colour-enriched open field) on the expression of exploratory behaviours? 3) Do zebrafish larvae at 6 days post fertilization (dpf) possess the ability to discriminate colour as adult zebrafish do? 4) What is the pattern of natural preference/avoidance among six different colour zones (i.e. yellow, red, green, orange, black, and blue) and how does it compare to the adult pattern of preference/avoidance?

Materials and Methods

Ethical Note

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), Directives 86/609/EC

and 2010/63/EU, and were performed only after a positive recommendation of the Animal Experiments Committee of Leiden University 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 7h AM: lights off at 21h 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). Adult zebrafish were returned to the animal facility after egg production.

Zebrafish eggs were obtained by random mating between sexually mature individuals. Briefly, on the day (16h) before eggs were required, a meshed net allowing eggs to pass through but preventing adult fish from accessing/eating them, 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 7h AM) and age was set as post fertilization day (dpf) 1. Five eggs were transferred in each well of a 6-well plate, which contains 10 ml of egg water (0.21 g/l Instant Ocean Sea Salt and 0.0005% (v/v) methylene blue). The well plates were kept in a separate climate room maintained at a temperature of 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/stress, the medium was not refreshed except on 2 dpf where the medium was completely replaced by fresh egg water. Non-fertilized eggs were also removed and replaced by healthy eggs from the same clutch. Larvae were allowed to develop undisturbed under this condition until behavioural testing at 6 dpf.

Open Field Test

Standard Open Field Test Apparatus

The standard open field test apparatus consisted of a standard plastic Petri dish (9.2 cm in diameter), which was virtually divided into six equally sized radial compartments. The bottoms of each of the six radial compartments were transparent but their outside perimeters were covered with white tape to block external cues. A virtual circular zone (2 cm in diameter) was also delineated in the centre and served as a starting location (Figure 6.1A). The apparatus was filled with 20 ml of egg water, which was replaced between each individual trial.

Colour-Enriched Open Field Test Apparatus

The colour-enriched open field test apparatus also consisted of a standard plastic Petri dish (9.2 cm diameter) that was divided into six equally sized radial compartments (Figure 6.1A). The bottoms and walls of each of the six radial compartments were equipped with different colour photographic filters (Lee Filters, Hampshire UK), which were maintained in place underneath the bottom and outside the walls of the Petri dish, respectively with the use of transparent tape. Specifically, the colour filters (purchased at Art & Light Photography, Germany) used in the current study included red (cat # bright red 026), blue (cat # Tokyo blue 071), green (cat # Fern green 122), yellow (cat # yellow 101), orange (cat # Orange 105), and black (cat #IR87 infrared black). Absorbance spectra of the colour filters can be found in the supplementary Figure 1. In addition to the coloured radial segments, a circular zone (2 cm in diameter) was also delineated in the centre but left uncoloured (with no photographic filter). This zone also served as a starting location. Note that during behavioural testing, one standard and one colour-enriched open field apparatuses were placed on top of the light/infrared platform and were recorded simultaneously using the Viewpoint ZebraLab of Viewpoint S.A., Lyon, France (Figure 6.1A). The infrared camera (35 frames/s) of ZebraLab was located above the experimental set up at a distance allowing the capture of movement of the larval zebrafish within both test apparatuses simultaneously. The experimental setup was illuminated with diffuse lights from fluorescent light tubes located on the ceiling. The juxtaposition of the colours used and relative position of both fields were changed in a pilot experiment and found that these have no effect on the locomotor activity and zone preference of the zebrafish.

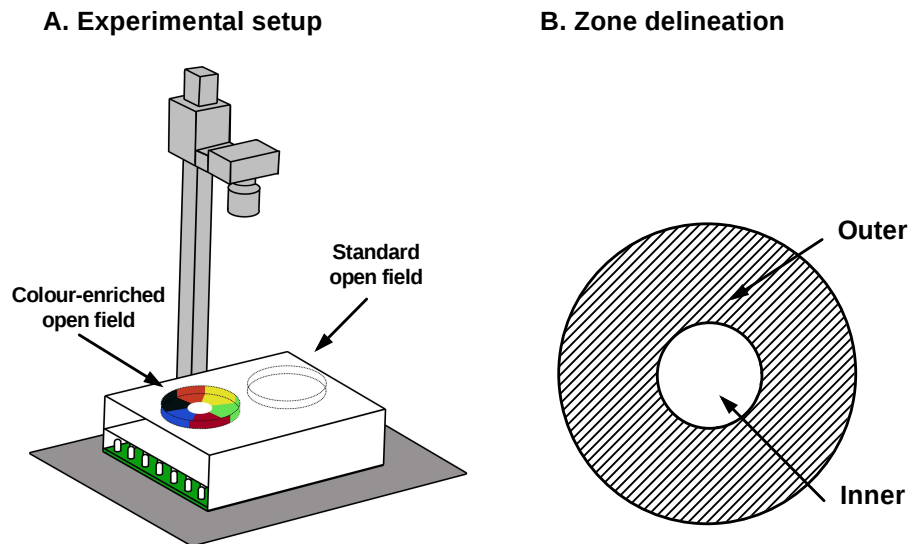


Figure 6.1 **Experimental procedure.** A) The standard open field apparatus (left) consists of standard plastic Petri dish (9.2 cm in diameter) virtually divided into six equally sized radial compartments (radial (2010 #1254) open field test apparatus (right) is identical to the standard open field except 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 set up. The duration of the test is 15 minutes. 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 field apparatuses, the inner zone consists of the centre area (white area, 2 cm in diameter) while the outer zone consists of the remaining area, including all the radial zones surrounding the centre zone (hatched pattern). Note that centre avoidance is measured in the inner zone only and is reported both as the % of TDM in the inner zone as well as % of time spent in the outer zone.

Experimental Procedure

To obtain accurate tracking of the swimming behaviour of individual fish, we first adjusted the settings of the automated video-recording system before the beginning of the experiment. This was done by using a test Petri dish with larvae of an equivalent life stage, which served to establish the tracking threshold of the camera. We next delineated the contour of each of the six radial zones (Figure 6.1A) in addition to the outer and inner

zones within the Petri dish (see Figure 6.1B). 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. Briefly, the experimental procedure was performed as described below:

On the day of testing, larvae (6 dpf) were directly transferred to the central zone of each of the different open field apparatuses using a plastic Pasteur pipette (VWR International B.V., The Netherlands). Automated video recording relying on both white and infrared lights was provided by the specialized light/infrared platform comprised in the ZebraLab behavioural system (View Point S.A., Lyon, France). Larval zebrafish were tested individually and not in a group. However, both fields were tested simultaneously. Automated video recording of behaviour started upon entry in the test apparatus. The duration of the test was 15 minutes and the chronolog function of the ZebraLab software was used to record and analyse swimming behaviour. During the recording, the experimenter was outside the view of the larval zebrafish to avoid disturbance of behavioural responses. A total of 90 (n=45 for standard open field test and n=45 for colour-enriched open field test) zebrafish larvae (6 dpf) were used in this experiment. All larvae were rapidly euthanized with an overdose of tricaine (MS-222) and disposed of according to local regulations following completion of the behavioural testing.

Behavioural Endpoints

All measures described below were automatically recorded and analysed with the ZebraLab software (ViewPoint S.A., Lyon, France). The behavioural endpoints measured were:

1) General locomotor activity: Total distance moved (TDM) over time across the whole test apparatus but also across all different zones of both standard and colour-enriched open field apparatuses was measured. The findings were used to determine general locomotor activity over time (3 x 5 min-blocks) and space (inner vs outer zones as well as radial zones with and without colours).

2) Habituation learning: this is one of the simplest forms of non-associative learning. It is defined as the reduction of a behavioural response when an animal is exposed to a continuous stimulus/environment [164,392]. Habituation learning was measured by statistically comparing the mean TDM (mm) obtained on the first 5 min-block with that of the last 5-min block. Habituation of locomotor activity is considered to have occurred when locomotor activity measured on the last 5-min block was significantly lower than the

locomotor activity measured on the first 5-min block. These findings will be used to determine whether the complexity of the environment (i.e. presence or absence of colours) differentially affected the pattern of habituation learning.

3) Centre Avoidance: The percentage (%) of the TDM in the inner zone of the test apparatus was used to determine centre avoidance (see Fig. 1B). Specifically, centre avoidance was calculated as the ratio between TDM in the outer zone and TDM over the whole test arena (including inner and outer zones). The % of TDM in the outer zone was obtained by multiplying this ratio by a factor of 100 as depicted in the formula below:

$$\text{Centre avoidance (\% TDM inner zone)} = [(TDM \text{ inner}) / (TDM \text{ outer} + \text{inner})] \times 100$$

This calculation was performed in order to correct for individual differences in locomotor activity as recommended by Bouwknecht and colleagues [368].

Centre avoidance can also be presented as the percentage of time spent in the inner zone as shown in the formula below. However caution must be applied with the use of the latter calculation since erroneous conclusions can be obtained if animals do not display sufficient levels of locomotor/exploratory activity [368].

$$\text{Centre avoidance (\% time spent inner zone)} = [(time \text{ inner}) / (\text{test duration (i.e. 900 seconds)})] \times 100$$

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. Delineation of the inner and outer zone is shown in Figure 1B. In the present study we measured centre avoidance using both calculation methods shown above.

4) Colour zone preference/avoidance: The % TDM within each of the 6 radial zones and well as in the starting location (neutral central zone) was used to determine colour preference and central zone preference, respectively (Figure 1A). As for centre avoidance, colour 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 centre zone. The % of TDM in the each colour zone as well as central zone was obtained by multiplying this ratio by a factor of 100 as depicted in the formulas below:

$$\text{Colour zone preference (\%)} = [(TDM \text{ radial zone}) / (TDM \text{ whole apparatus})] \times 100$$

$$\text{Central zone preference (\%)} = [(\text{TDM central zone})/(\text{TDM whole apparatus})] \times 100$$

Furthermore, in order to ascertain that preference for a given zone was related to the colour properties of the open field apparatus and not to the spatial properties of the room, we also performed these calculations for the standard open field, which was colourless. We predicted a random pattern of exploration (chance level is set at 14.28% per zone) with no specific preference for any of the radial zones (which would correspond to the colour zones in the colour-enriched open field) the standard open field. Such results would allow us to rule out biases in data interpretation related to spatial properties of the room.

5) Number of visits per zone: To gain further information of the pattern of exploration and zone preference, we also measured the number of entries made to each of the radial zones as well as the central zone for both type of open field apparatuses.

6) Latency to leave the start location measured in seconds (s): We measured the amount of time required to leave the central zone and initiate exploration of any of the radial zones. This measure was used to assess eagerness or hesitation to explore an environment that varies in its degree of complexity (i.e. presence or absence of colours).

7) Freezing behaviour: The time spent (%) in immobility over the total duration of the test was measured as an index of anxiety-like behaviour [298,369,371]. 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:

$$\text{Freezing (\%)} = [(\text{duration of freezing behaviour (s)})/\text{total duration of the test (i.e. 900s)}] \times 100$$

Statistical Analysis

Statistical analyses and graphs were performed using GraphPad Prism version 5 for MAC OS X (GraphPad Software, San Diego California USA). All data was 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 field environments on total activity level (Figure 6.2C), centre avoidance (Figure 6.3A-D), latency to begin exploration (Figure 6.5A), and freezing behaviour (Figure 6.5B). One-way ANOVA test

was performed to analyse habituation of locomotor activity over 5 min-time blocks (Figure 6.2 A and B). A Dunnett's post hoc test was used to analyse multiple comparisons. Two-way ANOVA analyses with OPEN FIELD type (i.e. standard open field and colour-enriched open field) 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 zone preference (Figure 6.3 E and F) as well as frequency of visits/colour zone (Figure 6.4). Significant main effects were further decomposed using pair-wise comparisons with a Bonferroni's correction, for multiple comparisons. Data are presented as mean $\pm$ SEM, and a probability level of 5% was used as the minimal criterion of significance.

Results

Temporal Patterns of Locomotor Activity are Differentially Influenced by Varying Degrees of Environmental Complexity

Standard open field (Figure 6.2A): one-way ANOVA analysis for repeated measures reveals no main effect of TIME BLOCKS [$F_{(2, 132)}=1.99$, $p=0.1396$]. These results show that levels of locomotor activity did not vary over time (time blocks of 5 min).

Colour-enriched open field (Figure 6.2B). One-way ANOVA analysis for repeated measures reveals a significant main effect of TIME BLOCKS [$F_{(2, 132)}=6.5010$, $p=0.0020$]. Dunnett's post hoc analysis indicated that locomotor activity levels in the second time block (min 6-10) were significantly more elevated than in the first time block (min 0-5) ($p<0.01$). However, habituation of locomotor activity (i.e. significant decreases in locomotor activity between the first and last time blocks) was not observed in any of the environmental contexts tested (i.e. standard and colour-enriched open field).

Total activity level (standard and colour-enriched open fields; Figure 6.2C): Student's T-test (two-tailed) reveals a significant influence of environmental complexity (i.e. presence of colour) on general locomotor activity between the standard open field and the colour-enriched open field [$T_{(88)}= 2.153$, $p=0.0341$]. Specifically, the total activity level was significantly lower in the colour-enriched open field relative to the standard open field.

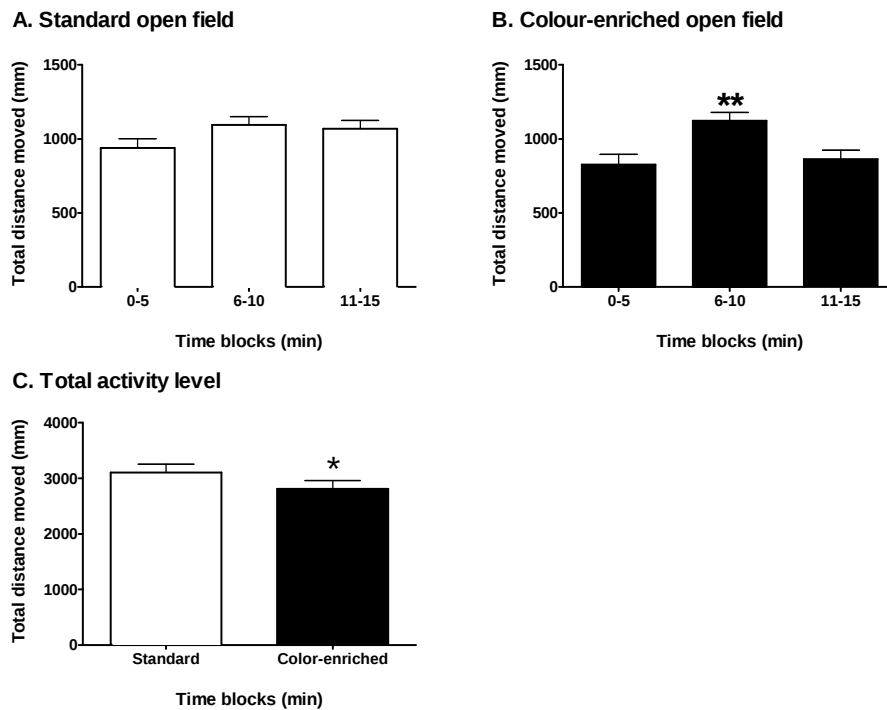


Figure 6.2 **Patterns of locomotor activity.** Analysis of the temporal pattern of locomotor activity (irrespective of zones) shows that while levels of locomotor activity did not vary over time in the standard open field (panel A), locomotor activity levels were particularly elevated in the second time block relative to the other time blocks in the colour-enriched open field (panel B). However, habituation of locomotor activity did not occur in none of the open field types. Panel C shows that the total level of locomotor activity over the duration of the test (15 min) and irrespective of zones was significantly lower in the colour-enriched open field relative to the standard open field. To calculate the statistical power of the experiment with a sample size of 45 we compared the 0-5 and 6-10 pair and the 6-10 and 11-15 pair using the program at <http://www.quantitativeskills.com/sisa>. Using $\alpha=0.05$ and two-sided tests, for the first comparison an absolute difference of 130 in the dependent variable (total distance travelled) corresponds to a statistical power of 80.38%. For the second comparison a difference of 130 gives a power of 89.46%. For a difference of 150 the power increases to 90.12% and 96.01% for the first and second comparison, respectively. If we consider a power of 80% as an acceptable level for these types of experiments, our experiment is suitable for showing differences between treatment of 130 (c.12%) or more. This means that if the real difference is 130 or more we will draw the wrong conclusion (not significant) in less than 20% of the cases. Statistical icons: * $p < 0.05$, ** $p < 0.01$.

Patterns of Zone Preference/Avoidance are Differentially Influenced by Varying Degrees of Environmental Complexity

Centre avoidance (measured as % TDM in outer zone): Centre avoidance, which was measured as the percentage TDM in the outer zone during 15 min in the standard open field, are shown in Figure 6.3A. A student's T-test analysis reveals that zebrafish larvae moved significantly more in the outer zone relative to the inner zone in the standard open field (Figure 6.3A) [$T_{(88)} = 16.32$, $p < 0.001$]. A student's T-test (two-tailed) analysis performed for the colour-enriched open field reveals that similar to what was observed in the standard open field, significantly higher levels of movement were observed in the outer zone relative to the inner zone in the colour-enriched open field (Figure 6.3B) [$T_{(88)} = 2.236$, $p = 0.0279$]. However, preference for the outer zone was much less pronounced than what was observed in the standard open field (Figure 6.3B).

Centre avoidance (measured as percentage time spent in outer zone): Centre avoidance, which were measured as the % time spent in the outer zone during 15 min in the standard open field, are shown in Figure 6.3C. A student's T-test analysis reveals that zebrafish larvae also spent significantly more time the outer zone relative to the inner zone in the standard open field (Figure 6.3C) [$T_{(88)} = 20.85$, $p < 0.0001$]. A student's T-test (two-tailed) analysis performed for the colour-enriched open field reveals that contrarily to what was observed in the standard open field, larvae spent significantly more time in the inner zone relative to the outer zone in the colour-enriched open field (Figure 6.3D) [$T_{(88)} = 4.934$, $p < 0.0001$]. The results show that while larvae tested in the standard open field display the expected pattern of exploration (i.e. low exploration of inner zone and high exploration of the outer zone), larvae tested in the colour-enriched open field display a pattern of exploration that is rather random.

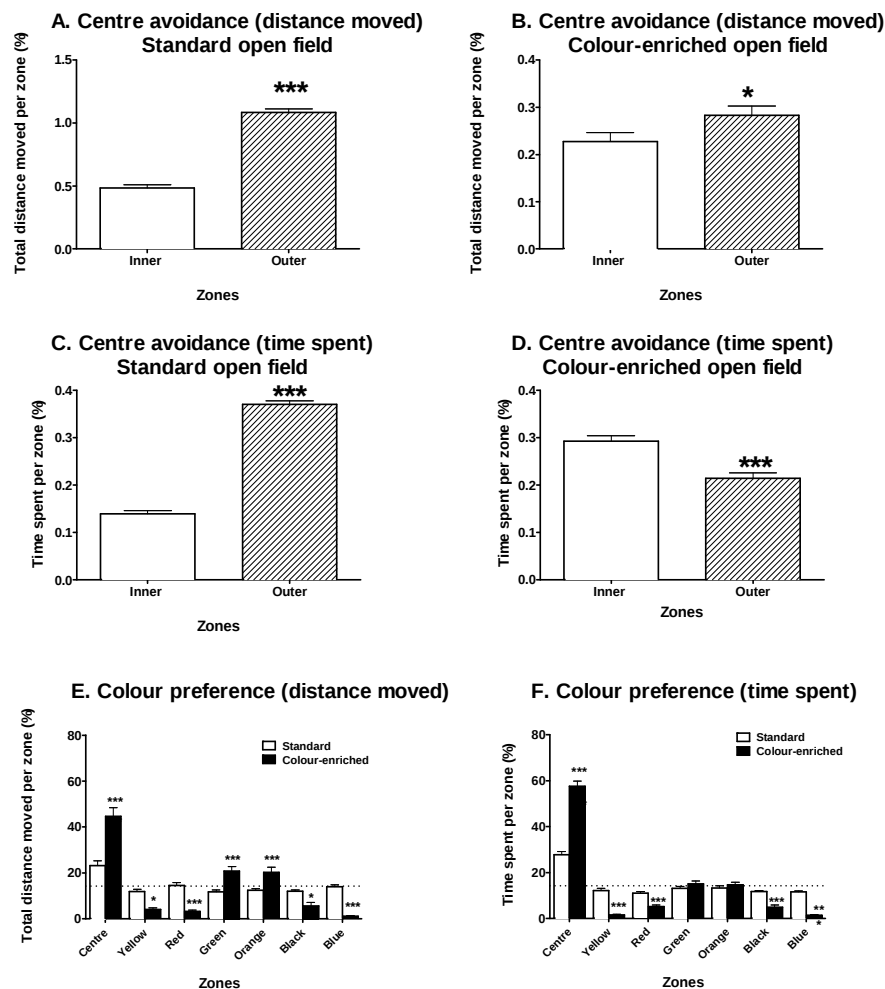


Figure 6.3 **Pattern of zone preference/avoidance.** Zebrafish larvae tested in the standard open field (panel A) displayed the expected pattern of exploratory behaviour by avoiding the centre zone and showing a marked preference for the outer zone, thus engaging in centre avoiding behaviour. Larvae tested in the colour-enriched open field (panel B) only displayed a marginal preference for the outer zone suggesting that the pattern of exploration of both zones (inner and outer) appears to be random. Larvae tested in the standard open field (panel C) also spent significantly more time in the outer zone than in the inner zone. In contrast, larvae tested in the colour-enriched open field (panel D) spent more time in the inner zone than in the outer zone. These findings corroborate the findings shown in panels A and B and reinforce the observation that the patterns of exploration in the colour-enriched environment have significantly deviated from the normally expected patterns of zones (inner and outer) exploration. E) Colour zone preference/avoidance measured as the

% of TDM per zone. Larvae tested in the colour-enriched open field displayed preference for the centre as well as the orange and green zones while avoiding the colours yellow, red, black, and blue. Note that the pattern of exploration of the (colourless) radial zones in the standard open field, which would be the spatial equivalent of the colour zones in the colour-enriched open field, was random (close to chance level set at 14.28%), thus excluding spatial biases. F) Colour zone preference/avoidance measured as the % of time spent per zone. The results largely corroborate the findings in panel E and further support the suggestion that zebrafish larvae are able to discriminate colours and display a natural preference for green and orange as well as avoidance for yellow, red, black and blue.

Colour preference/avoidance as measured by percentage TDM per zone. Colour zone preference/avoidance was assessed by measuring the TDM (%) in each of the 6 radial zones as well as in the central zone (colourless) (Figure 6.3E). A two-way ANOVA analysis reveals a significant interaction between COLOUR ZONES and OPEN FIELD types [$F_{(6, 616)} = 43.95$, $p < 0.0001$]. 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 larvae tested in the standard open field and larvae tested in the coloured-enriched open field. Specifically, larvae tested in the colour-enriched open field moved significantly more in the centre ($p < 0.001$) as well as the orange and green (all $p < 0.001$) zones as compared to larvae tested in the standard open field. However, larvae tested in the colour-enriched open field moved significantly less in the yellow ($p < 0.01$), red ($p < 0.001$), black ($p < 0.05$), and blue ($p < 0.001$) zones compared to larvae tested in the standard open field.

Colour preference/avoidance as measured by percentage of time spent per zone. Colour zone preference/avoidance was also assessed by measuring the time spent (%) in each of the 6 radial zones as well as in the central zone (colourless; Figure 6.3F). A two-way ANOVA analysis reveals a significant interaction between COLOUR ZONES and OPEN FIELD types [$F_{(6, 616)} = 88.67$, $p < 0.0001$]. 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 % time spent per zone between larvae tested in the standard open field and larvae tested in the coloured-enriched open field. Specifically, larvae tested in the colour-enriched open field spent significantly more time in the centre ($p < 0.001$) as compared to larvae tested in the standard open field. However, larvae tested in the colour-enriched open field spent significantly less time in the yellow ($p < 0.001$), red ($p < 0.001$), black ($p < 0.001$), and blue ($p < 0.001$) zones compared to larvae tested in the standard open field. No differences between open field types were observed for the orange and green zones.

Number of entries per zone. The frequency of visits per zone is presented in Figure 6.4. A two-way ANOVA analysis reveals a main effect of COLOUR ZONES [$F_{(6, 511)} = 11.01$, $p < 0.0001$] but no main effect of OPEN FIELD type [$F_{(6, 511)} = 0.02$, $p < 0.9748$]. Bonferroni post hoc analysis indicates that the number of entries to the centre were significantly higher than in any other zones of the test apparatus for both larvae tested in the standard open field and larvae tested in the colour-enriched open field ($p < 0.0001$). Note that there were no significant differences between open field types on the number of entries per zone suggesting that the larvae visited all zones equally, regardless of open field type, and selectively and deliberately avoided or preferred specific zones depending on their colours.

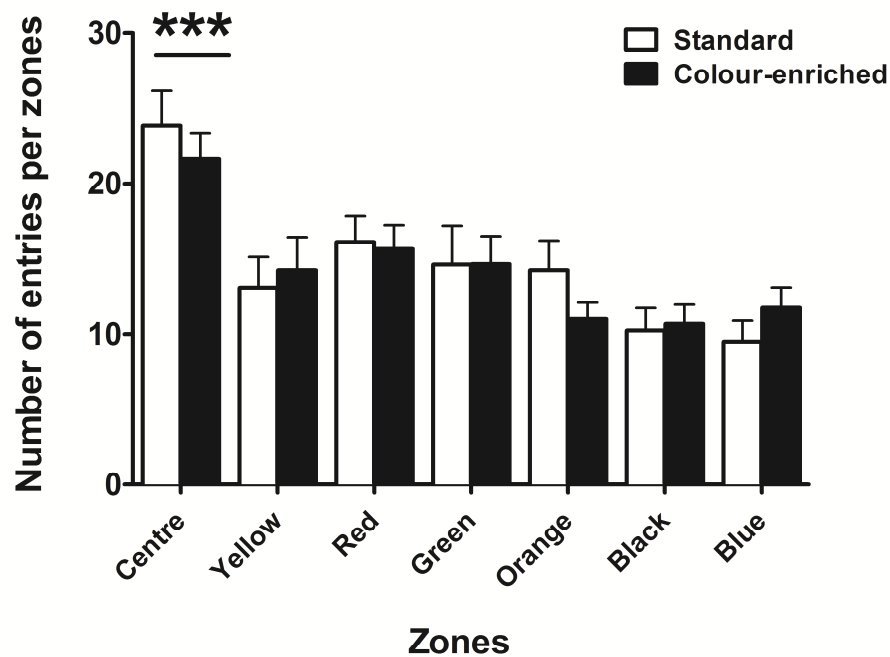


Figure 6.4 **Frequency of visits.** Analysis of the frequency of visits in each of the colour zones shows that regardless of the open field type, larvae visited the centre zone at a higher frequency than the other colour zones, which were otherwise equally visited. These findings show that preference/avoidance for a given zone is the result of voluntarily engaging in more/less exploration (locomotor activity) and spending more/less time within a particular zone.

Environmental Complexity Influences Measures of Anxiety-Like Behaviours

Latency to begin exploration. Onset of radial zone exploration is shown in A. Analysis of the latency to leave the centre zone and visit any of the colour zones was analysed using a student's T-test and revealed that larvae tested in the colour-enriched open field displayed a significantly longer latency to leave the centre zone relative to larvae tested in the standard open field [$T_{(88)} = 5.234$, $p < 0.0001$] (Figure 6.5A).

Freezing behaviour. Time spent (%) in immobility was used as an index of freezing behaviour. The data are presented in Figure 6.5B and analysed using a student's T-test. We report that when compared to larvae tested in the standard open field, larvae tested in the colour-enriched open field displayed significantly more time in state of relatively immobility [$T_{(88)} = 7.549$, $p < 0.0001$].

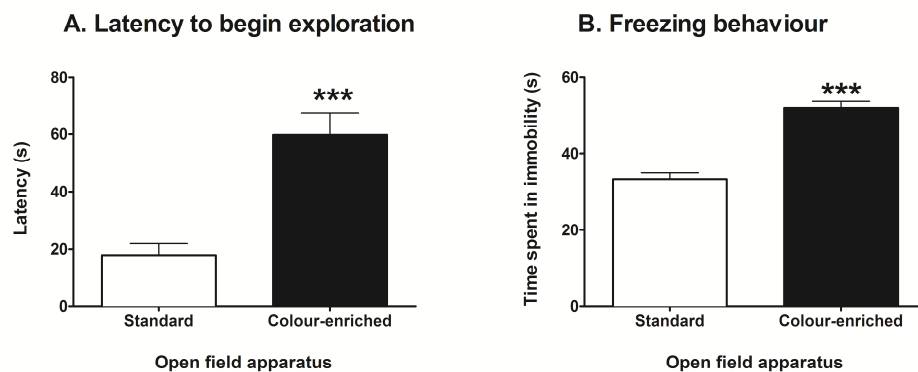


Figure 6.5 **Patterns of anxiety-like behaviours.** A) Analysis of the latency to leave the centre zone to explore any of the radial zones is significantly higher in the colour-enriched open field than in the standard open field. B) Expression of freezing behaviour which represents a physical state where larvae are in almost immobile (except for movement required for respiration) shows that larvae tested in the colour-enriched open field display significantly more freezing behaviour than larvae tested in the standard open field.

Discussion

Development of a Novel Open Field Test for Zebrafish Larvae

Standard open field

The term ‘standard open field’ refers to the most commonly used version of the open field test (mostly in rodents), which typically consists of a forced exposure to a novel and large open space (e.g. square, rectangular, or circular) [369,372,393]. The terms ‘novel’ and ‘large’ are important here since the open field apparatus should be sufficiently different (novel) and considerably larger than the home environment in order to elicit the expression of the typical anxiety-like behaviours usually observed in this test [369]. The walls of the open field apparatus are usually black or white but otherwise colourless, and no objects are present in the test apparatus. The definition of ‘standard open field’ was therefore fulfilled in the current study since the use of a large Petri dish with white walls was sufficiently larger and unfamiliar (i.e. more novel) than the home environment (i.e. well of a 6-well plate). Furthermore, criterion for forced exploration of the open field was also fulfilled since larvae were individually taken from their home environment and forced-placed in the open field apparatus for 15 minutes.

Pattern of exploratory behaviours in the standard open field

We observed that zebrafish larvae as young as 6 dpf displayed exploratory behaviours that are similar to those observed in adult zebrafish [173,394] as well as other species [369,372,373,376,395] in the wild as well as in laboratory settings. Specifically, upon exposure to the standard open field zebrafish larvae central zone rapidly and move close to walls of the field, a behaviour known as thigmotaxis. This behaviour represents the propensity to avoid the centre of an arena and stay or move in the proximity of the boundaries (for instance the walls) of a novel environment [376,395]. Thigmotaxis is a validated index of anxiety since clinically effective anti-anxiety drugs can significantly attenuate its expression [395-397]. In the current study, zebrafish larvae had very short latency to leave the start location (inner zone) to subsequently swim in the peripheral zone (outer zone) of the test apparatus (>75%) for most of the duration of the test (15 min). In the wild, centre avoidance is believed to be adaptive in serving as a strategy to facilitate the search for shelter, protection and/or escape routes [376,395]. However, true thigmotaxis is lacking here due to complexity of the colour-enriched field. Centre avoidance in open field and centre preference in colour-enriched field suggest that zebrafish larvae tend to display thigmotaxic behaviour.

Social separation plays a vital role in the movement and behaviour of the animal in an open field test [279,398,399]. This bias was rectified by random selection of the larvae tested in the standard open field and colour-enriched field to ensure that there is no effect of social interaction.

Habituation learning

Temporal analyses of locomotor activity patterns are also typically extracted from the open field test as measures of habituation learning. Habituation learning represents the simplest form of non-associative learning existing in animal biology [400]. Habituation learning occurs as a result of decrease of exploratory activity as a function of repeated exposure to the same environment [164,400-402]. While we previously showed that adult zebrafish readily (within 5 min) display habituation learning in the open field test [173], an equivalent behavioural pattern is not apparent in zebrafish larvae as young as 6 dpf, at least over a period of 15 minutes. Specifically, detailed analysis of the temporal patterns of locomotor activity over the whole open field apparatus reveals that no significant decrease in levels of locomotor activity was observed over time. The reason for the difference between larval and adult zebrafish is not clear but does not appear to be related to an inability for habituation learning *per se* since previous studies have reported habituation of the acoustic startle response in zebrafish larvae of equivalent age [164,403]. Zebrafish larvae are also able to habituate to repeated visual stimulus of light and dark (unpublished data). Therefore, it is more likely that longer test duration is required for habituation learning to develop in young zebrafish larvae. More studies are required to clarify this issue.

Environmental Complexity Alters Patterns of Exploratory Behaviours Commonly Observed in the Open Field Test

Another important goal of this study was to assess the impact of environmental complexity (e.g. presence of colours) on pattern of exploratory behaviours commonly observed in the standard open field test. The use of colours is ecologically relevant since several species of fish including zebrafish use colours for the purpose of food foraging, conspecific recognition, shoaling, as well as predator avoidance [377,380,404-407]. Here we tested the impact of an array of six colours including yellow, red, green, orange, black, and blue on exploratory and avoidance behaviours. Our interest in using colours as a mean to increase environmental complexity stems from previous studies from our laboratory where we showed that black seems particularly aversive for zebrafish larvae. Specifically we showed

that zebrafish larvae displayed strong dark-avoidance behaviour in the light-dark preference test [374] and engage in thigmotaxis in response to a sudden exposure to darkness (that is not due to nightfall) [408]. In these tests, dark-avoidance behaviour and thigmotaxis serve as indexes of anxiety-like behaviours in larval zebrafish since commonly used anxiolytics and anxiogenics significantly attenuated and increase this behaviour, respectively [374,408]. In the current study, we wanted to extend these findings and investigate whether avoidance behaviours toward other colours also occur, and if so, how exposure to a larger panel of colours affected patterns of exploratory behaviours and anxiety-like behaviours commonly observed in the open field test.

Pattern of exploratory behaviours in the coloured-enriched open field

A differential pattern of exploratory behaviours was observed in larvae tested in the colour-enriched open field relative to those tested in the standard open field. First, we observed that while onset of exploration (i.e. leaving start location) in the standard open field was initiated within a relatively short period of time (i.e. 17 seconds), exploration in the coloured-enriched open field was delayed and began much later (i.e. 60 seconds). Such delay in the onset of exploration may stem from an enhanced risk assessment/appraisal of the neighbouring colour zones that took place in the first time block (0-5 min), thus further supporting the notion that zebrafish larvae can discriminate colours [172,385,391].

Furthermore, the delayed onset of exploration may also underlie the alterations that were observed in the temporal pattern of locomotor activity. Although the total level of overall locomotor activity was significantly reduced in larvae tested in the colour-enriched open field relative to those tested in the standard open field, we observed a significant burst (increase) of locomotor activity in the second time block (min 6-10) relative to the other time blocks. As in the standard open field, habituation learning was not observed in the colour-enriched open field reinforcing the suggestion that a longer period of time is required in order to observe habituation learning in this test in zebrafish larvae.

Centre Avoidance

Interestingly, zebrafish larvae didn't display centre avoidance; rather, they move much of the time in the centre when tested in the colour-enriched open field. Since larvae only expressed a slight preference for the outer zone of the colour-enriched open field, the results suggest that the patterns of exploration of the inner and outer zones appear to be

rather random. These findings indicate that more complex/stressful environments such as the colour-enriched open field appear to influence the natural pattern of zone preference to such an extent that larvae seem to ‘lose’ the commonly observed preference in this test and chose to stay in the centre. This phenomenon is not unprecedented. We previously showed that brief exposure to acute stress also abolishes ‘normal’ zone preference in adult zebrafish exposed to the open field test [173]. This is also in agreement with previous studies showing that centre avoidance or thigmotaxis can be modified by stressful and anxiety-provoking contexts [376,395].

Colour discrimination and natural colour preference/avoidance

Another goal of the current study was to determine whether zebrafish larvae could discriminate colours, and if so, to assess what pattern of preference/avoidance towards specific colours were prevalent. Ability for colour discrimination as well as preference/avoidance towards certain colours has been shown in adult zebrafish [377,379-381] as well as in other fish species [382] but has not been studied yet in larval zebrafish. Nevertheless previous reports suggest that larval zebrafish are capable of colour discrimination [172,385,391]. This is further supported by the demonstration that larval zebrafish have a well-developed visual system including all four types of cones and rod photoreceptors by the age of 6 dpf [172,385,391]. In agreement with these reports, the findings reported in the present study also support the contention that zebrafish larvae have the capacity for colour vision and discrimination. We also report that the natural pattern of colour preference/avoidance among a choice of 6 colours consists of avoidance towards yellow, red, black, and blue and preference towards green and orange.

Ruling out spatial biases

It is noteworthy that except for the exploration of the centre zone, the pattern of exploration of the radial zones in the standard open field was random (i.e. around chance level set at 14.28%) as expected. These findings allow us to rule out any spatial biases related to the pattern of exploration of the colour zones in the colour-enriched open field. Therefore, we can assume that the differential patterns of exploration we observed here are related to the colour properties of the colour-enriched open field rather than to spatial properties of the testing room. Therefore, we can assume on the basis of our results that larval zebrafish as young as 6 dpf display a natural preference for green and orange as well as specific avoidance of yellow, red, black, and blue.

Comparison with adult zebrafish behaviour

Previous studies of natural colour preference/avoidance in adult zebrafish have reported preference towards red and aversion towards blue colours [377,379]. The results presented in the current study contribute to extend these findings to larval zebrafish, where a clear preference for green and orange combined with avoidance behaviour towards yellow, red, blue, and black was observed. While our findings of an aversion towards blue [377,379] and black [173,374] are in agreement with previous reports, surprisingly we did not observe the expected bias towards red in the present study. However, zebrafish is known to learn to approach blue significantly faster than red when escaping from a moving net [278] or using a food reward [409], considering this blue facilitation as an "unconditioned bias for shorter wavelengths, found also in other several species like goldfish [410], anuran tadpoles [411], turtles [412]. However the short wavelength bias can be modified, at least temporarily, by pairing the less preferred alternative with food[409].

The reasons underlying this discrepancy are not clear but are likely related to ontogeny differences and foraging habits of this species. For instance contrary to what is commonly observed in several other fish species, bias toward red is only relevant for foraging but not in mate choice in zebrafish [377,379,380,382,406,407]. Specifically, the colour red represents an important cue in foraging given that the main component of the zebrafish diet comprises organisms rich in carotenoids (red colour pigments). The lack of a bias towards red observed in the current study might have occurred as a result of insufficient early-life exposure to nutrients rich in red pigments. Since the larvae (6 dpf) used in the current study were still dependent on their yolk sac for nutrition, larvae were not fed with any exogenous source of food prior and during the experiment. This action may have prevented any associative learning between red (or any other colours) and foraging, resulting in avoidance rather than preference towards red. In the case where preference towards red would be 'hard wired' rather than resulting from associative learning, immature circuitry would be likely to underlie the lack of natural bias towards red. In any cases, the results presented in this study are not necessarily in disagreement with that of other studies [377,379,380] but rather suggest that a red bias is likely to develop as a result of experiencing food enriched in red pigments or simply will emerge at a later life stage. Further studies are required to clarify this issue.

Another possible cause underlying the discrepancy in results could be related to the methodologies used between the different laboratories to assess innate/natural colour preference. For instance method using place preference tests, which involve choosing a

colour from a set of two colours, may lead to false positive or negative results. Specifically, as stated in Avdesh and colleagues [380], a choice for a given colour could be due to either hedonic properties of one colour or aversive properties of the other colour. For instance, two colours could both be aversive, but one less so than the other resulting in a 'false' colour preference. Inclusion of a neutral zone (colourless) among the possible choices ameliorates this problem, as demonstrated by the use of a T-maze test by Avdesh and colleagues [380]. Based on that principle, we believe that the methodology used in our study, which included a larger choice of colours as well as a neutral (colourless) choice, might be even more sensitive than previous methods.

Finally, relatively very little is known regarding patterns of natural preference/avoidance towards yellow, orange and green although one study reported that yellow was less preferred than green when tested in a two-colour choice context [380]. The significance of these results is not clear and difficult to compare to the current set of findings. To our knowledge, preference for orange and green have not been reported before. We suggest that preference for these colours might also be related to the foraging habits (e.g. orange related to diet rich in carotenoids and microcrustaceans) [406,407,413] as well as the biological niche of aquatic species (e.g. green related to algae and other aquatic vegetation) [407]. More studies are required to clarify these issues.

More Complex Environments are Challenging and Enhance the Expression of Anxiety-Like Behaviours

Overall, the findings presented in the current study suggest that the colour-enriched open field, which contains at least two colours known to be aversive to zebrafish (i.e. blue and black [173,374,377,379,380]), may be perceived by larval zebrafish as a more stressful/anxiety-provoking environment than the standard open field. Evidence supporting this notion comes from our findings that exposure to the colour-enriched open field appears to exert inhibitory influences, at least to some extent, on exploratory function as shown by reduced overall locomotor activity in the colour-enriched. These findings are also in agreement with previous studies in rodent models showing that diverse type of stressful/aversive environments are associated with overall inhibition of exploratory behaviour (i.e. reduced locomotor activity) [369,371,372]. We also show that exposure to the colour-enriched open field is associated with increased freezing behaviour (a well-known index of anxiety-like behaviour) [298,369,371,372,395,414], delayed onset of exploration (likely due to an enhanced risk assessment/appraisal of the situation), disrupted temporal patterns of locomotor activity, and highly attenuated preference for a given zone

of the test apparatus (i.e. outer zone) that is typically preferred in this test [369,371,372,395]. These findings are in line with previous findings from our laboratory showing that brief acute stress prior exposure to an open field disrupt ‘normal’ pattern of zone preference. Taken together, these findings support our claim that the colour-enriched open field represents indeed a stressful/challenging context relative to the standard open field [173].

Concluding Remarks

The findings presented here confirm that exposure to unfamiliar/ novel environment experienced in the standard open field tests can evoke relatively simple and robust anxiety-like behavioural responses in zebrafish such as centre avoidance. Addition of choice of colours, among which yellow, red, blue, and black appear to be particularly aversive to zebrafish larvae enhances the anxiety-provoking nature of the open field test. However, pharmacological manipulations using commonly used anxiolytic and anxiogenic drugs remain to be performed to ascertain the anxiety-like nature of the behaviours observed in the standard open field (centre avoidance) and colour-enriched-open field (avoidance towards yellow, red, blue, and black).

In the current study, we not only developed and adapted a traditional rodent behavioural assay that serves as a gold standard in preclinical drug screening but we also provide a version of the same test that affords the possibility to investigate the impact of environmental stress on behaviour in larval zebrafish while being the first assay for assessment of colour discrimination and pattern of natural colour preference/avoidance in larval zebrafish. Exposure to environmental stress and behavioural paradigms are often combined in the field of stress research for several purposes. For instance, exposure to stress has been used to reveal the function of particular genes or to enhance/attenuate the expression of certain behaviours, magnify (or enhance the chance to uncover) properties of a given drug, or to simply document behavioural stress responses of a given individual. Given the outstanding advantages related to the use of zebrafish embryo for state-of-the-art genetic studies, the combination with the behavioural assays developed here will certainly contribute in the future to advance knowledge on molecular mechanisms underlying behaviour stress and anxiety state (elicited by colour aversion) as well as learning and memory (related to colour discrimination and behavioural conditioning) relevant to drug discovery.

In sum, the study provides a detailed method amenable to automation and immediately applicable to stress and cognition research using zebrafish models. In the future, zebrafish models will improve preclinical drug screening methodologies towards the goal to uncover novel effective, neuroactive drugs.

