Behavioural Brain Research 439 (2023) 114228

Contents lists available at ScienceDirect

Behavioural Brain Research

journal homepage: www.elsevier.com/locate/bbr

Short communication

Zebrafish (*Danio rerio)* shoaling in light and dark conditions involves a complex interplay between vision and lateral line

Shayna-Lee Chaput [a], Warren W. Burggren [b], Peter L. Hurd [a,c], Trevor J. Hamilton [c,d,*]

[a] Department of Psychology, University of Alberta, Edmonton T6G 2E9, Alberta, Canada
[b] Developmental Integrative Biology Research Group, Department of Biological Sciences, University of North Texas, Denton TX76205, USA
[c] Neuroscience and Mental Health Institute, University of Alberta, Edmonton T6G 2H7, Alberta, Canada
[d] Department of Psychology, MacEwan University, Edmonton T5J 4S2, Alberta, Canada

ARTICLE INFO

Keywords:
Behavioral neuroscience
Cobalt chloride
Group behavior
Lateral line
Shoaling
Zebrafish
Vision

ABSTRACT

We know little about how - or even if in some species – fish shoal in darkness. We hypothesized that 'dark shoaling' occurs in zebrafish and therefore must depend upon lateral line sensory input. Shoaling in groups of five adult zebrafish was analyzed with motion tracking software. We measured average inter-individual distance, time near the arena wall (thigmotaxis zone) and total distance traveled under normal room light, and in near-complete darkness (infrared light at 850 nm). These observations were repeated in fish treated with cobalt chloride ($CoCl_2$), which ablates lateral line function. In untreated controls, dark shoaling was reduced compared to in light, but nonetheless still present. Elimination of lateral line sensory input by $CoCl_2$ treatment similarly reduced, but did not eliminate, shoaling under both light and dark. Our findings indicate that normal zebrafish shoaling in light or dark requires both visual and lateral line inputs, with neither alone sufficient for normal shoaling.

Zebrafish (*Danio rerio*) are highly social, like many other fish species, forming shoals – a behavior in which they swim alongside one another in a group [1,2]. Shoaling is distinct from "schooling", in which a group of fish swim in the same direction in a highly coordinated fashion [1,2]. Many sensory systems, including vision, hearing, olfaction, and lateral line sensation, presumably could function in concert to provide the necessary information for an individual zebrafish to remain within the outlines of its shoal. The lateral line, which provides important mechanosensory input reflecting disruptions in water flow and pressure near the fish's body, has long been implicated in detection of food, predators, obstacles, and the speed and direction of water currents, as well as contributing to shoaling behaviors in fishes [2–6]. Past studies show that both lateral line sensory input and vision are important for shoaling in fish under high and low light level conditions [1,2,7]. However, very little is known of zebrafish shoaling behavior in near or complete darkness. In total darkness, both larval and adult zebrafish show intermittent cycles of sleep-like state [8]. Anecdotal observations also indicate that zebrafish in their awake cycle in darkness may still be capable of some form of shoaling, because when illumination is suddenly turned on in a previously dark environment, zebrafish are sometimes aggregated in a shoal. However, these 'dark shoals' are loosely organized and characterized by higher interindividual distances than in light conditions [9]. These observations of less coordinated dark shoaling in zebrafish suggest that normal shoaling in light may involve some degree of visual sensory input, as well as sensory input from the lateral line and possibly other sensory organs [10].

Experiments have not been conducted under combinations of light regimes and lateral line ablation designed to further reveal mechanisms by which adult zebrafish shoal in the dark. We hypothesized that lateral line ablation would decrease, but not eliminate, the ability of zebrafish to shoal in the dark. To test this hypothesis, we quantified aspects of shoaling behavior in groups of wildtype zebrafish under both light and dark conditions, and with and without chemical disruption of the lateral line by cobalt chloride ($CoCl_2$) exposure. Adult male and female (~50:50) wild-type, short-fin zebrafish (n = 155) were obtained from Aquatic Imports (Calgary, AB, Canada) and housed in 10 L polypropylene tanks in a three-tier bench-top, recirculating system (Aquatic Habitats, Aquatic Ecosystems, Inc. Apopka, FL, USA) and were held for at least 60 days prior to experimentation. Water chemistry in habitat, treatment and testing tanks was kept uniform; pH was maintained between 6.5 and 8.0 using sodium bicarbonate, non-iodized salt, and acetic acid, and temperature was maintained between 26 and 30 °C. Water in

* Corresponding author at: Neuroscience and Mental Health Institute, University of Alberta, Edmonton T6G 2H7, Alberta, Canada.
E-mail address: trevorjameshamilton@gmail.com (T.J. Hamilton).

https://doi.org/10.1016/j.bbr.2022.114228
Received 24 August 2022; Received in revised form 8 November 2022; Accepted 23 November 2022
Available online 24 November 2022

the behavioral testing arena was replaced after every five trials [11]. The habitat room fluorescent lights operate on a 12 h light: 12 h dark cycle between 08:00 and 20:00. Fish were fed fish pellets (Gemma Micro 300, Skretting by Nutreco, Westbrook, USA) and dry brine shrimp (Omega One Freeze Dried Mysis Shrimp Nutri-treat; OmegaSea LLC, Sitka, USA), once daily after testing on experimental days. All fish tested were experimentally naïve and at least 9 months of age. Lighting was provided with incandescent lamps above and beside the arena (28 cd/m^2, measured with a cal Spot photometer; Cooke Corp. CA, USA). All experiments were performed in full compliance with the *ARRIVE Guidelines* and the NRC's *Guide for the Care and Use of Laboratory Animals*.

$CoCl_2$, a nonspecific calcium channel antagonist, is routinely used to

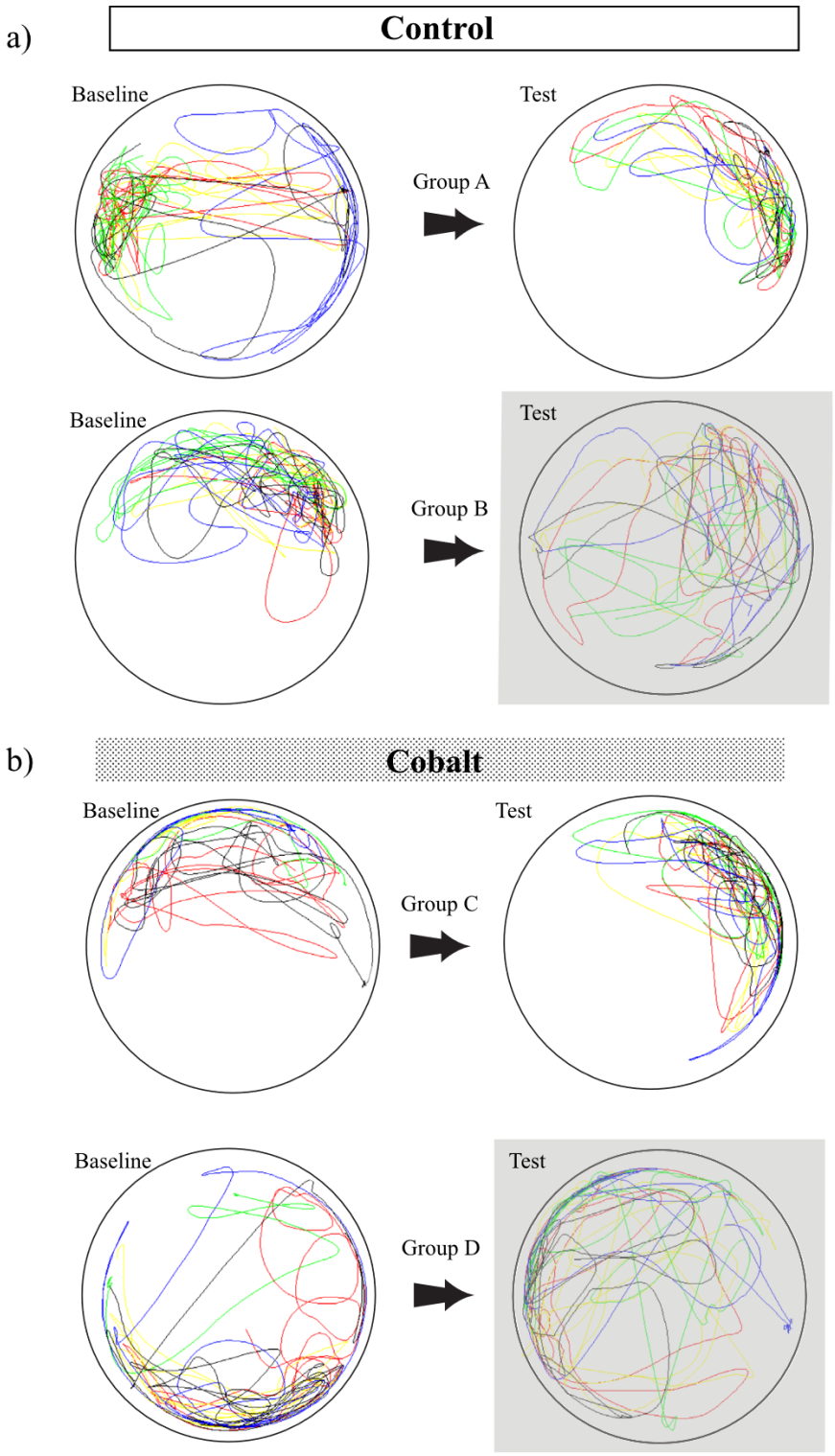

Fig. 1. Track plots of individual fish (colored lines) from one representative shoal in each of the four experimental groups during the Baseline and Test trials (first 30 seconds of the trial). Groups C and D received $CoCl_2$ prior to the Baseline trial. Groups B and D were tested in the dark during the Test trial. (Note that the 10 min Acclimation period between Baseline and Testing periods is not depicted).

chemically ablate the neuromast hair cells of the lateral line [12–15]. $CoCl_2$ exposure involved transferring zebrafish to 3 L polypropylene tanks containing $CoCl_2$ solution (1.5 L of RO water, 0.1 mM $CoCl_2$ [after 14], and ~0.18 g sodium bicarbonate to buffer pH to +/−0.02 of habitat pH). $CoCl_2$-exposed groups (designated groups C and D, see below) were immersed in the treatment solution for 3 h while being monitored visually for adverse effects. Noteworthy is that $CoCl_2$ also eliminates olfaction [12], but it is highly unlikely that olfaction per se would be involved in shoaling behaviors given the latency time of olfaction relative to rapid shoaling-related movements. No fish demonstrated behaviors that would indicate distress due to $CoCl_2$. Control shoals (groups A and B) underwent identical procedures without the presence of $CoCl_2$.

Fish were removed from their habitat tanks and randomly assigned to one of four experimental groups of five fish. These groups served as the unit of analysis in the behavioral test. Four groups were created as part of a 2 × 2 factorial design. The crossed treatment groups for this design were (1) $CoCl_2$ effect, with $CoCl_2$-treatment vs. No-$CoCl_2$ control, and (2) Illumination level, with visible light vs. "darkness" (with only faint infrared illumination – see description below) (Fig. 1). Each of the four resulting treatment groups were designated as:

- Group A ("control" + "light", n = 8);
- Group B ("Control" + "dark", n = 7);
- Group C ($CoCl_2$ + "light", n = 8); and
- Group D ($CoCl_2$ + "dark", n = 8).

Eight replicate runs were performed for each of the treatment groups, except for Group B, which had seven replicates, for a total of 155 fish recorded.

Behavioral trials, each 30 min in length, began with the transfer of a shoal of fish into the circular open field test arena (34 cm diameter, ~107 cm circumference), filled to a depth of 6 cm with normal habitat water [11,16]. This provided adequate area for shoaling and exploration (Fig. 1), while limiting large vertical movements to accommodate the depth of field for the video camera. The arena walls were made of translucent white light-scattering plastic walls. Arena illumination for light conditions was provided from four equally spaced locations around the arena, which minimized visual inhomogeneities. During darkness, two infrared light sources (850 nm; ICAMI) located on either side of the arena were used to provide the minimum infrared light to allow the motion-tracking software to track individual fish movements. Infrared (IR) illumination remained on during every test to control for any potential effects of IR light and to maintain identical IR illumination across groups. Illumination solely with infrared light (typically 800–950 nm) to enable video recording is generally defined as 'darkness' in studies of adult zebrafish locomotive behavior, including shoaling [e.g. 17–20].

Each behavioral trial was divided into three periods. First a period designated as "Baseline" from 0 min to the 10 min point was used to determine shoaling parameters under full light conditions (essentially, a control period). At the end of the Baseline period, the lights in the arena were either left on (Groups A and C) or turned off (Groups B and D) for another 10 min. The fish were then left undisturbed without measurement for an "Acclimation" period from time 10 min to time 20 min. This was designed to allow fish to acclimate to the light condition following Baseline measurements. Then, at the end of the Acclimation period, a "Testing" period from time 20 min to time 30 min was administered, during which time the effects of the light condition on shoaling were determined. Noteworthy is that at least in zebrafish larvae, most cone adaptation occurs within 5 min [21]. To summarize, Groups A and C remained in the light during all time periods during the behavioral trials (Fig. 1A), while Groups B and D were in the light only during the Baseline period, and then in darkness during Acclimation and Testing periods (Fig. 1B).

Individual fish movements as part of a group were recorded using EthoVision XT motion-tracking software (version 10.0, Noldus, VA, USA) which is a commonly used procedure for quantifying zebrafish shoaling [see 16 for additional references]. A key variable measured was time (sec) spent in the 'thigmotaxis zone', which is the outermost circular zone of the test arena, with the distance from the wall based on the average length of a large individual adult zebrafish ~5.5 cm, measuring 23 and 34 cm for the inner and outer diameter, respectively. Also measured was the total distance traveled by every fish in the shoal (cm), and interindividual distance (mean distance between one fish and all others in the shoal: cm) (Fig. 1).

Two-way ANOVAs were used to test for main treatment effects. Post-hoc Tukey tests were used to compare groups within these analyses. These post-hoc tests revealed no significant differences beyond those consistent with the ANOVA main effects, with the sole exception of two significant post-hoc effects described below. All statistics were carried out using R v.4.04 (R Core Team, 2021) and GraphPad Prism (v. 9, San Diego, CA, USA). (Refer to Supplementary material for data and statistical analysis.).

As expected, there were no significant differences between groups A and B (groups not treated with not $CoCl_2$-treated) during the Baseline period, under visible light in interindividual distance ($F_{1,\ 27} = 0.178$, $p = 0.730$, eta-Squared (η^2) = 0.005, Fig. 2a), time in thigmotaxis zone ($F_{1,\ 27} = 0.470$, $p = 0.980$, $\eta^2 = 0.012$, Fig. 2b), or total distance traveled ($F_{1,\ 27} = 0.128$, $p = 0.994$, $\eta^2 = 0.004$, Fig. 2c). During the test period, which tests the effects of vision alone, groups A and B showed a large effect of light treatment on inter-individual distance within the shoal, with fish positioning themselves more than twice as far from each other when shoaling in the dark ($F_{1,27} = 165.1$, $p < 0.0001$, $\eta^2 = 0.820$, Fig. 2a).

Treatment with $CoCl_2$, which renders the lateral line dysfunctional, altered shoaling behavior under visible light. Specifically, under Baseline conditions shoal dispersion, assessed by inter-individual distance, was ~25% greater in the $CoCl_2$-treated groups (C and D) than the control groups (A and B) ($F_{1,27} = 6.82$, $p = 0.015$, Fig. 2a). Further, whether in light or dark, $CoCl_2$-treated fish (groups C and D) spent ~15 % less time in the thigmotaxis area than shoaling fish in untreated groups (A and B: $F_{1,27} = 12.1$, $p = 0.002$, $\eta^2 = 0.194$, Fig. 2b). Total distance traveled by the shoal under visible light was however unaffected by $CoCl_2$ treatment ($F_{1,27} = 1.85$, $p = 0.190$, $\eta^2 = 0.063$, Fig. 2c). Reassuringly, we found no significant differences between groups C and D ($CoCl_2$-treated groups later tested in light and dark, respectively) in the Baseline period (which is to say, before the dark tested group had the lights turned off) for interindividual distance ($F_{1,27} = 6.82$, $p = 0.975$, $\eta^2 = 0.194$, Fig. 2a), time in thigmotaxis ($F_{1,27} = 12.1$, $p = 0.938$, $\eta^2 = 0.305$, Fig. 2b) or total distance traveled ($F_{1,27} = 1.85$, $p = 0.877$, $\eta^2 = 0.063$, Fig. 2c). However, in the test phase we found significant interactions for both inter-individual distance where $CoCl_2$-treated fish showed a more muted effect of light condition ($F_{1,27} = 8.66$, $p = 0.007$, $\eta^2 = 0.043$, Fig. 2a) and total distance traveled where the effect of turning the lights off was magnified in $CoCl_2$-treated fish ($F_{1,27} = 4.72$, $p = 0.039$, $\eta^2 = 0.104$, Fig. 2c). Interestingly, $CoCl_2$-treated fish in the dark also spent more time near the arena wall (thigmotaxis) compared to $CoCl_2$ fish in the light, providing additional evidence of lateral line disruption.

Our study has confirmed that vision is involved in zebrafish shoaling by examining shoaling behaviors both in light and in dark, eliminating visual stimuli both with, and without, disruption to the lateral line. The overall size of the shoal was greatly enlarged in dark conditions. This increase in inter-individual distance suggests that vision is required for zebrafish to maintain tight shoals. Nonetheless, we show the lateral line has a role in shoaling under both light and dark conditions, based on examining fish with chemically disabled lateral line systems. Specifically, disabling the lateral line with $CoCl_2$ significantly reduced shoal tightness, demonstrating that zebrafish shoaling behaviors even in full light are reliant, at least in part, on the lateral line system. The negative effect of disabling the lateral line depends upon the availability of supplementary visual information.

Certainly, vision is of primary importance to shoaling in many fishes

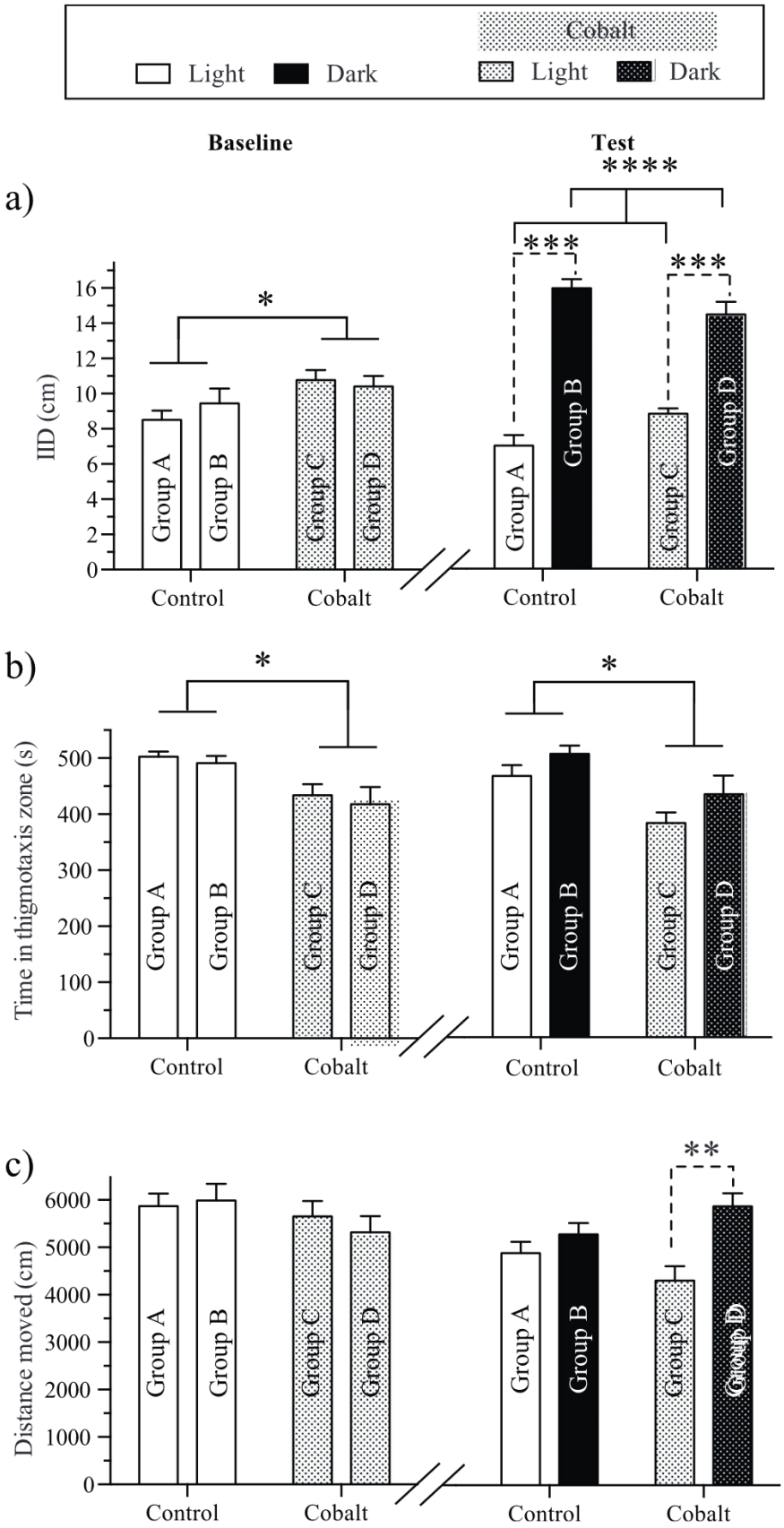

Fig. 2. Effects of $CoCl_2$ and darkness on zebrafish shoaling. (a) Average interindividual distance (IID), (b) time in the thigmotaxis zone, and (c) total distance traveled by the shoal. Mean values ± 1 se.m. are presented. Asterisks indicate significant differences, as follows: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

[1,7], with not only the silhouette but also the color pigment variants and overall color patterns being important in selection of shoal mates [22]. Our experiments confirm the importance of vision in zebrafish. Yet, interestingly, the other measured indicators of shoaling behavior (time in thigmotaxis zone, total distance traveled) were not significantly affected by removal of visual input (i.e. darkness), suggesting senses other than vision were involved in maintaining at least these unaltered shoaling variables.

The *combination* of removal of visual cues by darkness and of lateral line disruption with $CoCl_2$ did not completely disrupt shoaling behavior based on what is expected from a purely random dispersion (IID: ~24 cm). While inter-individual distance and total time spent in the thigmotaxis zone was decreased, shoaling was not greatly disrupted by removal of what are widely assumed to be the two major senses involved in shoaling. Interestingly, the total distance traveled during lateral line disruption in the dark was increased, an effect not observed under only visual or lateral line deprivation. This finding, which might indicate zebrafish are searching more actively for the wall or nearby neighbors, was unexpected and warrants further investigation. Noteworthy is that the changes in inter-individual distance might be greater in a larger behavioral arena.

Our findings suggest that since some shoaling behavior persists with the absence of vision or lateral line input, then additional senses may be involved in shoaling. Hearing has been implicated in fish shoaling [23], but there was little evidence of interaction between noise and darkness in three-spined stickleback [24]. Olfaction could be a possible sense to consider, although the diffusion through water of chemical stimuli that would stimulate olfactory receptors is extremely slow compared to the very rapid position adjustments provided by pressure changes sensed by the lateral line. This would appear to eliminate any role for olfaction in shoaling in the present experiments, further supported by the fact that $CoCl_2$ also disrupts olfaction in numerous fresh-water fishes [12].

In considering senses involved in shoaling, it is noteworthy that zebrafish larvae (4–5 days post fertilization) can detect low levels of near infrared light at 860 nm but not at 960 nm [25], though this capacity is unproven in adults. Infrared light of ~850 nm was used for recording in "darkness" in the current study, so it is possible – but not yet tested - that adult zebrafish may to a limited extent be able to visually detect shoal members even in 'darkness'. Another consideration is that we did not quantify the polarization of the group (a measure of their schooling as opposed to the less organized shoaling behavior), the former which may or may not have been altered by the sensory manipulations in this study.

Zebrafish shoaling has been revealed to be a complex interplay between both eyes and lateral line input. Neither organ alone is sufficient for normal shoaling, but the relative contributions under the varying conditions normally found in their natural habitat remain to be described. Future experiments should consider a range of intermediate levels of light and, if technically possible, different levels of chemical inactivation of the lateral line, to ascertain whether there are graded interactions of vision and lateral line mechanoreceptor input. Finally, while the zebrafish is a popular animal model, it is not automatically representative of fishes in all aspects of its biology. Investigation of the relative contributions of the eyes and lateral line to shoaling should be expanded to additional fish species.

Funding

This work was supported by NSERC Discovery Development (#05426) grant to T.J.H. and US NSF grant #2103499 to W.B.

CRedit authorship contribution statement

SC: Formal analysis; Funding acquisition; Investigation Software; Methodology; Roles/Writing – original draft; Visualization; Writing – review & editing. **WB**: Conceptualization; Methodology; Project administration; Roles/Writing – original draft; Writing – review & editing. **PH**: Data curation; Methodology; Roles/Writing – original draft; Supervision; Validation; Writing – review & editing. **TH**: Conceptualization; Formal analysis; Methodology; Project administration; Resources; Roles/Writing – original draft; Supervision; Validation; Visualization; Writing – review & editing.

Declarations of interest

None

Data availability

Data is in supplementary information.

Acknowledgments

We thank Aleah McCory (Animal Care Technician) for help with daily husbandry and aquarium maintenance.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbr.2022.114228.

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