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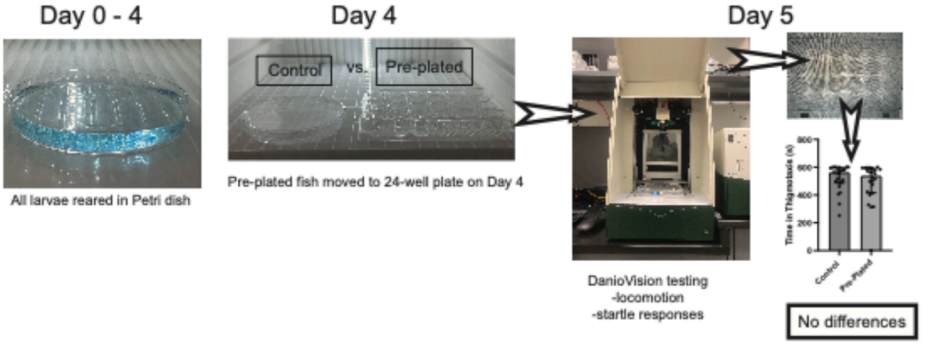
Plating larval zebrafish prior to the day of experimentation has no impact on spontaneous swimming and startle responses

Matthew M.M. Harper [a,1], Ethan V. Hagen [b,1], Yanbo Zhang [b], Trevor J. Hamilton [a,c,d,*]

[a] Department of Psychology, MacEwan University, 10700-104 Ave. NW, Edmonton, Alberta Canada
[b] Department of Psychiatry, University of Alberta, Edmonton, AB, Canada
[c] Neuroscience and Mental Health Institute, University of Alberta, Edmonton, AB, Canada
[d] Allen Discovery Center for Neurobiology in Changing Environments, University of California San Diego, USA

GRAPHICAL ABSTRACT



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ABSTRACT

Behavioural testing in larval zebrafish often involves pipetting the larvae into well plates for individual testing. Transferring larvae into plates the day prior to experimentation can increase efficiency of testing. Furthermore, pharmacological and toxicological studies can require a prolonged dosing period requiring the larvae to be pre-plated into the well plate the day prior to experimentation. Here, we compared the behavioural impact of pre-plating larval zebrafish at 4 days post-fertilization (dpf) to fish transferred at 5 dpf on the day of testing. Motion-tracking

Related research article None

* Corresponding author.
E-mail address: trevorjameshamilton@gmail.com (T.J. Hamilton).

[1] Equal author contribution

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software was used to examine locomotion and zone preference, and responses to light, dark, and mechanical startle stimuli. We found no significant differences in distance moved, time spent in the thigmotaxis zone (outside edge of the arena), high mobility, immobility, light startle, dark startle, and mechanical startle responses. This data suggests that pre-plating larval zebrafish one day prior to testing does not have a significant impact on behaviour in a spontaneous swim task, dark startle test, light startle test, or mechanical startle test. Pre-plating larval zebrafish can increase the efficiency of behavioural testing.

- Compare plating larvae one day prior to testing to plating day of testing.
- Test the behaviour in a spontaneous swimming test, and measure light-, dark-, and mechanical-startle responses.
- There were no significant differences in locomotion or startle responses.

Specifications table

Subject area	Pharmacology, Toxicology and Pharmaceutical Science
More specific subject area	Larval Zebrafish Behaviour
Name of your method	Pre-plating larval zebrafish for efficient pharmacological administration
Name and reference of original method	
Resource availability	Noldus EthoVision (https://noldus.com/ethovision-xt) Graphpad Prism (https://www.graphpad.com) 24 well plates (https://www.thermofisher.com) Tritech Digitherm Incubator (https://www.tritechresearch.com/incubators-overview.html) Eppendorf pipettes (https://www.eppendorf.com)

Background

The use of larval zebrafish to examine the effects of toxicological and pharmacological agents is gaining popularity [1]. Standardization of procedures is paramount as is the development of efficient procedures for testing. In adult zebrafish, the amount of handling by researchers can impact stress levels of zebrafish [2,3] but less is known about larval zebrafish. Long term exposure to a confined space from 3 days post fertilization (dpf) to 6 dpf and onwards does have an impact on the structure and function of neurons [4], however pre-plating larval fish into individual wells at 4 dpf, the day prior to testing at 5 dpf, has not yet been investigated. With larval zebrafish developing at such a rapid rate [5,6], it is important to streamline all testing and dosing methods to allow minimal developmental differences across groups and testing times. Neurodevelopmental alterations of behaviour have been shown as a consequence of exposure to a wide variety of compounds across days of exposure [7,8] therefore it is important to determine whether isolating (i.e., pre-plating) a larval zebrafish the day before exposure on its own could have an impact on behaviour. Zebrafish are considered larvae from 3 to 30 dpf [9] when they can be placed into a range of well plates (i.e., arena sizes) depending on the desired testing procedures, age of the larvae, and aims of the study. When studying pharmacological/toxicological impacts on larval zebrafish behaviour the compound of interest is administered, and behaviour is recorded with a motion-tracking software system within a set exposure time. Variables that are commonly studied in larval zebrafish include spontaneous swimming [10–12], and startle responses due to sudden darkness [13], light [14,7] and response to a tapping stimulus [15,16]. Measurement of movement at 5 dpf is indicative of overall nervous system functionality and can be quantified by examining overall swimming activity, burst behaviour, and escape responses [17]. At this age larvae navigate their surroundings with a sustained 'beat-and-glide' swimming pattern [18] in search of food, conspecifics, and escape from predators. Larvae as young as 5 dpf demonstrate thigmotaxis, known as 'wall-hugging' behaviour, shown to be reduced by anxiety reducing drugs (anxiolytics) like diazepam and increased with anxiety increasing drugs (anxiogenics) like caffeine [19], validating this as a measure of anxiety-like behaviour. Larval zebrafish can elicit rapid escape responses to evade predators, that can be experimentally induced by touch, acoustic stimuli, illumination changes, and mechanical vibration [20]. In particular, startle responses are elicited with increasing intensity of flashes of light [14,21], and are also generated when fish experience the transition from light to dark [21]. Behavioural testing in larval zebrafish is relatively high throughput and can yield large quantities of data.

Here, we describe a method to place individual larvae into the wells one day prior to experimentation (at 4 dpf) and utilize a behavioural test battery to examine locomotion and startle responses. We focused on swimming behaviour and startle responses as they are common high throughput measures in pharmacology and toxicology [17]. The behaviours of focus in the study were: distance moved, time spent in the thigmotaxis zone (the outside edge of the arena), time spent highly mobile (high mobility), time spent immobile (immobility), and response to dark, light, and mechanical stimuli. We found no significant differences in pre-plated larvae at 4 dpf compared to larvae pipetted into the wells at 5 dpf. This suggests that fish can be placed into the wells one day prior to experimentation, allowing for more efficient data collection on the subsequent testing day. This method can also be used to streamline dosing on acute test days since the media can be replaced prior to testing along with the addition of the compound of interest. Therefore, pre-plating can be used with acute exposure and 24-hour exposure to help efficiency on the day of testing.

Method details

Adult zebrafish were housed in 10 L polycarbonate tanks in a Tecniplast ZebTEC multilinking habitat system (Tecniplast Group, Toronto, ON, CAN) at an approximate 1:1 male/female sex ratio. The system water was continuously circulated and filtered through 100 % polyester pleated mechanical filters followed by 5 μm activated carbon filters and then radiated with UV light. Habitat water was maintained by an automatic water exchange and 5-step filtration process in conjunction with non-iodized salt, sodium bicarbonate, and acetic acid buffering. The pH was maintained at 7.07 ± 0.04, while the water temperature was maintained at 28.0 °C ± 0.3 ° and conductivity at 1003 ± 9.6 μS. Fish were fed twice daily (am/pm) with Gemma Micro 300 fish flakes (Skretting, Tooele, UT, USA). An automated 14-hour light/dark cycle was adhered to (7:00 am to 9:00 pm). Daily husbandry and water quality tasks were completed by a MacEwan University Animal Care Coordinator.

Larval zebrafish were bred in the MacEwan zebrafish lab. The breeding procedure involved placing two males and three females into a divided 3 L breeding tank with a sloped breeding insert. Inside the breeding tank a clear dividing wall was used to separate the males and females and a small green artificial plant was placed into each side of the tank for enrichment purposes. All breeding fish were fed prior to breeding selection and were not bred 2 weeks prior to selection. Fish were selected based on visual health and sex markers (size, colouration, etc.) [22]. Fish chosen for breeding were all from the same age cohort (~8-month-old). Once fish were placed in the separated breeding tanks, they were placed on a breeding shelf in the habitat room. We utilized 3 different breeding groups per day and collected one clutch of eggs from each breeding group. The habitat room was maintained at 27 °C with a set light-dark cycle (light on 7:00, light off 21:00). The breeding shelf was wrapped in an aquarium background to provide visual enrichment for the fish. Fish were placed on the shelf at 16:00 and left overnight. The following morning between 9:00–10:00 the separator was removed allowing the fish to spawn. Fish were allowed ~30 min to spawn before the eggs were collected. After breeding, adult fish were returned to their housing tanks and fed. The fertilized eggs were placed into a petri dish then the debris and habitat water were rinsed and replaced with fresh zebrafish media (dH_2O, NaCl, KCl, CaCl, $MgSO_4$, and methylene blue). Zebrafish embryo media is made from adding 20 mL of a 50X E3 solution (NaCl: 7.3 g, KCl: 0.325 g, CaCL: 1.1 g, MgSO4: 2.025 g, dH2O: 500 mL) to 1 L

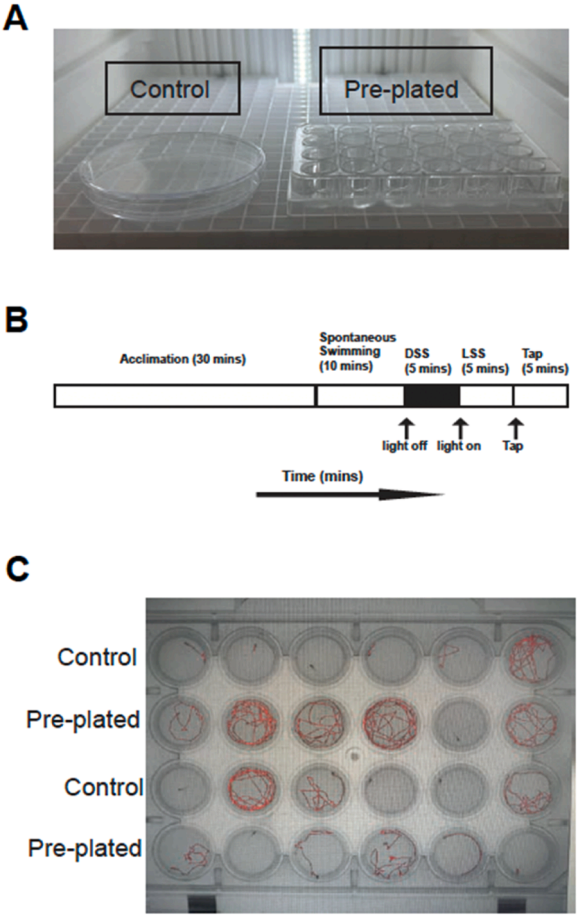

Fig. 1. Experimental set up. (A) Incubation set up on day 4 (4 days post-fertilization larvae) with an example of the petri dish that normally houses the fish (control) and the 24 well plate used in the pre-plating experiment. (B) Experimental timeline during data recording in DanioVision. (C) Representative image of larval behaviour testing on day 5. Red lines are individual tracks from each larva in the well, recorded with EthoVision.

dH2O and 200 μL of 0.05 % methylene blue to make the 1x zebrafish embryo media, resulting in a 0.00005 % concentration of methylene blue. This concentration of methylene blue is within the Canadian Council on Animal Care guidelines [23], although it has recently been found to alter oxidative energy metabolism [24], but not development or behaviour [25]. Embryos were placed in a Tritech Research DigiTherm incubator (Tritech Research, Inc, CA, USA) which maintained the same light/dark schedule mentioned above with an internal temperature of 28.5 °C. The embryo media was changed daily, and unviable embryos were removed. Embryo viability was confirmed using a dissecting microscope.

On the 4th day, 24 larval fish were selected from the available clutches and were used to half fill each 24-well plate with 1 larva per well (12 larvae per plate) in 900 μL of media (Fig. 1A). Larval activity and health were checked via dissecting microscope and a light tap to the petri dish to assess responsiveness of the larvae prior to pipetting. The other half of the larval fish ($n = 24$) used in the experiment remained in the petri dish for another24 h. Larvae from the petri dishes were plated into the remaining 12 wells per plate on the day of testing. Prior to testing, the pre-plated fish had their media replaced with new media. Larvae were again inspected for gross activity levels and healthy morphology under a dissecting scope. All well plates were filled to 1800 μL of embryo media prior to testing to allow for sufficient movement of the larvae.

Behavioural testing

Behavioral testing was conducted using a Noldus DanioVision system and a PC equipped with Noldus EthoVision XT 17 behavioural tracking software (Wageningen, the Netherlands) to track larvae at 5 dpf and produce light, dark, and tapping stimuli (Fig. 1B,C). The testing period phases are described in detail below. Briefly, it consisted of an acclimation period with the internal light on and set to 100 % luminosity within the DanioVision settings (10,000 lx, validated with a CalSpot Photometer), a spontaneous swimming period, a sudden transition from light to dark, known as a 'dark startle stimulus' (DSS) [26], a dark transition period (a period of darkness after the initial DSS), a 'light startle stimulus' (LSS), a light transition period (a return to lighted conditions after the dark transition period), and a tapping stimulus to assess the 'mechanical startle' (pre-equipped in the DanioVision system). Distance moved, time spent in the thigmotaxis zone, high mobility, and immobility were assessed during spontaneous swimming, the dark transition period, and the light transition period. These variables were used to assess potential differences in larval behaviour during a variety of light conditions due to pre-plating. Distance moved was quantified as the total movement of the center point detected in EthoVision for each larvae. The mobility threshold detects percentage changes in pixels of the entire body of the larva, from one frame to the next. Immobility was defined as the period in which the change in object area was below 5 %, commonly set at this level [27,22]. Similarly, the high mobility threshold was set at 60 %, defined as the period in which the change in object area was above 60 %. The arena (16.2 mm diameter) was divided in two by a virtual circle placed in the center of the arena (8.1 mm diameter) with the outer zone from the circle to the arena wall being the 'thigmotaxis' zone.

Acclimation period

The 24-well plates were filled with 12 pre-plated larvae and 12 control larvae that were plated on testing days; the specific arrangement of these groups in the wells was alternated between the two rounds of testing. Filled plates were transferred to the DanioVision system to acclimate with the interior light on for 30 min. During this time, video was recorded, but behaviour was not tracked by EthoVision XT.

Spontaneous swimming assessment

Spontaneous swimming was tracked with the interior light on for a period of 10 min. The variables of focus were the time distance moved (mm), time spent in the thigmotaxis zone (s), high mobility, and immobility .

Dark startle response

Immediately following the spontaneous swimming period, the larvae were presented with a DSS and a dark transition period that lasted for 5 min. To quantify DSS, we measured the recorded movement 5 s before and after the startle and subtracted before from after. The variables of focus were the time in thigmotaxis, distance moved, high mobility, and immobility to assess startle response to sudden darkness and a period of sustained darkness.

Light startle response

Following the DSS test, after 5 min in darkness, the larvae were presented with an LSS and a light transition period that lasted for 5 min. The light stimulus was set to 100 % within the DanioVision settings. To quantify LSS, we measured the recorded movement 5 s before and after the startle and subtracted before from after. The variables of focus were the time in thigmotaxis, distance moved, high mobility, and immobility to assess startle response to sudden brightness and a return to light conditions.

Tap startle response

Immediately following the light/dark startle response testing, a tapping startle response assessment began with an '8-intensity' tap,

which is the highest setting in DanioVision. The mechanical stimulus was produced with a metal solenoid "tap stimulus" that was programmed to impact the underside of the well plate. To quantify mechanical startle, we measured the recorded movement 5 s before and after the startle and subtracted before from after.

Statistical analysis

Variables were first assessed with the D'Agostino-Pearson omnibus normality test. Parametric data were analyzed with a Welch's *t*-test and non-parametric data were analyzed with a Mann Whitney *U test*, with P values <0.05 considered significant. All statistics were analyzed with GraphPad Prism (v10, San Diego, CA, USA).

Method validation

To test whether pre-plating larval zebrafish the day prior to experimentation would have any behavioural impact, we tested for differences in spontaneous swimming, light startle response, dark startle response, and mechanical startle responses. All data were tested for normality, and all data were non-parametric. Mann-Whitney tests were used to compare pre-plated larvae to larvae plated the day of testing.

Fig. 2 shows the spontaneous swimming variables for both the control fish and pre-plated larvae. There was no significant difference between controls and pre-plated larvae in distance moved ($p = .862$, $U = 279$, Fig. 2A), nor in time in thigmotaxis zone ($p = .255$, $U = 232$, Fig. 2B), nor in high mobility ($p = .439$, $U = 250$, Fig. 2C) and no significant difference in immobility ($p = .910$, $U = 282$, Fig. 2D).

We also compared the variables listed above in the 5-minute dark transition period following spontaneous swimming, and the 5-minute light transition period following the dark transition. During the dark transition, there was no difference between control and pre-plated larvae in distance moved ($p = .846$, $U = 278$, Fig. 3A), time in thigmotaxis zone ($p = .627$, $U = 264$, Fig. 3B), high mobility (p

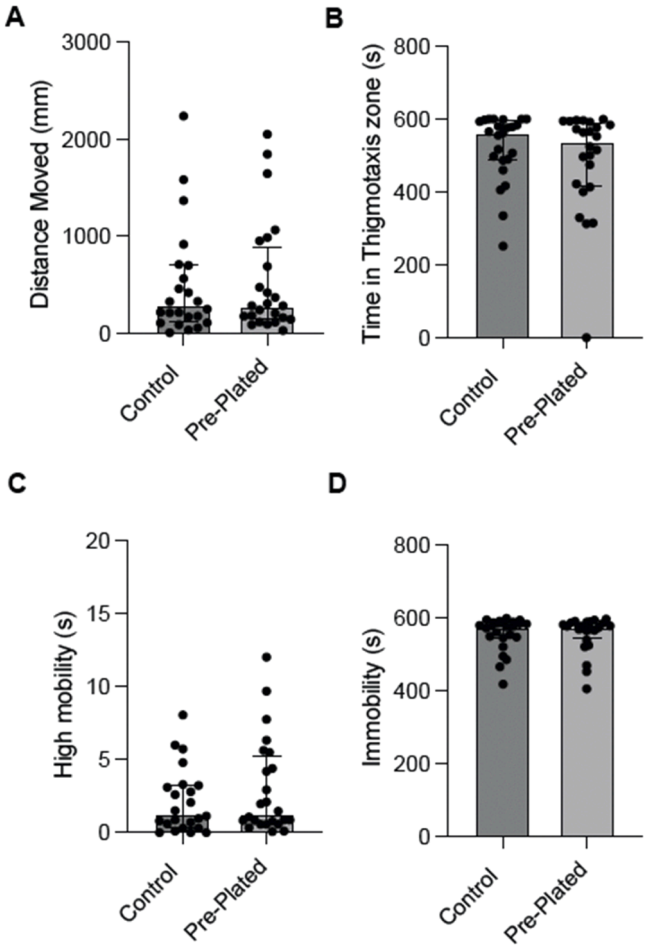

Fig. 2. Spontaneous swimming in the light. (A) Total distance moved during spontaneous swimming. (B) Time in thigmotaxis zone. (C) Time spent highly mobile. (D) Time spent Immobile. Data was analyzed with a D'Agostino & Pearson test for normality followed by a Mann Whitney test.

= .343, $U = 241.5$, Fig. 3C) or immobility ($p = .878$, $U = 280$, Fig. 3D). In the 5-minute light transition period immediately following, the pre plated fish showed no behavioural difference to controls in distance moved ($p = .418$, $U = 248$, Fig. 4A), time in the thigmotaxis zone ($p = .857$, $U = 279$, Fig. 4B), high mobility ($p = .553$, $U = 259$, Fig. 4C) or immobility ($p = .404$, $U = 247$, Fig. 4D).

We tested the variables of interest during three different stimuli used to induce a startle response: a 'dark startle', a 'light startle', and a 'mechanical' startle. A startle 'magnitude' was calculated by subtracting the 5-second pre-startle distance moved immediately before the stimuli from the 5-second post-startle distance moved immediately after the stimuli. This magnitude was used as the startle response. The dark and light startle responses occurred at the onset of the 5-minute transition periods. After the sudden change in illumination from light to dark during the 'dark startle', there was no difference in startle response between pre-plated larvae and controls ($p = .720$, $U = 270$, Fig. 5A). The light suddenly returned during the 'light startle', there was again no difference in startle response between pre-plated larvae and controls ($p = .370$, $U = 244$, Fig. 5B). At the end of the 5-minute light transition period, a single tapping stimulus was used to assess the mechanical startle response. There was no difference in the startle response between the two groups when the mechanical startle was used ($p = .214$, $t = 1.264$, df=37.5, Fig. 5C).

Limitations

This study compared fish plated at 4 dpf to fish placed into the wells at 5 dpf in 24-well plates. It is not known whether this would also apply to fish placed into 48- or 96-well plates, nor whether this would apply for fish placed into wells at 3 dpf. A second limitation is the variability in movement seen across zebrafish studies [17]. Larval zebrafish in this study moved at 29.1 mm/min and 28.7 mm/min (median scores) when they were moved into the plate on the day of experimentation and pre-plated on day 4, respectively. Distance moved during spontaneous swimming tests varies between researchers with some reporting lower movement; ~10 mm/min distance travelled in light conditions [20]. In another study with larvae transitioned from darkness to light, distance moved immediately increases to about 40 mm/min, then decreases to 12–25 mm/min over the next five minutes (in 6 dpf larvae; [17]). Relatively high average distance moved was found by Tuz-Sasik et al., at around 100 mm/min in light conditions [16]. Differences in spontaneous movement can be due to a variety of factors including strain and genetic background differences, husbandry procedures, testing arena size, water temperature, time of day, day/night cycles, and illumination intensity during development and testing [20,16,17,28]. Any of these factors alone or in combination, that are different from our procedures, may influence the effect of pre-plating.

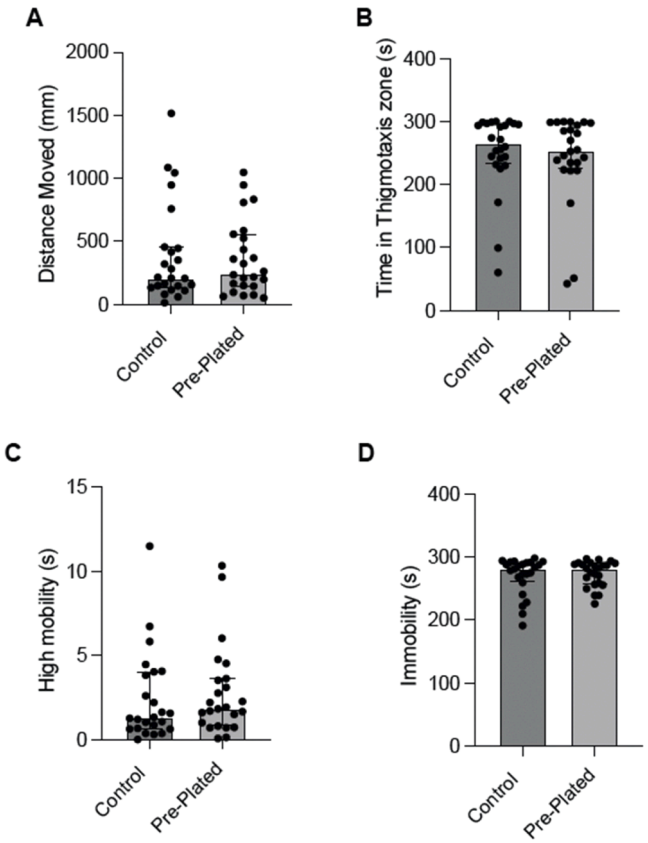

Fig. 3. Behaviour after the dark stimulus. Fish were recorded for 5 min with the light off. (A) Total distance moved. (B) Time in thigmotaxis zone. (C) Time spent highly mobile. (D) Time spent Immobile. Data was analyzed with a D'Agostino & Pearson test for normality followed by a Mann Whitney test.

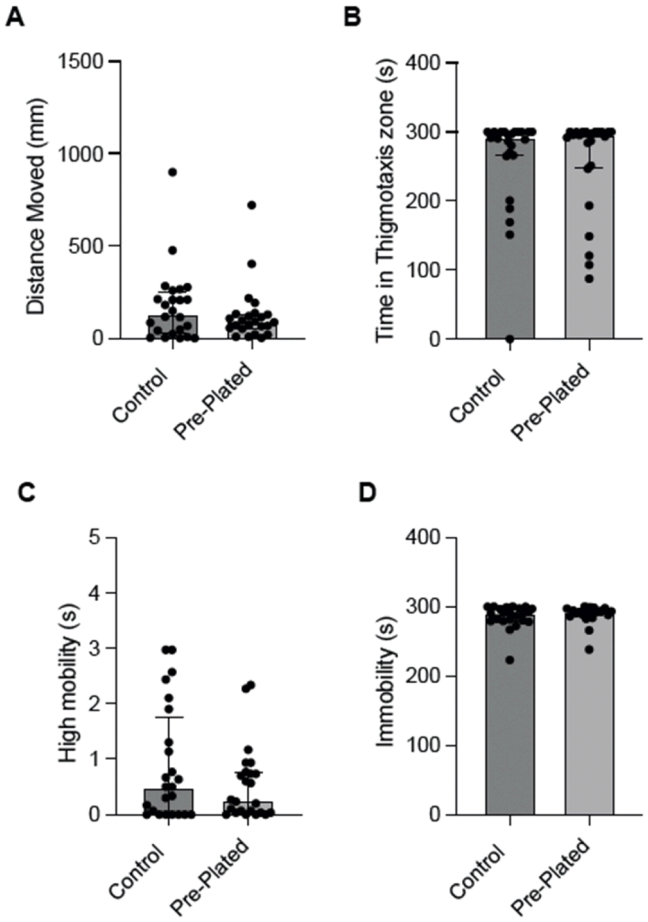

Fig. 4. Behaviour after the light stimulus. Fish were recorded for 5 min with the light on. (A) Total distance moved. (B) Time in thigmotaxis zone. (C) Time spent highly mobile. (D) Time spent Immobile. Data was analyzed with a D'Agostino & Pearson test for normality followed by a Mann Whitney test.

Ethics statements

Experiments were performed under MacEwan University Animal Ethics Board (AREB) under protocol number 101853 in compliance with the Canadian Council on Animal Care (CCAC) experimental guidelines and were carried out in compliance with ARRIVE guidelines for animal research.

Supplementary material *and/or* additional information [OPTIONAL]

Supplementary material includes data used to generate graphs.

CRediT authorship contribution statement

Matthew M.M. Harper: Conceptualization, Methodology, Data curation, Formal analysis, Writing – original draft. **Ethan V. Hagen:** Conceptualization, Methodology, Data curation, Formal analysis, Writing – original draft. **Yanbo Zhang:** Supervision, Writing – review & editing. **Trevor J. Hamilton:** Supervision, Conceptualization, Funding acquisition, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

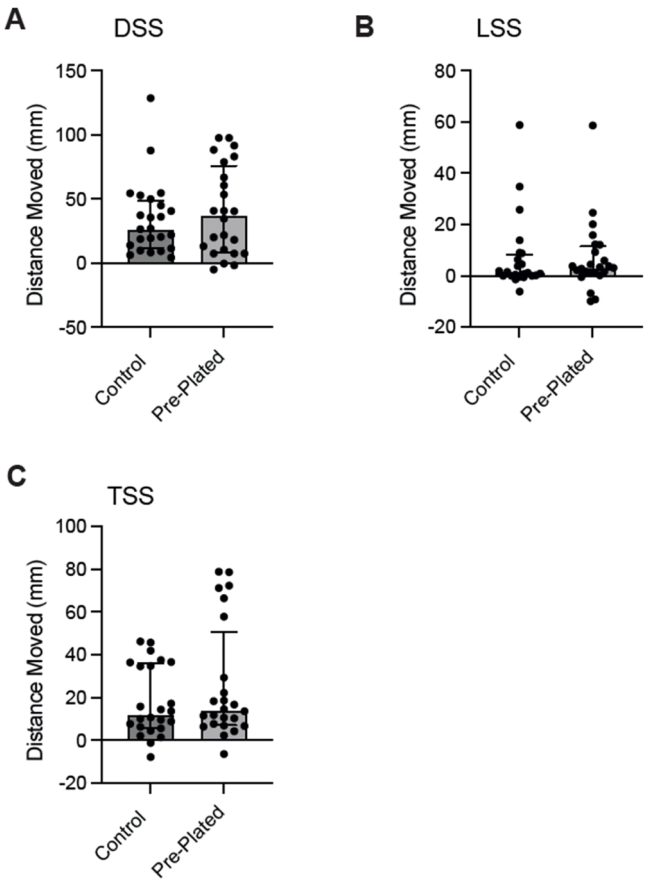

Fig. 5. Distance moved following a startle response. (A) The response from the transition to dark was calculated by subtracting the distance moved 5 s the before dark startle stimuli (DSS) from 5 s after dark startle stimuli. (B) The response from the transition to light was calculated by subtracting the distance moved 5 s the before light startle stimuli (LSS) from 5 s after light startle stimuli. (C) The response from the mechanical, tap stimulation (TSS) was calculated by subtracting the distance moved 5 s before tap startle stimuli from 5 s after tap startle stimuli. Data was analyzed with a D'Agostino & Pearson test for normality followed by a Mann Whitney test.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mex.2025.103730.

Data availability

Data is included in supplementary material

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