A Unified Approach to Investigating 4 dpf Zebrafish Larval Behaviour through a Standardised Light/Dark Assay

Courtney Hillman[1], James Kearn[2], and Matthew O. Parker[1,3]

[1]Surrey Sleep Research Centre, University of Surrey, Guildford, UK

[2]Defence Science and Technology Laboratory (DSTL), UK

[3]School of Pharmacy and Biomedical Sciences, University of Portsmouth, Portsmouth, UK

Author note

Correspondence concerning this article should be addressed to Matthew O. Parker, PhD. Surrey Sleep Research Centre, University of Surrey, Guildford, GU2 7XH, UK. Email: matthew.parker@surrey.ac.uk, phone number: +44 7986 205349

Abstract

Zebrafish have emerged as a dynamic research model in the domains of psychiatry, neuroscience and behaviour. Working with larvae ≤ 4 days post-fertilisation (dpf) offers an avenue for high-throughput investigation whilst aligning with the 3Rs principles of animal research. The light/dark assay, which is the most used behavioural assay for larval research, lacks experimental reliability and standardisation. This study aimed to formulate a robust, reproducible and standardised light/dark behavioural assay using 4 dpf zebrafish larvae. Considerable between-batch and inter-individual variability was found which we rectified with a normalisation approach to ensure a reliable foundation for analysis. We then identified that 5-minute light/dark transition periods are optimal for locomotor activity. No discernible impact of acclimation (length or lighting conditions) on larval performance was observed. Overall locomotion was found to be significantly greater in the early afternoon compared to morning and evening, but time of day of testing had no impact on light/dark transitions. Next, we confirmed the pharmacological predictivity of the standardised assay using ethanol which, as predicted, caused hyperlocomotion at low concentrations and hypolocomotion at high concentrations. Finally, the assay was validated by assessing the behavioural phenotype of hyperactive transgenic (*adgrl3.1*$^{-/-}$) larvae, which was rescued with psychostimulant medications. Our standardised assay not only provides a clear experimental and analytical framework to work with 4 dpf larvae, but also facilitates between-laboratory collaboration using our normalisation approach.

Keywords: Zebrafish, light/dark transition test, behaviour, standardised testing, locomotor activity, 3Rs of animal research

Introduction

Zebrafish (*Danio rerio*) larvae are an increasingly popular research model with a breadth of application and tractability for toxicological and pharmacological research (Cassar et al., 2020). Due to their rapid breeding and small size, working with larvae provides potential for high-throughput approaches, with the added benefit that larvae up to 5 days post-fertilisation (dpf) are exempt from protection by national legislation (e.g., the (Animals (Scientific Procedures) Act 1986) 1986) in the United Kingdom (UK) and the European Union (EU) Directive 2010/63/EU of the European Parliament). Hence, 4 dpf larval research has significant interest for meeting the criteria of the 3Rs (replacement, refinement and reduction) of *in vivo* animal research (Crilly et al., 2018). Indeed, as a first-tier model, 4 dpf zebrafish larvae can reduce the number of protected animals required at later testing stages as well as minimising the suffering of experimental animals when testing toxic substances. Therefore, from a 3Rs perspective, zebrafish larvae are a unique and appealing research model.

The light/dark assay remains the most used for studying behaviour in zebrafish larvae due to its cost and time-effectiveness as well as its high face and pharmacological predictivity (Haigis et al., 2022). The protocol manipulates the innate tendency for larvae to display hyperactivity following a sudden change from light to dark conditions (Bachour et al., 2020). Some have suggested that this locomotor response represents a stress or alarm response, and it has been used for modelling anxiety-like phenotypes in larvae (Rock et al., 2022; Steenbergen et al., 2012). Its popularity stems from its ease of manipulation for toxicity and drug analysis and the robust behavioural response observed (Haigis et al., 2022). As seen in Table 1, there is, however, a lack of consistency between light/dark assay experimental protocols in 4 dpf larvae. The lack of experimental consistency can impact the behavioural responses observed and hence the reproducibility of results between laboratories and researchers (Pesavento et al., 2022). This lack of standardised testing and variability in protocols has been highlighted previously by Legradi (2015). However, no standardised or reproducible assay has been published to date.

Table 1
List Of Previous Publications Using 4 dpf Zebrafish Larvae and The Different light dark Parameters Used.

Author and year of study	Age of larvae (dpf)	Acclimation period	Transition period
Y. Wang, 2023	4	15 min in light	10 min light to dark x 3 (60 min total)
Okeke, 2022	4	10 min in dark	10-min light to dark to light x1 (30 min total)

Scarlett, 2022	4	10 min in dark	10-min light to dark x 2 (40 min total)
Mundy, 2021	4	5 min in light	5-min light to dark to light followed by 15-min dark x1 (30 min in total)
Hasumi, 2020	4-5	N/A	15-min light to dark x 6 (180 min total)
Basnet, 2019	4-5	30 min in dark	10-min light to dark x 6 (120 min)
Walter, 2019	3 – 5	10 min in light	5-min dark to 5-min light to 15-minute dark (25 min total)
Best, 2017	4	Overnight acclimation in dark + 1 hour	7.5-min light to dark x8 (60 min total)
Fraser, 2017	4-5	20 min in light	10-min dark to light x1 (20 min total)
Liang, 2017	4	840 min in light	10-min dark to light x4.5 (50 min total)
Thit, 2017	4	N/A	5-min dark to light x 9 (90 min total)
Liu, 2015	3 – 9	210 min in dark	30-min light to dark x 3 (180 min total)
Zhao, 2014	4 – 6	20 min in dark	10-min in dark into 10-min light to dark x 3 (70 min total)
Beker van Woudenberg, 2013	4	15 min in light	15-min dark to light to dark (45 min total)
Padilla, 2011	4, 5 and 6	20 min in dark	10-min light to dark x 2
Jin, 2010	4	20 min in dark	10-min light to dark x 3.5 (70 min in total)

Key; dpf: days post-fertilisation, min: minutes.

Here, we aimed to develop a standardised robust and reproducible light/dark behavioural assay using 4 dpf zebrafish larvae and to validate this assay using pharmacological and genetic manipulations. After assessing different parameters to determine the optimal experimental conditions, we validated the assay pharmacologically in two-ways. Wild-type embryos were exposed to ethanol and genetically modified (*adgrl3.1* knock-out *(adgrl3.1$^{-/-}$))* larvae were used to study behavioural interventions of two Attention-Deficit Hyperactivity Disorder (ADHD) drugs. Ethanol was selected due to its well-known ability to induce low concentration hyperactivity and high concentration hypoactivity, which we postulated would impact the dark phase responses (McLellan, 2017). Furthermore, it is widely available and is commonly used in larval light/dark assay research (Davis et al., 2021; Irons et al., 2010; MacPhail et al., 2009). The *adgrl3.1$^{-/-}$* mutant line demonstrates phenotypes typical of ADHD and therefore we hypothesised that the larvae would display hyperactivity in the dark phase (Sveinsdóttir et al., 2023) which would be rescued/reversed with methylphenidate (MPH), a standard stimulant drug used to treat ADHD. We also assessed atomoxetine (ATO), another common ADHD drug known to induce sedation in patients, which we postulated would result in hypoactivity (Bastiaens, 2007). In addition to the validation, we also aimed to provide a consolidated method of analysis and interpretation of the behavioural data, including radar

charts and heat maps of different swim patterns, which will be of use to the zebrafish research community.

Materials and Methods

Animal husbandry

AB wild-type (WT) zebrafish were bred in-house from the University of Portsmouth colony. Breeding adults in the colony were housed in ~50:50 male:female groups of 8-10 per 2.8 L tank, or up to 20 per 6 L tank, on a re-circulating system (Aquaneering, Fairfield Controlec Ltd., Grimsby, UK). Sample sizes (~n = 16) were calculated based on power analyses from effect sizes observed in our pilot studies. *adgrl3.1*$^{-/-}$ larvae were used for the genetic phenotype experiment. The *adgrl3.1*$^{-/-}$ line was created by CRISPR-Cas9 genome engineering as previously described (Sveinsdóttir et al., 2023) and maintained via homozygous in-cross at the University of Portsmouth fish facility (with matched-age WT [AB] controls). Room and tank water temperatures were maintained at 25-27°C, pH 8.4 (±0.4), on a 14:10-hour light/dark cycle with the lights switched on at 08:30. Fish were fed on ZM fry food from 5 dpf until adulthood when they were moved onto a diet of flake food and live brine shrimp 3 times/day (once/day on weekends). Larvae were bred for experimentation either by adding marbles to the base of the tank overnight (to induce spawning) or by pair breeding. Larvae were reared in a translucent incubator (28.5^{o}C) in the fish facility until the day of testing, where they were moved into our behavioural testing room and placed in a small countertop incubator at 28°C. On completion of behavioural testing, larvae were culled using Aqua-Sed (Aqua-Sed™, Vetark, Winchester, UK) in accordance with manufacturer guidelines. All experiments were approved by the University of Portsmouth Animal Welfare and Ethical Review Board. Genetically altered fish were used under license from the UK Home Office (Animals (Scientific Procedures) Act, 1986, Licence number PP8708123).

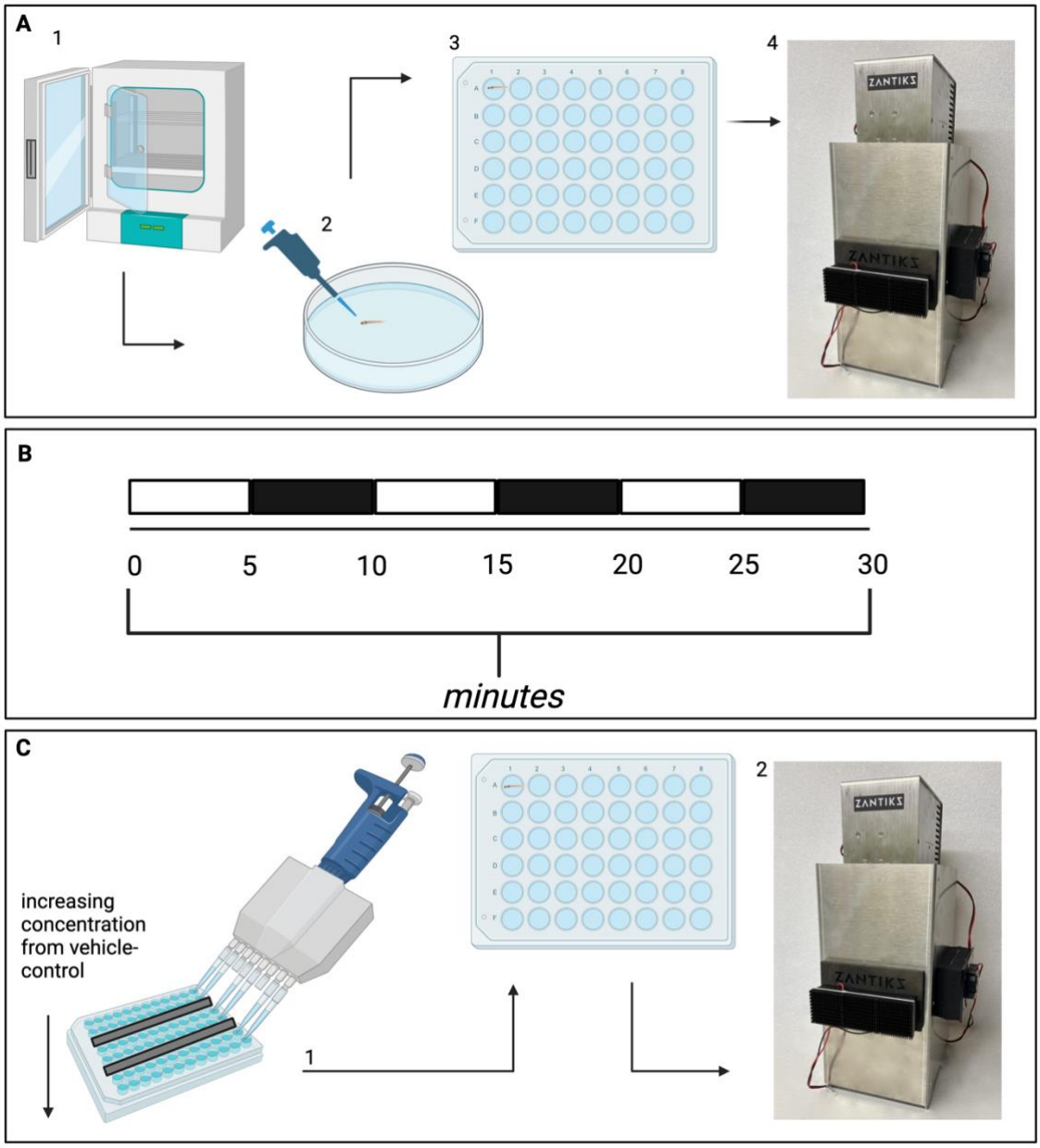
Behavioural apparatus

All behavioural testing was carried out between 09:00 and 18:00 (Mon-Fri) in Zantiks MWP units (Zantiks Ltd, Cambridge, UK). Previous literature determined that 48-well plates are the optimal format for consistent and robust behavioural responses and high-throughput analysis (Widrick et al., 2023). However, our preliminary studies suggest any well-plate size is suitable *(see SD 1).* Therefore, with respect to previous literature and both high-throughput and time-efficiency, all behavioural testing took place using 48 well plates. Filming was carried

out directly from above the plates, which allowed live monitoring within the behavioural system.

Figure 1.

The experimental protocol for the standardised light/dark assay including drug exposure procedures



Note. (A) the fish are transferred from the incubator to a 48 well plate and placed in the behavioural unit with the lights on for 30-minutes of acclimation prior to the recording. (B) Breakdown of the light/dark recording which incorporates 5-minute light phase to dark phase transitions for 30-minutes. (C) Overview of drug exposure procedure from a 96-well dosing plate to the fish. Key; L/D: Light/Dark. All figures were created with Biorender.com.

Standardisation of Light/Dark Assay

A breakdown of the experimental procedure is seen in Figure 1. At 08:30 the larvae Petri dishes were transferred into an incubator in our behavioural testing room at 28°c to allow the fish to acclimate to the new environment for 30 minutes, prior to experimentation. 225 µL of aquarium water and a randomly selected larva were transferred into each well of a 48-well plate using trimmed pipette tips (~ 2 mm removed from the tip end of a 1000 µL pipette tip). The well plate was transferred to the Zantiks MWP unit, and the overhead lights in the unit were switched on for 30-minutes for acclimation (Fig. 1A). Following acclimation, the light/dark tracking script was run for a 30-minute baseline recording. This comprised of three sets of five-minute light phase (white light, 350 lux) to dark phase transitions (Fig. 1B). The MWP unit was temperature controlled throughout experimentation at 28.02^{o}c ± 0.07.

The Light/Dark Assay for Chemical Exposures

The drug exposure protocol incorporated the standardised protocol (*Section 2.3 of this paper*). Following the baseline recording, the plate was removed from the MWP, and a multi-channel pipette was used to administer 25 µL of different ethanol concentrations *(CAS number: 64-17-5, tested at 0.5 – 4.0 %)* from a 96-well plate to achieve the desired final exposure concentration (Fig.1C). The 48-well plate was returned to the unit and the larvae were tracked during the light/dark alternations (Fig 1B).

The Light/Dark Assay for Genomic Phenotype Evaluation

Assays with adgrl3.1$^{-/-}$ larvae incorporated the standardised protocol and both WT and adgrl3.1$^{-/-}$ larvae were used (see Section 2.3). Following the baseline recording, the plate was removed, and a multi-channel pipette was used to administer 0.05 – 20 µM MPH *(CAS number: 298-59-9) and* 2.93 - 10.2 µM ATO *(CAS number: 82248-59-7)* (Fig. 1C). The 48-well plate was returned to the unit and the larvae were subjected to the light/dark protocol (Fig 1B).

Optimisation of the Light/Dark Assay

Alterations in a range of parameters were investigated as part of the standardisation of the light/dark assay. This included assessing previously published protocols (Acevedo-Canabal et al., 2019; Bachour et al., 2020; Haigis et al., 2022), for determining the effect of altering the length of light/dark transition periods. The length of acclimation period and lighting conditions were assessed as well as the effect of the time of day upon behavioural responses.

Statistics

The data files were imported into Excel for data processing and analysis (Microsoft Excel, 2019) where they were normalised to baseline to overcome variability between laboratories in the same facility, between different facilities and larvae batch variability (*see SD2*). Normalisation was carried out by determining the percentage of movement compared to the overall baseline mean response of all the larvae. Normalised data was imported into GraphPad Prism (GraphPad Prism version 9.0.0 for Windows, GraphPad Software, San Diego, California USA). All graphs, figures and analyses were performed with GraphPad software, except the radar charts which were built in Excel. Data was assessed for distribution using the Shapiro-Wilk test and Levine's test for homogeneity. If the data was normally distributed, statistically significant differences were assessed using a two-way analysis of variance (ANOVA) with light/dark phase and concentration or strain of fish as the variables assessed using a Dunnett's multiple comparison test as a post-hoc test. A one-way ANOVA was used where appropriate, and the Tukey's multiple comparison test was employed. Non-normally distributed data was analysed using the non-parametric Kruskal-Wallis and a Dunn's multiple comparison post-hoc test. Post-hoc data is reported in the figure legends. The significance values are $p^{****} < 0.0001$, $p^{***} < 0.001$, $p^{**} < 0.01$, $p^{*} < 0.05$.

Radar charts and heat maps were produced by adapting an approach described by Haigis (2022) and Steele (2018). Briefly, using Microsoft Excel, the percentage of baseline data was analysed as either darting (>2000 %/s), steady swimming (690 – 2000 %/s), slow swimming (0.1 - 690 %/s) or zero (0 %/s) movements, the values of which were adapted from Haigis (2022), Steele (2018) and Scarlett (2022) according to relative movement in our experiments. Each observation had an addition of one to overcome the limitations of percentage determination using a zero response. The endpoints evaluated were total movements, darting swimming movements, steady swimming movements, slow swimming movements and the absence of movements (zero movements). The mean response for each of these observations was calculated for controls. The percentage of movement compared to vehicle-controls was calculated for each drug concentration and a base 10 logarithmic conversion was performed. The radar charts were created in Microsoft Excel and the heat maps were produced in GraphPad Prism.

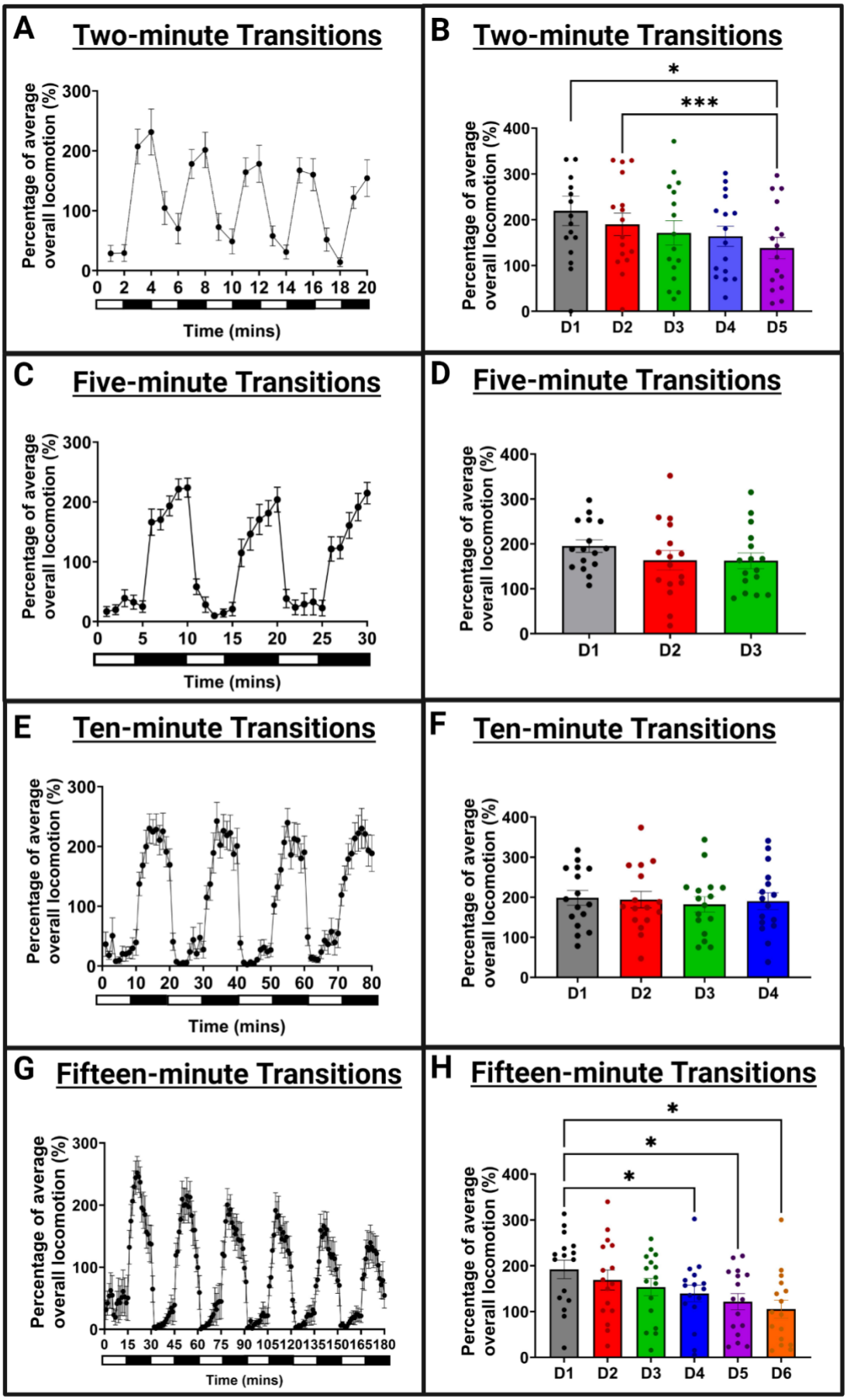
Results

Five-minute light/dark protocol produces a robust response in 4 dpf zebrafish larvae allowing for high throughout behavioural analysis

Previous publications have lacked consistency with respect to the length and number of light/dark transition periods for assays using 4 dpf zebrafish larvae (Table 1). Therefore, we assessed the optimal length of light to dark transition periods by replicating the protocols employed by Acevedo-Canabal (2019), Bachour (2020) and Haigis (2022), as well as assessing our own protocol. There were reproducible and robust dark phase locomotor responses with all transition periods. In addition, the five-minute (one-way ANOVA: $F_{(2, 45)} = 1.068$, $p = 0.3521$) and ten-minute transition (One-way ANOVA, $F_{(3, 60)} = 0.1225$, $p = 0.9465$) protocols, had no significant change in locomotion across successive dark phases (Fig. 2D and 2F). In both the two-minute (one-way ANOVA: $F_{(1.813, 27.20)} = 7.896$, $p = 0.0026$) and fifteen-minute transition (One-way ANOVA, $F_{(3.914, 58.71)} = 5.970$, $p = 0.0005$) protocols, however, we observed a significant reduction in locomotion across successive dark phases (Fig. 2B and 2H). With the two-minute transition period, locomotion in dark phase five and six is significantly less than dark phase one (Fig. 2B). Similarly, with the 15-minute transition period, dark phase four, five, and six locomotion are all significantly less than dark phase one (Fig. 2H).

Figure 2

Effect of varying light and dark phase length on larval behavioural response

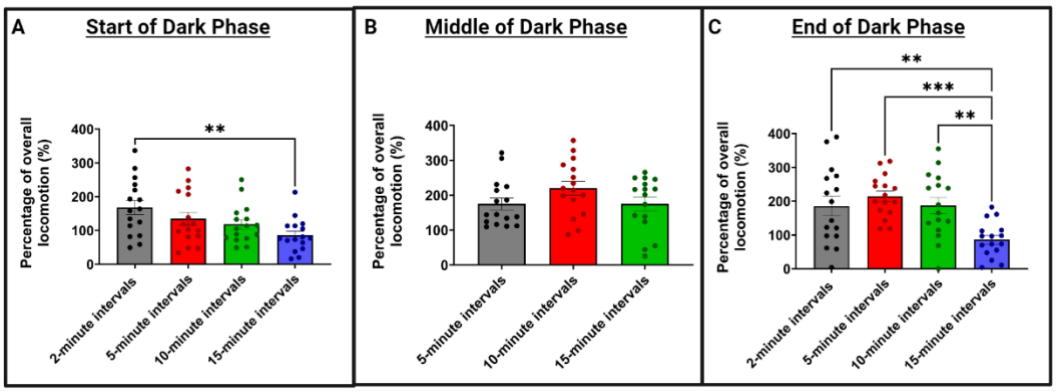

A
Two-minute Transitions
Percentage of average overall locomotion (%)
Time (mins)
B
Two-minute Transitions
*
***
D1
D2
D3
D4
D5
C
Five-minute Transitions
D
Five-minute Transitions
E
Ten-minute Transitions
F
Ten-minute Transitions
D6
G
Fifteen-minute Transitions
H
Fifteen-minute Transitions

Note. Light/dark locomotion displayed as a percentage of baseline response are represented in minutes for (A) two-minute transitions (C) five-minute transitions (E) ten-minute transitions and (G) fifteen-minute transitions. The mean dark phase responses are numbered as D1-D6 for (B) two-minute transitions, (D) five-minute transitions, (F) ten-minute transitions and (H) fifteen-minute transitions. Tukey's multiple comparison test for (B) shows significant reduction in locomotion for D5 compared to D1 (p = 0.00115) and for D5 compared to D2 (p = 0.0006). Tukey's multiple comparison test confirmed significant reduction in locomotion for (H) for D4-6 and D1 (p = 0.0346, p = 0.0235 and p = 0.0140, respectively). Data is represented as mean ± SEM. A,C,E G dots represent averaged responses, B, D, F, H dots represent individual fish responses.

We next looked in detail at locomotion within the dark phases, and calculated the locomotion within the first minute, half-way through, and during the final minute of the dark phase (Fig. 3) (n.b., the two-minute period was only sufficient for a single dark phase analysis). There was a significantly reduced initial dark phase locomotion during the fifteen-minute protocol compared to the two-minute transition period (one-way ANOVA: $F_{(3, 60)} = 4.002$, p = 0.0115) (Fig. 3A). No significant difference in locomotion is seen half-way through the dark phase regardless of length of transition period (one-way ANOVA: $F_{(2, 45)} = 1.909$, p = 0.1600) (Fig. 3B). However, by the end of the recording period, the two-minute, five-minute and 10-minute locomotion's are significantly greater than the fifteen-minute protocol (one-way ANOVA: $F_{(3, 60)} = 7.021$, p = 0.0004) (Fig. 3C).

Figure 3

Time-dependent effects of varying light and dark phase length on larval behavioural responses



Note. Locomotion displayed as a percentage of baseline response represented as mean dark phase responses for (A) the first minute of each dark phase (B) the median minute of each

dark phase and (C) the final minute of each dark phase for the two-minute, five-minute, ten-minute and fifteen-minute transition protocols. Tukey's multiple comparison test showed significant reduction in start of dark phase locomotion for the two-minute versus fifteen-minute protocol (p = 0.0064). Significant reduction in locomotion was shown by the end of the dark phase for the two-minute, five-minute and ten-minute versus fifteen-minute protocol (p = 0.0087, p = 0.0004, p = 0.0071, respectively). Data is represented as mean ± SEM. Dots represent individual fish responses.

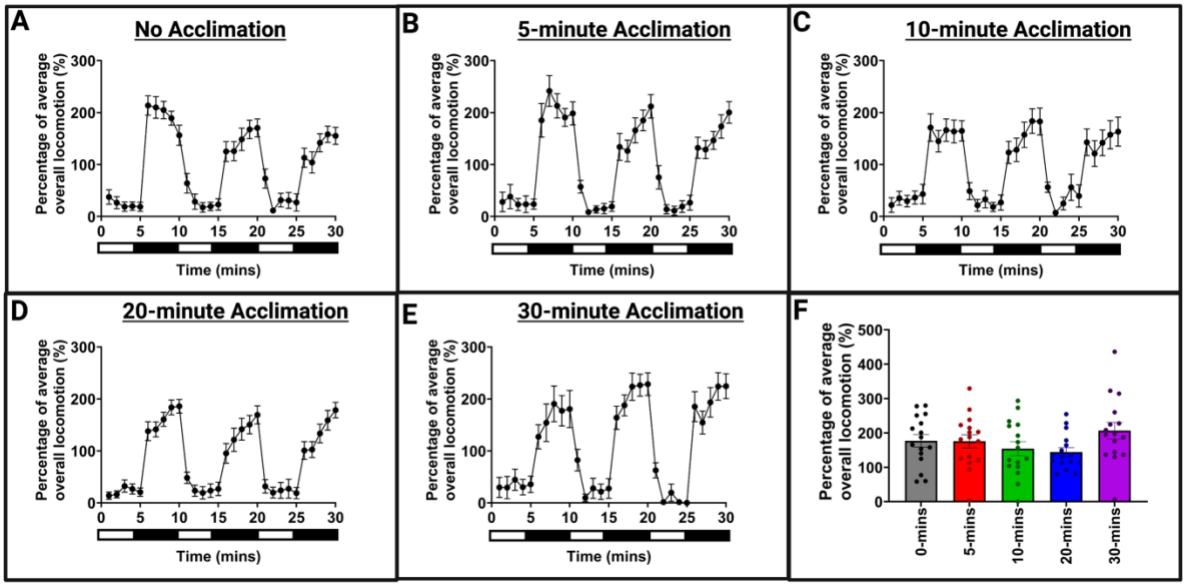
In view of the sustained increased locomotion throughout the recording period with the five-minute transition protocol, and relatively higher throughput potential, we proceeded with further testing using the five-minute transition protocol.

The acclimation period does not affect locomotor behavioural responses in the larval light/dark assay

The importance of both the conditions and length of acclimation period used prior to behavioural evaluation with fish has been previously reported (Makaras et al., 2021). Larval light/dark research lacks standardisation of the length and lighting conditions of the acclimation period (Table 1). The effects of the length and lighting conditions of acclimation within the behavioural monitoring unit on light/dark assay locomotor responses were addressed by comparing no acclimation, five-minute, ten-minute, 20-minute or 30-minute acclimation periods (Fig. 4), as well as varying the lighting conditions (Fig. 5). Our findings in Figure 4 show no significant effect of acclimation period length on locomotor responses (one-way ANOVA: $F_{(4, 75)} = 1.537$, $p = 0.2001$). Despite the lack of difference between the subsequent light/dark response with differing acclimation periods, we chose a 30-minute acclimation period for all further experimentation as this fitted with our experimental schedule; however, our data suggests that if labs use shorter/longer times this should have little impact on their data.

Figure 4

The light/dark response is unaffected by acclimation period length

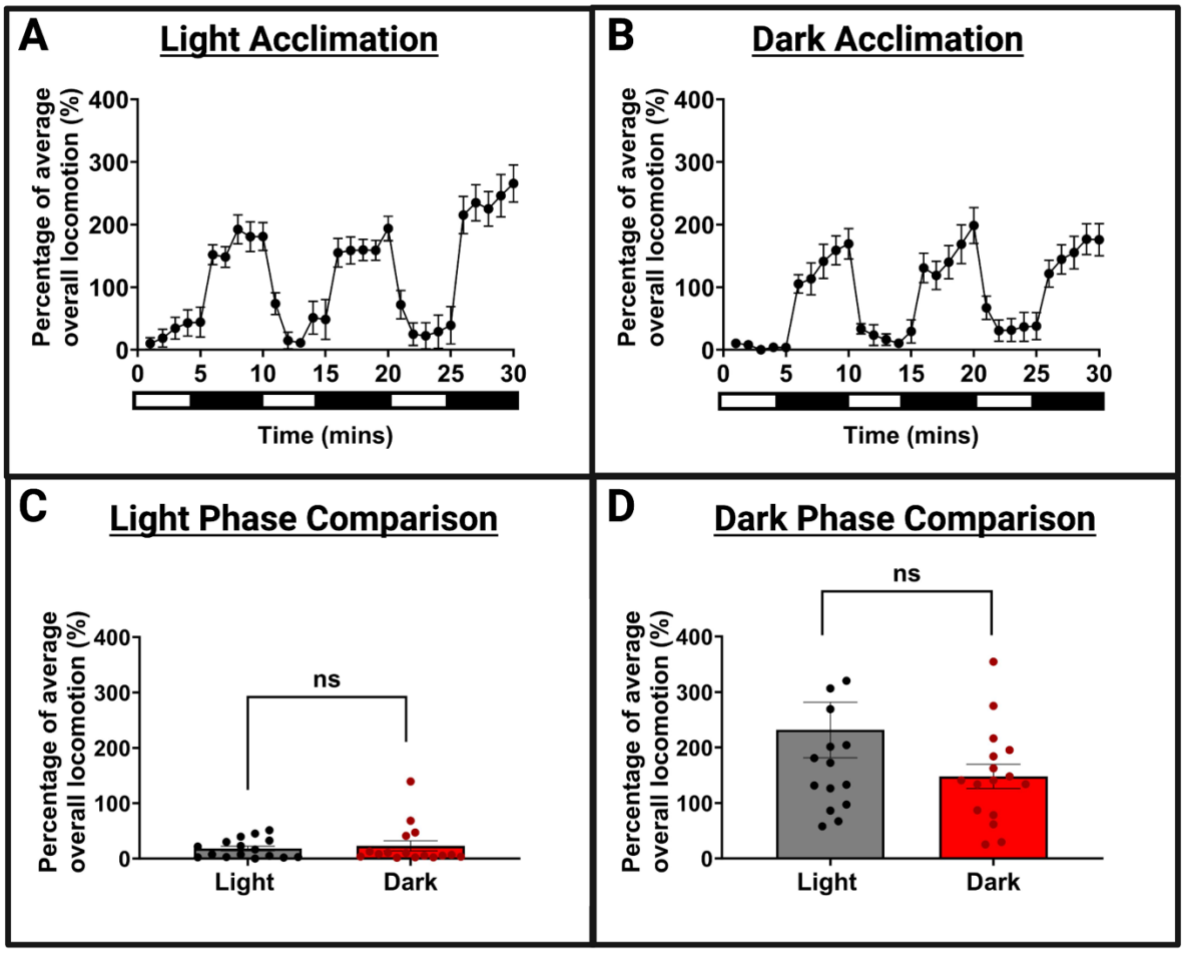


Note. The light/dark mean locomotor responses represented in minutes for (A) no acclimation, (B) five-minute acclimation in the light, (C) ten-minute acclimation in the light, (D) 20-minute acclimation in the light and (E) 30-minute acclimation in the light. (F) represents the mean dark phase locomotor responses for each acclimation period length. Data is represented as mean ± SEM. A - E dots represent averaged responses; F dots represent individual fish responses.

Varying the lighting conditions (light or dark) during the acclimation period revealed no significant effect on light (unpaired student's t-test: $t(30) = 0.4878$, $p = 0.6292$) or dark phase (unpaired student's t-test: $t(30) = 1.531$, $p = 0.1362$) locomotion (Fig. 5). Again, in our laboratory, light acclimation was selected for all further experimentation to maintain consistent conditions to the embryos' rearing and daytime light schedule. However, the data suggests that should other labs choose to use dark acclimation, this should have little effect on the data.

Figure 5

Lighting conditions in the acclimation period do not affect the subsequent light/dark assay behavioural response



Note. Larvae were acclimated in the light (A) or dark (B) for 30 minutes and then underwent the standardised light/dark assay (section 2.3 of this paper). No difference in locomotion in both the light phase (C) or dark phase (D) was observed. Data is represented as mean ± SEM. A - B dots represent averaged responses, C - D dots represent individual fish responses.

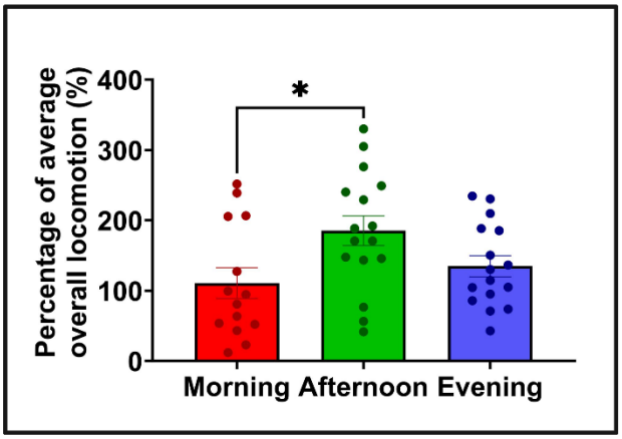
4 dpf zebrafish larvae are more responsive to dark phase transitions in the light/dark assay after 12 pm

It has previously been reported that the time of day affects larval zebrafish behavioural responses using the light/dark assay (Fitzgerald et al., 2019; Kristofco et al., 2016; MacPhail et al., 2009). Therefore, we next examined the effects of time of day on the magnitude of the light/dark assay response. Figure 6 shows the dark phase locomotor responses of the light/dark assay at three different times of the (working) day: morning (09:00 – 11:59), afternoon (12:00 and 14:29) and evening (14:30– 17:00). Our findings reveal that time of day has a significant effect upon larval light/dark assay locomotion (one-way ANOVA, $F_{(2, 43)}$ =

3.813, p = 0.0299); dark phase responsiveness was significantly greater in the afternoon compared to the morning (Fig. 6).

Figure 6

Time of day of the light/dark assay recording affects behavioural responses observed in 4 dpf zebrafish larvae



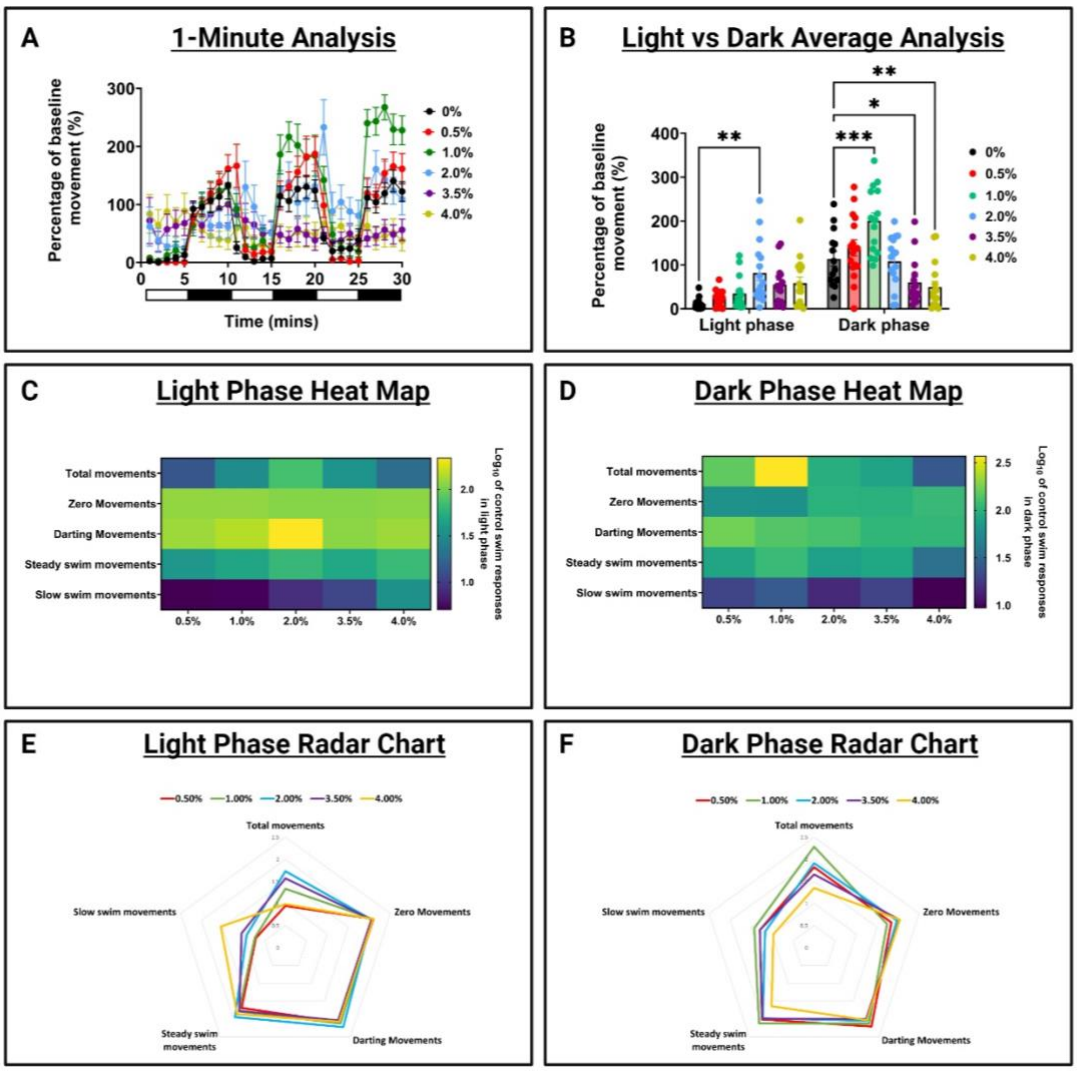
Note. The larvae were recorded at different times of the day with the same standardised test to determine the significance of time of day upon dark phase locomotion. The Tukey's multiple comparison post-hoc test confirmed significant increases in dark phase locomotion in the afternoon (12:00 pm – 14:29 pm) compared to the morning (9:00 am – 11:59 am) (p = 0.0275). No significant difference on locomotion was found between recordings done in the evening (14:30 – 17:00 pm) and either the morning (p = 0.6642) or the afternoon (p = 0.1575). Data is represented as mean ± SEM. Dots represent individual fish responses.

The light/dark assay is suitable for assessing the effects of chemicals on larval behaviour

We next validated the assay in the context of behavioural pharmacology by examining larval behavioural responses to different concentrations of ethanol. Ethanol was selected due to its widespread availability and common use in larval light/dark assay research (Davis et al., 2021; Irons et al., 2010; MacPhail et al., 2009). We also sought to identify meaningful and effective interpretations of the light/dark assay data (Fig. 7). By applying these straightforward analyses, distinctive concentration-dependent altered locomotor responses in both light and dark phases were revealed.

Figure 7

Concentration-dependent effects of ethanol on larval behaviour in the light/dark assay



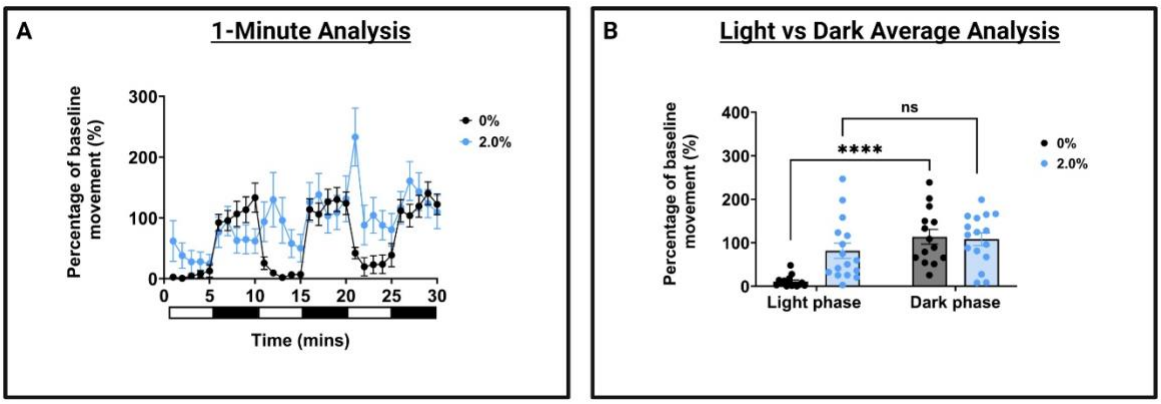
Note. The minute analysis (A and G) and light vs dark average (B and H) are represented as mean percentage of baseline movement over the 30-minute recording period. The Dunnett's post-hoc test confirmed significantly increased light phase locomotion compared to control larvae with 2.0% ethanol (p = 0.0018). In the dark phase there was a significant increase in locomotion compared to controls for 1.0% ethanol (p = 0.0001). 3.5% and 4.0% ethanol exposure resulted in decrease dark phase locomotion compared to controls (p = 0.0316, p = 0.0064, respectively). The heat map (C) and radar chart (E) show light phase responses and (D) and (F) show dark phase responses. The Dunnett's multiple comparison test confirmed reduced light phase total movement compared to controls for 1.0%, 2.0% and 3.5% ethanol (p = 0.0264, p < 0.0001, p = 0.0004, respectively). 4.0% exposed larvae also had significantly

increased slow swim movements compared to controls ($p < 0.0001$). In the dark phase, total movement was reduced for 4.0% exposed larvae compared to controls ($p = 0.0139$). Data is represented as mean ± SEM. (A) dots represent averaged responses, (B) dots represent individual fish responses.

Figure 7A and B show that ethanol exposure has a significant effect upon both light and dark phase locomotion (two-way ANOVA (interaction between concentration and light phase): $F_{(5, 177)} = 13.17$, $p < 0.0001$). Specifically, low concentrations of ethanol (1.0%) increase locomotion in the dark phase compared to control larvae. 3.5% and 4.0% exposed larvae had decreased locomotion in the dark phase compared to control larvae (Fig. 7B). The fish exposed to the 2.0% ethanol had significantly increased locomotion in the light phase compared to control larvae with relatively unchanged dark phase locomotion. We hypothesised that this altered locomotion was unresponsiveness to the light/dark stimuli (Fig. 8A and B). Further analysis confirmed our hypothesis with 2.0% ethanol exposed larvae having no significant locomotor difference between the light and dark conditions (Paired t-test, $t(15) = 1.386$, $p = 0.1859$).

Figure 8

Loss of distinctive light/dark response with exposure to 2.0% ethanol in zebrafish larvae



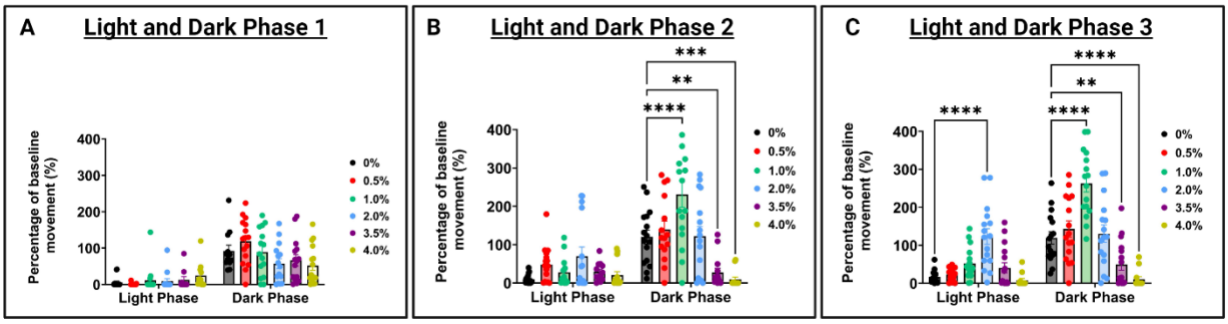
Note. The minute analysis (A) and light vs dark average (B) are represented as mean percentage of baseline movement over the 30-minute recording period. Šídák's multiple comparison test confirmed increased dark phase locomotion compared to light phase for control larvae ($p < 0.0001$). 2.0% ethanol had no significant between light and dark phase locomotion ($p = 0.6171$). Data is represented as mean ± SEM. A dots represent average responses, B dots represent individual responses.

We next furthered our analysis and interpretation of the data by adapting heat maps and radar charts (Haigis (2022) and Steele (2018)) to the larval light/dark analysis, which revealed a more intricate concentration-dependent behavioural response. The types of swim movements that are significantly increased following addition of low concentrations of ethanol are 'steady swim' and 'darting movements' (two-way ANOVA (dark phase swim responses): $F_{(4, 450)} = 20.13$, $p < 0.0001$) (Fig. 7D and F). 4.0%-exposed larvae had equivalent darting responses to control larvae; however, they had reduced total and steady movements (Fig. 7D and F). Furthermore, in the light phase all concentrations had reduced total movement compared to control larvae (two-way ANOVA (light phase swim responses): $F_{(4,450)} = 92.96$, $p < 0.0001$) (Fig. 7C and E). 2.0%-exposed larvae had increased darting response compared to controls and 4.0%-exposed fish had increased slow swim responses compared to the other concentrations. These specific swim observations we have highlighted here are not seen on the line graph and bar chart. Therefore, the radars and heat maps facilitate interpretation of the data and provide insight to more advanced swim responses which cannot be achieved with basic movement analyses.

Next, using the same data, we examined time-dependent effects of ethanol exposures for the first 10-minutes (two-way ANOVA: $F_{(5, 159)} = 3.603$, $p = 0.0041$), 10-20 minutes (two-way ANOVA: $F_{(5, 165)} = 9.048$, $p < 0.0001$) and 20-30 minutes (two-way ANOVA: $F_{(5, 172)} = 14.19$, $p < 0.0001$) post-exposure (Fig. 9). No concentration-dependent changes in locomotion were seen in the first 10-minutes post-exposure (Fig. 9A). The significant increase in locomotion with 1.0% ethanol is not observed until the second dark phase (Fig. 9B). Both 3.5% and 4.0% ethanol exposed larvae had reduced dark phase locomotion as well (Fig. 9B). By the final dark phase, the 2.0% exposed larvae are showed increased light phase locomotion (Fig. 9C). This demonstrates the potential of the assay to examine behavioural expressions of pharmacokinetics/dynamics and highlights the importance of evaluating time-dependence in addition to overall recording responses over the full 30-minutes.

Figure 9

Time-dependent effects of ethanol exposure upon larval light/dark behaviour



Note. Mean locomotor responses represented as a percentage of baseline movement for (A) light and dark phase one (0-10-minutes post-exposure), (B) light and dark phase two (10-20-minutes post-exposure) and (C) light and dark phase three (20-30-minutes post-exposure). Dunnett's post-hoc comparison test revealed reduced locomotion by the second dark phase for both 3.5% (p = 0.0025) and 4.0% (p = 0.0002) compared to controls. 1.0% exposed larvae had significantly increased dark phase locomotion compared to controls (p < 0.0001). By the final light and dark phase 1.0% (p < 0.0001) had significant increases in dark phase locomotion. The 2.0% ethanol exposed larvae had significantly increased light phase locomotion compared to controls (p < 0.0001). Both 3.5% (p = 0.0077) and 4.0% (p < 0.0001) ethanol exposed larvae had significant reductions in dark phase locomotion compared to controls. Data is represented as mean ± SEM. Dots represent individual responses.

The light/dark assay is suitable for assessing behavioural phenotypes of transgenic zebrafish lines

We next validated the assay by examining the phenotypes of *adgrl3.1*$^{-/-}$ larvae, which have previously been shown to express hyperactivity (Sveinsdóttir et al., 2023). In previous work, the noradrenergic reuptake inhibitor, MPH, a therapeutic drug that reduces hyperactivity in children with ADHD, has been shown to rescue the hyperactive phenotype. We examined the effects of this drug on the light/dark assay responses at concentrations determined from previous publications (Lange et al., 2012).

Our findings in figure 10 show a significant increase in dark phase locomotion in the *adgrl3.1*$^{-/-}$ larvae compared to WT larvae, which is partially rescued following addition of 5 and 20 µM MPH (Two-way ANOVA, (interaction between concentration and light phase): $F_{(7, 236)}$ = 8.180, p < 0.0001) (Fig. 10A and B). No significant changes were observed for the WT larvae treated with MPH compared to untreated WT fish in either light condition (Fig. 10A and B).

The *adgrl3.1*$^{-/-}$ larvae express phenotypic hyperactivity with increased dark phase total movement compared to WT (Two-way ANOVA, (dark swim responses): $F_{(4, 300)} = 11.92$, $p < 0.0001$) (Fig. 10D and F). They also exhibit decreased zero movements compared to WT larvae in the dark phase. Additionally, the light and dark radar and heat maps show that 20 µM MPH exposed *adgrl3.1*$^{-/-}$ larvae have significantly increased steady swim and slow swim responses with reduced total movement compared to untreated *adgrl3.1*$^{-/-}$ larvae (Two-way ANOVA, (light swim responses): $F_{(4, 300)} = 70.65$, $p < 0.0001$). 5 µM MPH exposed *adgrl3.1*$^{-/-}$ larvae display similar movement responses to 20 µM MPH exposed *adgrl3.1*$^{-/-}$ larvae, however, significantly increased total movements compared to untreated *adgrl3.1*$^{-/-}$ larvae, WT and WT MPH-exposed fish are also seen. The increased light phase swim responses cannot be seen with the line graph and bar chart (Fig. 10A and B). In the dark phase, 0.05 µM MPH exposed *adgrl3.1*$^{-/-}$ larvae produced comparable total movement responses to untreated *adgrl3.1*$^{-/-}$ larvae. These findings suggest that 20 µM MPH is optimal for reversing the hyperactive phenotype of *adgrl3.1*$^{-/-}$ larvae without impacting WT responses compared to the other tested concentrations. These observations highlight the value of including these additional analyses and interpretations of the larval light/dark data.

Like MPH, ATO is a noradrenergic reuptake inhibitor which reduces hyperactivity in children with ADHD and has previously been shown to reverse the hyperactive phenotype of *adgrl3.1*$^{-/-}$ larvae (Sveinsdóttir et al., 2023). However, studies have reported that a major side-effect of ATO in patients is sedation (Bastiaens, 2007). Therefore, here we examined the effects of ATO on the light/dark assay response at concentrations previously used with *adgrl3.1*$^{-/-}$ larvae with the objective to determine the translational relevance of our assay for evaluating complex effects such as sedation (Sveinsdóttir et al., 2023).

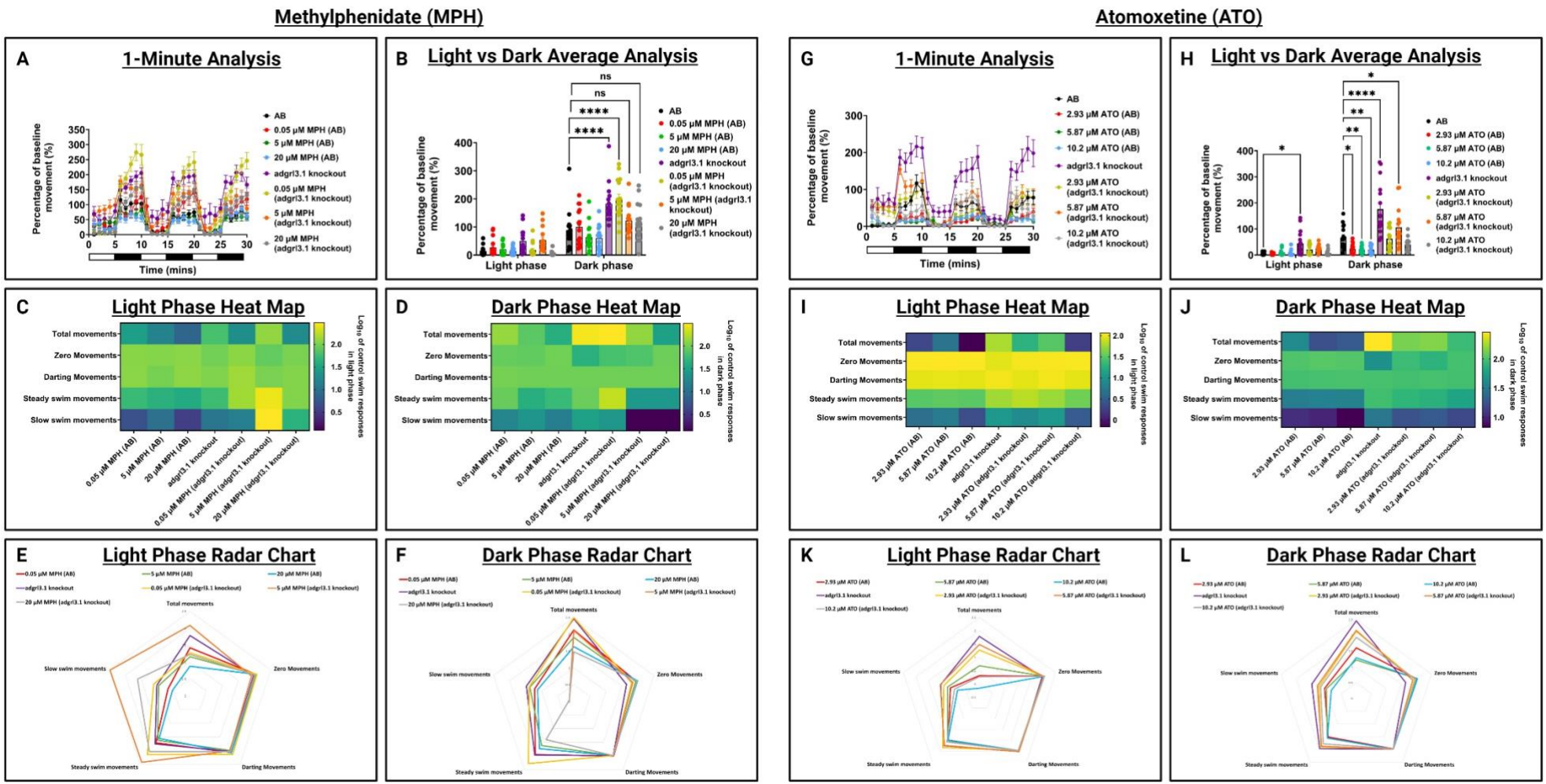
Our findings show that unlike MPH, the WT larvae exposed to ATO experienced significant reductions in locomotion compared to untreated WT larvae (Two-way ANOVA (interaction between concentration and light phase): $F_{(9, 299)} = 8.881$, $p < 0.0001$) (Fig. 10G and H). *adgrl3.1*$^{-/-}$ larvae exposed to 2.93 µM and 10.2 µM ATO had unchanged dark phase locomotion compared to untreated WT fish (Fig. 10G and H). However, 5.87 µM ATO exposed *adgrl3.1*$^{-/-}$ larvae had increased dark phase locomotion compared to untreated WT fish (Fig. 10G and H).

ATO exposure has a significant effect on both light (Two-way ANOVA, (swim responses): $F_{(4, 600)} = 112.1$, $p < 0.0001$) and dark phase (Two-way ANOVA, (swim responses):

$F_{(4, 600)} = 28.26$, $p < 0.0001$) swim phenotypes (Fig. 10I-L). The radar charts and heat maps demonstrate that the major differences between the WT and *adgrl3.1*$^{-/-}$ larvae exposed to ATO are in the total movement and zero movement responses. The WT larvae exposed to all three ATO concentrations experienced significantly reduced total movement compared to untreated WT larvae in both light and dark phases (Fig. 10I-L). Comparatively, the *adgrl3.1*$^{-/-}$ ATO treated larvae had comparable or slightly increased total movement compared to untreated WT larvae. Our ATO observations highlight the importance of including the additional analyses for depicting adverse effects of therapeutic substances in larval zebrafish and promotes the translational relevance of our assay.

Figure 10

The effects of AB wild type and transgenic adgrl3.1$^{-/-}$ *larval phenotypes on light/dark locomotion as well as the potential for assessing therapeutic agents*



Note. The minute analysis (A and G) and light vs dark average (B and H) are represented as mean percentage of baseline movement over the 30-minute recording period. The Dunnett's post-hoc test confirmed increased dark phase ($p < 0.0001$) locomotion for adgrl3.1$^{-/-}$ larvae compared to untreated WT. 0.05 µM MPH treated adgrl3.1$^{-/-}$ larvae had increased dark phase locomotion compared to untreated WT ($p < 0.0001$). No

difference was seen in light (p = 0.9990 and p = 0.9966) or dark (p = 0.4825 and p = 0.3684) phase locomotion for 5 µM and 20 µM MPH treated adgrl3.1$^{-/-}$ larvae compared to untreated WT, respectively. In the dark phase, 2.93 µM (p = 0.0102), 5.87 µM (p = 0.0048) and 10.2 µM (p = 0.0049) ATO reduced WT locomotion compared to untreated-WT larvae. The adgrl3.1$^{-/-}$ larvae had comparable dark phase locomotion to untreated WT, with both 5.87 µM (p > 0.9999) and 10.2 µM (p = 0.3039) but 2.93 µM had increased locomotion (p = 0.0260). The heat maps (C ad I) and radar charts (E and K) show light phase responses and (D and J) and (F and L) show dark phase responses. The Tukey's multiple comparison test showed increased light total movement for adgrl3.1$^{-/-}$ compared to untreated WT (p = 0.006). 5 µM MPH had increased total, steady and slow swim movements compared to WT (p < 0.0001, p = 0.0003, p < 0.0001, respectively). 20 µM MPH had decreased total movement compared to WT (p < 0.0001). Dark phase 5 µM and 20 µM MPH treated adgrl3.1$^{-/-}$ larvae had reduced slow movements compared to WT larvae (p < 0.0001). 5.87 µM (p = 0.0026) and 10.2 µM (p = 0.0071) ATO exposed WT had reduced total movements compared to unexposed WT. 2.93 µM ATO adgrl3.1$^{-/-}$ larvae had unchanged total movement in dark compared to untreated WT (p = 0.3429) but reduced compared to untreated adgrl3.1$^{-/-}$ (p = 0.0049). 2.93 µM (p = 0.0190) and 10.2 µM (p < 0.0001) WT larvae had reduced total movement compared to untreated WT. 5.87 µM (p = 0.0042) and 10.2 µM (p = 0.0105) ATO exposed adgrl3.1$^{-/-}$ larvae had reduced total movement compared to untreated WT larvae. Data is represented as mean ± SEM. (A and G) dots represent averaged responses, (B and H) dots represent individual fish responses.

Discussion

Here, we aimed to determine the optimal conditions for the light/dark behavioural assay in 4 dpf zebrafish larvae. Our primary objective was to develop a robust and reproducible light/dark assay for 4 dpf zebrafish larvae with an in-depth analysis plan, and to validate the assay both pharmacologically, and with genetically modified larvae. The premise of this study was for providing a standardised assay for future larval research.

There were several key findings here that should be highlighted. First, high levels of between-batch and inter-individual variability were found, which were corrected with normalisation. Second, we identified that a period of five-minutes for each dark and light period was optimal in terms of locomotor activity in 4 dpf larvae. The inclusion of an acclimation period (either in light or dark conditions) had no clear effects on performance. Lastly, we found that overall locomotion is significantly greater in the early afternoon (12:00–14:30) compared to morning and evening, but that testing at any period during the day had no effect on light/dark transitions. We confirmed the pharmacological predictivity of the assay using ethanol, which caused low concentration hyperlocomotion and high concentration hypolocomotion, as predicted. Finally, the behavioural phenotypes of transgenic (*adgrl3.1*$^{-/-}$) larvae were reported on, where hyperlocomotion behaviour was rescued with both MPH and ATO. We also demonstrate the translational relevance of our assay for observing potential adverse effects of therapeutic substances, with ATO exposure resulting in hypolocomotion in WT larvae. As well as validation of our assay with transgenic larvae and ethanol, we provided a comprehensive overview of alternative methods of analysis and interpretation of the data.

Large variations were identified in larval responses between different laboratories[1] running identical light/dark assay protocols with fish bred at the same facility (see SD 2). Larval zebrafish variability in behavioural responses is well established (Jacobs & Ryu, 2023) with both genetic and environmental factors impacting batch-batch variability (Xie et al., 2019). For instance, hatching time has a significant effect upon dark phase responsiveness in the light/dark assay; our time-of-day analysis identified significant effects upon the behavioural responses (Rock et al., 2022). Therefore, normalisation of the data is essential (Xie et al.,

[1] We tested larvae across two different locations during the experiment and noticed this difference when comparing the data.

2019). We established a normalisation technique where the dataset of interest is analysed as a percentage against the average baseline locomotor responses, which was able to overcome the variability identified across laboratories *(see SD 2).* We encourage normalisation of the light/dark data for facilitating statistical comparisons and collaboration between research laboratories (Xie et al., 2019).

Development of the assay involved confirmation of the optimal transition period length and number of repetitions. Researchers have previously used a wide range of different transition periods with further variation in the number of repetitions in the assay (Table 1). Here, we evaluated the protocols employed by other researchers (Acevedo-Canabal et al., 2019; Bachour et al., 2020; Haigis et al., 2022), as well as assessing our own developed protocol. Light to dark transitions were used, as our preliminary findings showed no significant difference between light to dark or dark to light transitions for larval locomotion *(SD 3).* We report that the ten-minute and five-minute protocols are both suitable for assessing behavioural responses in 4 dpf zebrafish larvae. We also suggest that the five-minute protocol is superior due to its time-effectiveness, enhanced throughput and sustained increased dark phase locomotion compared to the ten-minute protocol. The larvae exposed to the two-minute transition and fifteen-minute transition protocols had reduced dark phase locomotor responses by the final dark phases compared to the first (Fig. 2B and H). This indicates that the longer paradigms are unsuitable for 4 dpf light/dark assay studies due to reduced locomotion, which hinders the potential use of the assay for assessing drug effects for example. These findings are supported by Maeda (2021) who demonstrated that larvae exposed to 15-minute intervals had gradual decreases in dark phase locomotion. They did not however observe this with the five-minute protocol of the same length. A possible reason for the reduced locomotion observed could be habituation to the dark (Tuz-Sasik et al., 2022). Habituation is non-associative learning defined here as a decrease in dark phase locomotion following repeated exposure to the light/dark transitions (Tuz-Sasik et al., 2022). In the wild, repeated exposure to harmless stimuli causes high energy expenditure and motor fatigue, therefore, habituation ensures the fish conserves energy for survival (Tuz-Sasik et al., 2022). Another possible reason for the reduced dark phase locomotion may be exhaustion brought-on by excessive energy use (Tuz-Sasik et al., 2022). However, confirmation of these hypotheses requires further investigation.

Previous research has highlighted the importance of both the conditions and length of acclimation period used prior to behavioural evaluation with fish (Makaras et al., 2021). In these studies, the effect of changing the acclimation period conditions on locomotor responses was also observed. Contradictory to previous research, using our reported protocol, no significant effect upon locomotion in both light and dark phases when changing the length of acclimation (Fig. 4) or the lighting conditions was observed (Fig. 5). Discrepancies in our data compared to previous research may be a result of the age of fish used in the experiment as well as differing laboratory set-ups (Petersen et al., 2022). Our evidence therefore supports the use of any length of acclimation period for comparable and reproducible locomotor responses, highlighting the flexibility of the assay for experiments with tight time-constraints.

It has been suggested that the zebrafish circadian rhythm has no influence upon light/dark assay locomotion (Krylov et al., 2021). However, we observed increased dark phase locomotion in the afternoon compared to the morning when monitoring the influence of time of day on light/dark responsiveness (Fig. 6). A similar finding was reported by Fitzgerald (2019) and Kristofco (2016). Observations by MacPhail (2009) disagreed with these findings; they reported that morning recordings resulted in increased locomotor responses with a decrease in locomotor activity between 13:00 to 15:30. The discrepancy between our observations and those of MacPhail (2009) could be methodology-dependent, which highlights the importance of the experimental parameters of the light/dark assay with zebrafish larvae (Fitzgerald et al., 2019). Time of day appears to influence light/dark locomotion with 4 dpf larvae and therefore we strongly encourage incorporation of baseline recording and normalisation to baseline findings for consistent and comparable results throughout the day, alleviating the need to limit behavioural work to a certain time of day.

We aimed to further validate the assay by administrating ethanol, which we selected due to its robust concentration-dependent behavioural effects (low concentration hyperactivity and high concentration hypoactivity in the dark phase (Cohen et al., 1997)), its widespread availability and common global abuse (McLellan, 2017) and its frequent use in larval light/dark assay research (Davis et al., 2021; Irons et al., 2010; MacPhail et al., 2009). We also validated using an established transgenic line, *adgrl3.1*$^{-/-}$, which was employed because previous studies had shown robust hyperactivity compared to WT (Sveinsdóttir et al., 2023). We aimed to demonstrate the ability of our assay to distinctly establish specific drug

effects by demonstrating the common adverse effect, sedation, in the WT ATO exposed larvae (Bastiaens, 2007).

There are several ways that the larval light/dark assay data can be presented, and typically this takes the form of mean distance travelled (Haigis et al., 2022). Although this has advantages (e.g., it is quick and uses the raw data), we believe this approach has its limitations. First, as highlighted, when analysing the distance travelled data at face value there was major variation between different laboratories, and even batches within the same laboratory, using fish bred in the same facility and with identical experimental parameters. We postulate that this is associated with environmental stressors, transport, or other generic inter-batch variability (Xie et al., 2019). Secondly, different common swim phenotypes such as the freezing response cannot be accurately identified with just distance travelled data (Kalueff et al., 2013). These limitations prompted us to adopt a broader analytical approach (from Steele (2018)) and analyse responses as different swim parameters. Haigis (2022) also analysed the data in this format to produce a radar chart; however, they chose to normalise the data to control response and perform a $\log_2$ transformation. We similarly normalised the data to baseline responses, followed by analysis of the different swim parameters. Next, we normalised the individual swim responses to control larval response and performed a logarithmic transformation to overcome any exponential increases in swimming behaviour which was identified as impacting the readability of the radar charts and heat maps.

The swim parameters analysed here include total movement, darting movement, steady swimming movement, slow swimming movement and zero movements. We believe these swim phenotypes will facilitate more complex behavioural analysis with 4 dpf zebrafish larvae. We demonstrate that the radar charts and heat maps can distinguish more precise movement patterns and drug/genomic induced behavioural responses which cannot be ascertained solely from the line graph or bar chart (Fig. 7 and 10). Darting swimming is an important swim factor to analyse because it is an indicator of the initial dark escape/light-seeking response as well as a sign of anxiety-like responses (Haigis et al., 2022). Slow swimming responses highlight more clearly any reduced swim response whilst zero movements show the innate freezing common with zebrafish as well as complete loss of movement because of sedation, paralysis or death (Kalueff et al., 2013). A steady swim response was also included, which we postulate is continuous swimming which is not commonly observed in control 4 dpf larvae. As highlighted by, Haigis (2022), including analyses such as the radar chart permits identification

of behavioural responses which may go unnoticed in standard analyses. We believe our findings highlight the radar chart and heat map value in analysis and strongly encourage the inclusion of both to facilitate the readability and interpretation of future light/dark larval behavioural research.

Potential applications

Our validated assay promotes and encourages the use of 4 dpf zebrafish larvae for behavioural experimentation, which is beneficial from a 3R's of animal research perspective (Sneddon et al., 2017). The model can be used for studying potential therapeutic agents. For example, we used adgrl3.1$^{-/-}$ transgenic larvae to observe the effects of MPH and ATO, known anti-ADHD drugs, (Yang et al., 2018), as well as assessing the genetic behavioural phenotypes. There is also the potential for the model to be used for toxicology and ecotoxicology research (Haigis et al., 2022). Previous research has identified the use of the light/dark assay for assessing anxiogenic and anxiolytic substances (Facciol et al., 2019). However, here we suggest the assay can be used to screen for a range of pharmacologically induced behavioural parameters, including hyperlocomotion and sedation. Additionally, we believe a potential application for the assay would be to assess environmental effects on behaviour (e.g., water condition) (Rock et al., 2022). These potential applications we believe highlight the unique flexibility of this larval assay.

Limitations

We acknowledge several limitations. First, although we highlight excellent translational potential of the 4 dpf behavioural data, they remain a preliminary model only. Analysis of more complex behaviours is likely not possible at this age due to their underdeveloped CNS (Blader, 2000). Second, this method is reliant upon accurate behavioural tracking and therefore the tracking settings may need adjustment prior to investigation. Third, our new analytical method is more complex than simplistic averaging of distance travelled; however, a simple Excel macro overcomes this and facilitates analysis.

Conclusions

Here we present a standardised light/dark behavioural assay for use in 4 dpf zebrafish larvae, which was validated using exposure to ethanol with wild type AB and exposure to ADHD drugs with adgrl3.1$^{-/-}$ transgenic larvae. We report that the five-minute transition period is optimal for consistent and reproducible locomotor responses and that an acclimation period is not required for robust responses to be seen, which improves the throughput of the assay.

We present the influence of time of day on 4 dpf larval responses whilst providing a comprehensive analysis of different methods of interpretation and presentation of the behavioural data. Finally, this work has strong 3Rs applications. Despite some limitations due to their non-protected status, we strongly encourage the incorporation of 4 dpf studies prior to adult or mammalian behavioural studies in order to reduce the overall number of protected animals used in studies.

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Ethics approval: All experiments were approved by the University of Portsmouth Animal Welfare and Ethical Review Board, and under license from the UK Home Office (Animals [Scientific Procedures] Act, 1986, license number PP8708123).

Consent to participate: Not applicable.

Consent for publication: Not applicable.

Availability of data and materials: All data and analysis materials will be made available on the Open Science Framework https://osf.io/k7y2c/?view_only=61e23ea4bb2d452dab76646931e3f332

Code availability: Not applicable.

Authors' contributions:

Conceptualization: CH, JK, MP. Formal analysis: CH. Funding acquisition: JK, MP. Investigation: CH. Methodology: CH, JK, MP. Project administration: MP. Resources: JK, MP. Supervision: JK, MP. Visualization: CH. Writing – original draft preparation: CH. Writing – review & editing: CH, JK, MP.

Link to supplementary data: https://osf.io/k7y2c/?view_only=61e23ea4bb2d452dab76646931e3f332

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