



Effects of reduced visibility on fish behavior: low light is not equivalent to suspended sediments

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Abstract Human development has increased soil erosion, elevating suspended sediment levels across aquatic habitats. Poor visibility caused by suspended sediments can influence the antipredator responses, social preferences, foraging, and other behaviors of fishes, and is therefore a major contributor to human impacts on waters. Studying the impacts of suspended sediments in the laboratory requires regular water turbulence to prevent sediments from settling out of suspension, so some researchers instead alter lighting to mimic the impacts of suspended sediments. Here, we compared the social and movement behavior of zebrafish exposed to suspended sediments versus black pond dye, a potential easier-to-use alternative to suspended sediments. We also evaluated whether fluctuation in the visual environment further alters any potential behavioral impacts. We found that the

effects of pond dye differed from suspended sediments. Zebrafish moved more in pond dye than in suspended sediments, with the fish being on average 26% faster and 8% more active. Fluctuating visibility further amplified this effect. Fish also performed 126% more chases and 116% more follows in suspended sediments than dye. Our results suggest that black pond dye is not a substitute for suspended sediments and, further, that fluctuating visibility levels affect how fish respond to visual conditions.

Keywords Turbidity · Fluctuation · Visual clarity · Fish behavior · Suspended sediments · Dye

Introduction

Suspended particulates and dissolved organic matter attenuate the light entering aquatic environments by both absorbing and scattering light and are therefore primary contributors to the overall aquatic visual environment (Sandström, 1999; Davies-Colley & Smith, 2001). Suspended particulate matter primarily scatters light, creating a cloudy or hazy appearance in the water (sometimes referred to as ‘turbidity’; Davies-Colley & Smith, 2001). Such light scattering reduces the visual range of fish and other aquatic organisms (Chapman et al., 2014), interfering with their vision by masking, attenuating, and altering the contrast of visual signals (Sandström, 1999; Heubel & Schlupp, 2006; Fouilloux et al., 2022). Suspended

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particulates are prevalent throughout aquatic environments thanks largely to algae (Beck et al., 2019; Fouilloux et al., 2022; Rodrigues et al., 2023) and to suspended sediments (Utne-Palm, 2002; Borner et al., 2015; Fouilloux et al., 2022). With human activities such as dam construction and agricultural development altering the levels of suspended sediments (Syvitski & Kettner, 2011; Dethier et al., 2022) there is a growing interest in understanding the effects of suspended sediments on aquatic ecosystems (Søndergaard & Jeppesen, 2007; Kjelland et al., 2015; Rodrigues et al., 2023).

Most fish species rely on vision for tasks such as navigation (Guthrie, 1986), foraging (Kimbell & Morrell, 2016), predator avoidance (Seppälä et al., 2008), and identification of hetero- and conspecifics (Saverino & Gerlai, 2008), all of which suspended sediments can impede (Kimbell & Morrell, 2015; Sohel & Lindström, 2015). For example, suspended particulates reduce foraging efficiency of European perch (*Perca fluviatilis* Linnaeus 1758) on water fleas (Cladocera) (Nurminen et al., 2010), impair predator detection in three-spined stickleback (*Gasterosteus aculeatus* Linnaeus 1758; Sohel & Lindström, 2015), and dampen mating preferences for conspecifics in blacktail shiners (*Cyprinella venusta* Girard 1856; Glotzbecker et al., 2015). Fish therefore represent ideal subjects for understanding the effects of suspended sediments on behavior, both due to their visual nature and their importance to aquatic ecosystems.

Unfortunately, in the laboratory sediment tends to fall out of suspension over time, especially in still or calm waters, requiring operator effort or specialized equipment to maintain consistent suspended sediment levels. Common methods to deal with sediment settlement in the laboratory include adding sediment throughout the experiment (Van de Meutter et al., 2005; Suriyampola et al., 2018), completing the experiment before the sediment settles (Borner et al., 2015; Murray et al., 2016; Chamberlain & Ioannou, 2019), and increasing water turbulence with either air pumps or a mechanical stirring device (Sutherland, 2006; Gray et al., 2012; Kimbell & Morrell, 2015). Each of these methods presents its own drawbacks: addition of sediment alters the overall sediment levels, completion before sediment settling precludes longer-term experiments, and turbulence generally requires specialized equipment

or researcher intervention to maintain. Suspended sediments can also clog experimental equipment, requiring additional maintenance during studies. For example, in a long-term study by Gray et al. (2012) tank filters had to be removed during experiments to avoid sediments clogging the filters, requiring the researchers to conduct more frequent water changes to minimize waste buildup. Furthermore, the relative importance of maintaining consistent sediment levels during experiments is currently unknown: to our knowledge, no previous research has compared the effects of stable versus variable suspended sediment levels on aquatic organisms.

As an alternative to working with sediment, some researchers have instead attempted to “simulate” the visual effects of suspended sediments through manipulation of the lighting environment directly. For example, Swanbrow et al. (2012) tinted the glass of their aquariums to simulate the effects of ~30 nephelometric turbidity units (NTU) of suspended particulates on the antipredator behavior of fountain darters [*Etheostoma fonticola* (Jordan & Gilbert 1886)]. Sundin et al. (2010) used semi-transparent dividers between fish to simulate an unspecified level of suspended particulates to determine the effects of turbidity on pipefish (*Syngnathus typhle* Linnaeus 1758) mate choice. Duston and Astatkie (2012) compared feeding trials under light versus complete darkness to draw conclusions about the ability of larval striped bass [*Morone saxatilis* (Walbaum 1792)] to hunt under turbid conditions. However, the question remains as to whether or not these simulations truly replicate the effects of suspended sediments.

These alternative methods primarily reduce the amount of ambient light reaching the aquarium or absorb light as it travels through the water. However, although low light and suspended sediments both limit visibility, they do so in different ways. Suspended sediments primarily scatter light and thus shorten visual range (Ortega et al., 2020) while introducing noise to the visual signal (Fouilloux et al., 2022), whereas low light primarily reduces illuminance and alters the visible light spectrum (Davies-Colley & Smith, 2001; Fouilloux et al., 2022). Additionally, there is rather mixed empirical evidence for the hypothesis that aquatic animals respond similarly to both turbidity and low light. For example, crucian carp [*Carassius carassius* (Linnaeus 1758)] showed stronger reactions to predator cues under

turbid conditions than under colored water (Ranåker et al., 2012) while zebrafish [*Danio rerio* (Hamilton 1822)] held under low light conditions showed similar visual orientation behaviors to conspecifics held under turbid conditions (Suriyampola et al., 2018, 2020). However, to our knowledge no research to date has directly compared the broader, general behavioral responses of aquatic animals to scattered light (caused by suspended sediments) versus low light.

We tested how scattered versus low light conditions influence movement and social behavior of zebrafish by comparing the effects of suspended sediments and dye. Dyeing the water alters the light as it passes through the water in a similar manner to suspended sediments rather than altering the external light source before it reaches the water (Duston & Astatkie, 2012; Sundin et al., 2010; Swanbrow et al., 2012). However, unlike suspended sediments, dye does not settle out of water, which would make it an ideal substitute for suspended sediments in laboratory experiments should dye and sediment effects prove to have similar impacts on fish. Pond dye is also increasingly being used for ecosystem management of algae growth or to artificially improve the aesthetic appearance of water bodies despite relatively little research on its ecological effects, so research on dye's effects on aquatic organisms is important in its own right (Cuthbert et al., 2024).

In addition, we compared the effects of fluctuating versus stable visibility across both scattered and low light conditions in order to determine the importance of variable visibility on the overall impacts of low water clarity. Like most environmental conditions, in the real world visibility often changes over time. Most previous research on the effects of fluctuating environmental conditions has focused on thermal acclimation (Koussoroplis et al., 2017) and this body of work is revealing that fluctuating thermal conditions can have stronger impacts on animals that do stable temperatures (Morash et al., 2018). Evaluating the effects of fluctuating visual conditions on fish behavior is therefore not only important for understanding the importance of using stable versus fluctuating suspended sediment exposures in research, but also expands our understanding of the effects of fluctuating environmental conditions on ecosystems more broadly.

We used zebrafish as our study subjects because both their visual system (Bilotta & Saszik, 2001;

Fleisch & Neuhauss, 2006) and behavior (Kalueff et al., 2013) are well characterized, and because they are a highly social, shoaling species (Saverino & Gerlai, 2008; Dreosti et al., 2015; Suriyampola et al., 2015), making them good models to study impacts of impaired visibility on social behaviors. Furthermore, zebrafish originate from South Asia (including India and Bangladesh), a region known for frequent turbidity fluctuations brought on by rain (Panda et al., 2011; Suriyampola et al., 2015; Clift & Jonell, 2021) which we expect led to the evolution of behavioral adaptations to fluctuating visibility. These characteristics make zebrafish a particularly useful model study species for understanding adaptation to the effects of scattered light versus low light conditions on fishes more generally.

We predicted that low light conditions produced by dye (which absorbs light) and restricted visual range produced by suspended sediments (which scatters light) would produce contrasting behavioral responses in zebrafish because zebrafish are likely more adapted to suspended sediments. We tested this by conducting a series of three experiments. In Experiment 1, we used kaolin clay to create suspended sediment conditions and used frequent water agitation to maintain the sediment in suspension. In Experiment 2 we replicated the methods of Experiment 1, including water agitation, but instead of using suspended sediments we used black pond dye to create low light conditions. In Experiment 3 we again created low light conditions using black pond dye but did not include water agitation which allowed us to evaluate the effects of the water agitation itself. Within each experiment we also compared the responses of fish exposed to stable visual environments (both a clear control environment and a visually impaired environment) and a fluctuating visual environment (high amounts of sediment/dye alternated with low amounts of sediment/dye). This allowed us to determine if the fluctuating sediment levels common to both suspended sediments in research and natural environments have a stronger impact on fish behavior versus stable sediment and dye levels. Specifically, we tested how both scattered and low light conditions affected the fish's (1) movement speed and activity levels, (2) group coordination and cohesion, and (3) within-group social interactions.

Materials and methods

Ethics

Experimental procedures were approved by the Animal Research Ethics Board of McMaster University (Animal Utilization Protocol number 22–03–09) and were conducted in compliance with the guidelines set by the Canadian Council on Animal Care (CCAC). No fish died or were injured during any of the experiments.

Subjects and housing

All experiments took place at the Aquatic Behavioural Ecology Laboratory's (ABEL) aquatics facility at McMaster University (Hamilton ON, Canada). We used adult wildtype zebrafish purchased from a commercial supplier on February 11, 2022. Fish were initially maintained in a 386.4 l polyethylene plastic oval stock tank (Tuff Stuff Products, Terra Bella CA, USA) at room temperature (21.6–22.8 C) for 105 days before the first experiment. The light cycle was a 12:12 h light:dark cycle (lights on 9:00 AM and off at 9:00 PM) with a 30 min fade on/off period to simulate dawn and dusk. Light was provided using a combination of 13T8/MAS/48–840/1F2/P/DIM 10/1 Philips LED tubes and F32T8/TL835 Philips Fluorescent tubes (Koninklijke Philips N.V., Amsterdam, Netherlands), with a room illuminance of 796 lx. Fish were fed Zeigler Adult Zebrafish Diet (Zeigler Bros, Inc., Gardners PA, USA) until satiation once daily, six days a week prior to the experiment and every day during experiments. Between experiments fish were returned to the stock tank, where they were monitored for any potential delayed negative reactions from experimental conditions. We observed no adverse health or stress behaviors following experiments, and all fish appeared to resume normal behavior in the stock tank within one to two days.

Experimental apparatus

The same experimental tanks were used for all three experiments. Each experimental tank was a 29.5 l glass aquarium that was 25 cm wide×31 cm long×31 cm high. Each tank was fully isolated with no water flowing between tanks. The floors of all tanks were externally coated with opaque white

adhesive film to increase contrast for filming. The left and right sides of each tank were also an opaque white to prevent neighboring groups from visually interacting. The lighting environment was the same as in the stock tank (see above), with the opaque barriers limiting light entering from the covered sides. All experimental tanks, regardless of treatment, contained a single air stone but were otherwise bare. Throughout the course of all experiments, we maintained the water height at 8 cm to both minimize vertical movement during behavioral recordings and ensure that the fish could be easily observed even in the lowest visibility treatments.

Experiment 1: clay turbidity exposure

On May 27, 2022 we created 24 shoals consisting of four fish each, two male and two females ($n=120$ total fish), using haphazard selection from the stock tank. We weighed (mean mass±standard error; males= 0.77 ± 0.02 g, females= 0.91 ± 0.03 g) and photographed (with an iPhone 12 mini; Apple Inc., Cupertino, CA, USA) all fish during initial shoal creation (one female fish's photo was lost). A single observer (HMA) later analyzed the photos in ImageJ (Schneider et al., 2012) and used them to measure the standard length (SL) of all fish (mean length±standard error, males= 37.5 ± 0.3 mm, females= 37.6 ± 0.3 mm). After measurement, shoals were randomly assigned to both a tank and treatment condition. Fish sizes did not significantly differ across the treatment conditions (ANOVA; mass, $F=2.21$, degrees of freedom (df)=2, $P=0.12$; SL, $F=3.04$, df=2, $P=0.05$). We allowed the fish shoals to habituate to the experimental tanks and procedures for one day before starting the experiment. During this habituation period, we conducted one 50% water change at 9:00 AM and replicated the stirring regime (described below) used throughout the experimental treatments. Following the habituation period, the experiment lasted for two days.

To create suspended sediment conditions and reduce the visual range of the fish we used kaolin clay (Amson Naturals, Richmond Hill ON, Canada). Kaolin clay is considered safe for use with fish and is frequently used in suspended sediment studies (Leahy et al., 2011; Borner et al., 2015; Suriyampola et al., 2018; Chamberlain & Ioannou, 2019). To measure the approximate degree of light scattering, we used

an HI98703 Turbidity Portable Meter (Hanna Instruments Inc., Smithfield RI, USA) in nephelometric turbidity units (NTU). For reference, water in a drinking glass below 4 NTU would appear clear to the naked human eye (World Health Organization, 2017), an otherwise clear stream can increase to a turbidity level of 65 NTU following rainfall (Loperfido et al., 2010), and highly polluted waterways can regularly reach 77 NTU (Miguel-Chinchilla et al., 2019).

We recognize that turbidity measurements between different brands or models of nephelometric turbidity meters may sometimes vary and therefore represent only an approximation of light scattering and visual range (Rymaszewicz et al., 2017; Davies-Colley et al., 2021). Nephelometric turbidity measurements were used in our study both due to their historical importance in the field of fish ecology (Ortega et al., 2020; Rodrigues et al., 2023) and due to the technical challenges of using more reliable measurement techniques such as the “black disc” method (Davies-Colley, 1988; Kilroy & Biggs, 2002) or a beam transmissometer (Davies-Colley & Smith, 2001) during experimental proceedings.

To increase the scientific relevance and reliability of our turbidity measurements, we also measured the water clarity of each of our three treatment conditions using spectrophotometry (see below), a turbidity tube, and a modified version of the black disc and SHMAK clarity tube methods (Davies-Colley, 1988; Kilroy & Biggs, 2002). The turbidity tube was a 120 cm length graded, clear tube (Dynamic Aqua Supply Ltd., Surrey BC, Canada) with a 4.3 cm diameter Secchi disc at the bottom. We measured water clarity with the turbidity tube by holding the tube vertically, filling it with water, and then releasing water through the valve until the black and white wedges on the Secchi disc first became visible (Online Resource Table 1). The depth of the remaining water (in centimeters) represented water clarity, with a value close to 120 cm representing high water clarity and a value close to 0 cm indicating low water clarity. For the black disc test, we filled a 2.5 m long plexiglass aquarium with water and measured the maximum distance (in centimeters) at which a 2 cm diameter matte black disc suspended on a rod was just visible when viewed horizontally through the water (Online Resource Table 2).

We divided the fish shoals evenly between three treatment conditions: clear water, stable moderate suspended sediments, and fluctuating suspended

sediments where the suspended sediment level fluctuated between high and moderate sediment levels (total shoals per treatment, $n=8$). For brevity, we will refer to these as the clear, stable, and flux treatments throughout the remainder of the manuscript. For the clear and stable treatments, we maintained consistent visibility (as approximated with nephelometric turbidity measurements) throughout the experiment: 1.3 ± 0.1 NTU for the clear condition and 24.1 ± 1.0 NTU for the stable condition. For the flux treatment the turbidity fluctuated between a high value in the morning (low visibility, 44.0 ± 1.5 NTU) and a low value equivalent to the stable condition in the afternoon (moderate visibility, 23.5 ± 1.1 NTU). To reduce sediment settling rates and to maintain consistent turbidity levels, we stirred all tanks in a stereotyped zig-zag pattern with a plastic spatula every hour during the light cycle.

Across all tanks, we performed two 50% water changes every day: one in the morning (10:00 AM) and one in the afternoon (4:00 PM). For the stable and flux treatments we mixed clay and water in separate containers from the experimental tanks until we reached the target turbidity, then introduced the mixture into the tanks during the water change. For the clear control condition, we prepared the water identically to the stable and flux water except without adding clay. Thirty minutes after each water change, we checked the turbidity of two randomly chosen tanks per treatment (out of eight; total $n=6$) to track experimental turbidity.

Experiment 2: stirred dye exposure

The methods for this experiment, including the one day habituation period, were identical to Experiment 1 except where specified below. On July 28, 2022, we created 24 shoals of fish. In this experiment, male fish had an average body mass of 0.76 ± 0.02 g and SL of 39.3 ± 0.4 mm while female fish had an average mass of 0.87 ± 0.03 g and SL of 38.9 ± 0.3 mm. There was no significant difference in fish sizes across the three treatment groups (ANOVA; mass, $F=0.68$, $df=2$, $P=0.51$; SL, $F=1.34$, $df=2$, $P=0.27$). In this experiment, instead of clay we used black pond dye (Outdoor Water Solutions, Springdale AR, USA to create low visibility conditions. Pond dye is non-toxic for fish and similar pond dyes have been used in

other fish studies with no ill effects (Bristow et al., 1996; Ludwig et al., 2010; Tucker & Mischke, 2020). Because pond dye primarily absorbs rather than scatters light our turbidity meter could not effectively measure visible differences in pond dye concentrations (Rodrigues et al., 2023). We therefore primarily refer to the turbidity tube water clarity measurements below, with turbidity meter and black disc test measurements also available in Online Resource Tables 1 and 2. For reference, in kaolin clay a value of 60 NTU with the turbidity meter is approximately equivalent to 15 cm with the turbidity tube and 30 NTU is approximately 30 cm (Online Resource Table 1).

We again maintained consistent visibility conditions within the clear and stable treatments: >120 cm of visibility from the Secchi disc for the clear condition (no added dye) and 115 ± 0.9 cm of visibility from the Secchi disc for the stable condition (5 drops dye/28 l of water, $\sim 45 \mu\text{L}$ dye/L water). For the flux treatment, the dye fluctuated between a low visibility value in the morning (5 drops dye/14 l of water, $\sim 89 \mu\text{L}$ dye/l water; 90 ± 1.4 cm Secchi disc), and a high visibility value equivalent to the stable condition in the afternoon (5 drops dye/28 l of water, $\sim 45 \mu\text{L}$ dye/L water; 115 ± 0.9 cm Secchi disc). Though dye does not settle out of water, for consistency we followed the same stirring regime as in Experiment 1.

We were unable to sample the visibility of the tanks as frequently we did in Experiment 1 without impacting the water volume of the tanks because the turbidity tube requires a much larger water sample than the turbidity meter. However, during a 24-h pilot, dye visibility did not change noticeably, so unexpected changes in visibility throughout the experiment were unlikely (see Online Resource Table 3).

Experiment 3: unstirred dye exposure

In this experiment we replicated the methods of Experiment 2 but without the stirring regime to determine if there were any effects of stirring on fish behavior. We formed 24 shoals on August 11, 2022 using the same methods described in Experiment 2. Male fish had an average body mass of 0.79 ± 0.02 g and SL of 39.3 ± 0.3 mm (two photos/body length measurements were lost) while female fish had an average mass of 0.86 ± 0.03 g and SL of

38.9 ± 0.3 mm. There was no significant difference in body size between treatment groups (ANOVA; mass, $F=0.76$, $df=2$, $P=0.47$; SL, $F=1.64$, $df=2$, $P=0.20$).

Behavioral recordings

We filmed the behavior of all shoals one hour after each water change for a total of four recordings per shoal (96 videos per experiment), with one recording at midday (12:00 PM) and one in the evening (6:00 PM) on both days of the experiment. Recording methods were the same for all three experiments. We used Canon VIXIA HF R800 cameras (Canon Inc., Tokyo, Japan) mounted vertically above each tank and recorded for 15 min, with the first 5 min of each recording discarded due to potential human disturbance of the fish at the start of filming. Air stones were temporarily removed during filming to enhance visibility and then returned immediately after recording. For the dye experiments (Experiments 2 and 3) we additionally floated a transparent matte plastic film on top of the water during filming to diffuse glare from the overhead lights and soften the edges of any reflections, which interfered with our tracking program (see below). Due to the high contrast between the darker coloration of the fish against the white background of the tank floor, the plastic film did not interfere with the ability to observe the fish.

We used the automated tracking program TRex (version 1.1.6; Walter & Couzin, 2021) to track fish movement and positioning in our recordings. We manually used TRex's inbuilt preview window (TGrabs) to determine the threshold value for averaging out the background of the video versus detection of the fish (the background averaging threshold) and used the mean averaging mode for all videos except when fish movement was minimal, in which case the mode averaging method provided more accurate results. We calibrated the TRex outputs using TRex's inbuilt calibration tool and a known distance. All videos were manually reviewed following processing by TRex to verify successful position tracking. Due to difficulties with the dyed water producing more reflections that obscured the fish during filming and reducing the contrast between the dark fish and the white background, one video from Experiment 2 (stirred dye) and two videos from Experiment 3 (unstirred dye) were excluded from final analyses due

to poor tracking quality (hence 285 out of 288 total videos were used; see Discussion for full description of logistical difficulties filming dyed water). We then used the TRex coordinate and orientation outputs to calculate whole-shoal movement and positioning values for each video. We calculated the mean and maximum speed (cm/s), the proportion of time fish spent active (activity, range 0 to 1), the shoal area (cm²), the mean neighbor distance (cm), and the circular variance of fish orientation (polarity, range 1 to 0).

In brief, we calculated speed using the distance in centimeters that each fish moved between frames (~1 s/30 frames) and then took the mean and maximum values of each shoal for each recording. We calculated activity by manually reviewing videos to calibrate a speed under which fish were not engaged in active movement (1.5 cm/s), and then we classified the fish movement between each frame as “active” if the speed was greater than 1.5 cm/s and “inactive” if the speed was less than or equal to 1.5 cm/s. We then calculated the proportion of the time each fish spent active in relation to the total video time. We calculated shoal area and mean neighbor distance by drawing lines between the centroids of each fish; for shoal area, we used the non-crossing lines to create a polygon and calculated the polygon’s area and for mean neighbor distance we averaged the lengths of all lines (Fig. 1). Both shoal area and mean neighbor distance can be considered measures of shoaling or social cohesion (Shelton et al., 2014). We calculated polarity by taking the circular variance of the orientation of all fish (in radians) and averaging it; in other words, by determining how similar the angles of all fish were on a scale of one to negative one, where values close to one indicate all fish are facing similar directions and values close to zero indicate all fish are facing in dissimilar directions (Fig. 1). Polarity can be considered a measure of social coordination (Shelton et al., 2014). For all of these measures we manually corrected for noise in TRex’s outputs using rolling averages. The full code and explanation of calculations is available at <https://doi.org/10.5281/zenodo.18378650>.

We also used Behavioral Observation Research Interactive Software (BORIS; Friard & Gamba, 2016) to score three aggressive behaviors (aggressive contact, chasing, lateral display), and one affiliative (following) behavior. The behaviors were based on the definitions described by Kalueff et al. (2013). In brief,

we defined “aggressive contacts” as any physical contact (except spawning) between two fish where one fish pulled away or flinched from the other. “Chasing” was defined as the rapid pursuit of one fish by another fish where both fish exhibited rapid tail beating and stayed within 1.0–1.5 body lengths of each other. “Lateral display” was defined as a brief display where two fish lining up parallel to each other so the head of one fish was opposite the tail of the other fish with both fish quivering in place. “Following” was similar to chasing in that one fish followed in close proximity to another (up to two body lengths apart); however, the movement was slower and occurred without rapid tail beating. For lateral displays, chasing, and following we recorded both the number of behaviors and the mean duration of each behavior. Aggressive contacts were extremely brief, so we recorded only their number. For consistency with the TRex video scores, all three videos excluded from the TRex analyses were also excluded from the BORIS analyses.

Spectrophotometry

To measure the relative spectral composition of the light environment during each of our experimental conditions we measured the light absorbance of each treatment using an Ocean Optics Flame S-XR1-ES (200–1025 nm) spectrophotometer with a DH-Mini Deuterium Tungsten Halogen (200–2500 nm) light source passed through a plastic cuvette (Ocean Optics, Orlando FL, USA). We prepared three solutions of each treatment concentration (high concentration clay, low concentration clay, high concentration dye, low concentration dye) with clear water used as a reference sample. We sampled each solution five times except for solution 3 of the high concentration clay, which was only sampled four times (n=19 total samples). We used an integration time of 37,850 μs and averaged 20 scans per sample. We simultaneously measured the visibility of all solutions using a turbidity meter and turbidity tube following the methods described above (Online Resources Table 1). We recognize that due to light scattering by the suspended sediment treatments, our suspended sediments absorbance measurements represent only relative absorbance rather than the true absorbance of these samples. Hence, we present them here as representations of the absorption patterns of our treatment

Table 1 Statistical results for the linear mixed models of tested principal components

Term	Wald ²	df	P value
<i>PC 1, Social Cohesion</i>			
Treatment	0.21	2	0.90
Experiment	0.73	2	0.69
Time	2.93	1	0.09
Treatment × Experiment	3.33	4	0.50
Treatment × Time	6.27	2	0.04
Experiment × Time	1.62	2	0.44
Treatment × Experiment × Time	2.52	4	0.64
Term	Wald ²	df	P value
<i>PC 2, Movement</i>			
Treatment	28.22	2	< 0.0001
Experiment	62.96	2	< 0.0001
Time	21.82	1	< 0.0001
Treatment × Experiment	4.28	4	0.37
Treatment × Time	10.60	2	0.005
Experiment × Time	0.03	2	0.98
Treatment × Experiment × Time	6.98	4	0.14
Term	Wald ²	df	P value
<i>PC 3, Pursuit</i>			
Treatment	2.78	2	0.25
Experiment	28.58	2	< 0.0001
Time	5.65	1	0.02
Treatment × Experiment	1.26	4	0.87
Treatment × Time	0.86	2	0.65
Experiment × Time	4.64	2	0.10
Treatment × Experiment × Time	0.41	4	0.98
Term	Wald ²	df	P value
<i>PC 4, Hierarchy Establishment</i>			
Treatment	3.89	2	0.14
Experiment	2.29	2	0.32
Time	0.10	1	0.75
Treatment × Experiment	9.22	4	0.06
Treatment × Time	1.16	2	0.56
Experiment × Time	8.71	2	0.01
Treatment × Experiment × Time	1.83	4	0.77

In the first column “treatment” refers to the visual treatment condition (clear, stable, flux), “experiment” refers to the experiment (Experiment 1, stirred clay; Experiment 2, stirred dye; Experiment 3, unstirred dye), “time” refers to the time of day of the recording (midday, evening) and “x” is used to indicate an interaction (example: “experiment x time” indicates an interaction between the experiment and time factors). The Wald² in the second column is the test statistic. “df” in the third column stands for “degrees of freedom.” The fourth column is the p-value, where bold values indicate $P < 0.05$.

conditions and to provide a fuller characterization of our treatment visual conditions.

We processed the light absorbance spectra using R package ‘pavo’ (version 2.9.0; Maia et al., 2019).

To account for sensor noise, we averaged together all spectra from the same solution and smoothed the spectral curves using a span of 0.25. All spectra were plotted using the R package ‘ggplot2’ (version 3.5.1;

Wickham 2016, Fig. 2). The absorbance spectra of the clay treatment solutions was largely flat, with an average optical density (OD) of 0.07 ± 0.0003 for the high clay concentration and 0.05 ± 0.0003 OD for the low clay concentration. The absorbance spectra of the dye treatment solutions showed two peaks, one around 500 nm (green/cyan; high dye concentration 0.08 OD, low dye concentration 0.04 OD) and one around 630 nm (orange/red; high dye concentration 0.08 OD, low dye concentration 0.03 OD). The average OD for the high dye concentration was 0.05 ± 0.0006 and the average OD for the low dye concentration was 0.02 ± 0.0003 .

Statistical analyses

Statistical analyses were conducted in R (version 4.4.1; R Core Team, 2020) using a significance value of 0.05. All plots were created using the R package 'ggplot2' (version 3.5.1; Wickham, 2016). We conducted a Principal Component Analysis (PCA) using the base R function 'prcomp'. All data were centered and scaled within prcomp before analysis. We decided on the number of principle components (PCs) to include in our analysis by examining the loadings of all PCs and using those that best captured our research questions while maximizing the captured variance (Online Resource Fig. 1, Online Resource Tables 3 and 4). In total, we used four PCs which collectively accounted for 0.70 of the total proportion of variance (Online Resource Table 4). Shoal area

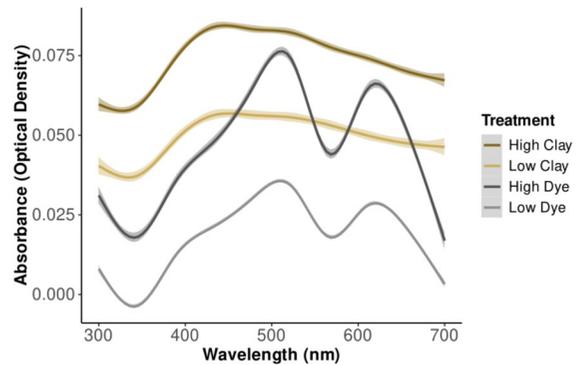


Fig. 2 Light absorbance spectra for all treatment concentrations of clay and dye. The clay treatments are represented by shades of tan, with dark tan representing the high concentration of clay and light tan representing the low concentration of clay. The dye treatments are represented by shades of grey, with dark grey representing the high concentration of dye and light grey representing the low concentration of dye. The envelopes around each line represents the 95% confidence intervals. The absorbance spectra of both clay concentrations are largely flat, with the high concentration centered around 0.07 optical density (OD) and the low concentration centered around 0.05 OD. The absorbance spectra of both dye concentrations show two absorbance peaks around 500 nm and 630 nm with peak OD of 0.08 for each peak. The remainder of the absorption for the high dye concentration is centered around 0.05 OD and the remainder of the absorption for the low dye concentration is centered around 0.02 OD

and mean neighbor distance primarily loaded onto PC 1, with shoal area and mean neighbor distance

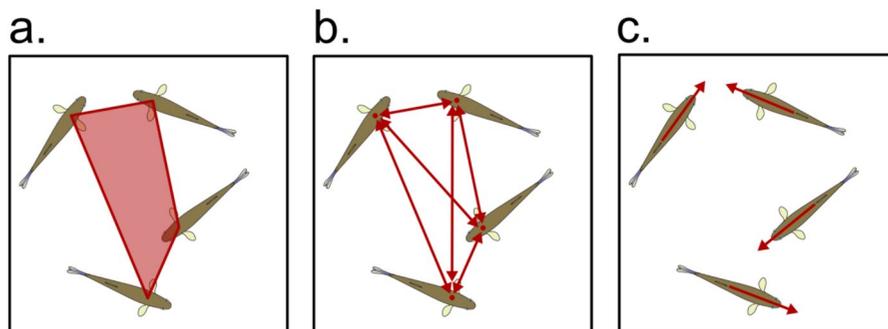


Fig. 1 Illustration of our measurements of shoal area, mean neighbor distance, and polarity. **a** illustrates the measurement of shoal area, where a line is drawn through the centroid of each fish to create a polygon, and the area of that polygon calculated. **b** shows the measurement of mean neighbor distance, where the distance between the centroids of each fish is calcu-

lated and then averaged together. **c** shows the measurement of polarity, where the angle of each fish is calculated and then the circular variance of the angles of all fish in the video is calculated on a scale of zero (all fish facing different directions) to one (all fish facing the same direction)

decreasing as PC 1 increased. We therefore consider PC 1 to be primarily a measure of social cohesion, with higher PC 1 values indicative of smaller distances between fish (higher cohesion). Mean speed, max speed, and activity primarily loaded onto PC 2, with all three values decreasing as PC 2 increased. We therefore consider PC 2 to be primarily a measure of fish movement, with smaller PC 2 values indicative of greater movement. Aggressive contacts, chasing, and following primarily loaded onto PC 3, with all three values increasing as PC 3 increased. We therefore consider PC 3 to be primarily a measure of pursuit or aggression, with higher PC 3 values indicative of greater pursuit. Lateral displays and polarity primarily loaded onto PC 4, with lateral displays increasing and polarity decreasing as PC 4 increased. Lateral displays are aggressive behaviors associated with hierarchy establishment (Schneider, 2011), and while polarity is not specifically associated with hierarchy establishment a negative relationship between polarity and lateral display is expected due to the reversed orientation that fish exhibit when performing the display. We therefore consider PC 4 to be primarily a measure of hierarchy establishment, with higher PC 4 values indicative of higher rates of hierarchy establishment.

We fit each PC with a linear mixed model using the 'glmmTMB' function in the R package 'glmmTMB' (version 1.1.9; Brooks et al., 2017). Treatment (clear, stable, and flux), experiment (clay, stirred dye, and unstirred dye), and time of recording (midday and evening) were included as fixed effects and shoal ID as a random effect to account for repeated measures. We used the 'simulateResiduals' function from the package 'DHARMA' (version 3.1.2; Hartig, 2022) to inspect the fit of each model before final analysis. We used the 'Anova' function in the package 'car' (version 3.1.2; Fox & Weisberg, 2019) to detect the significance of all main effects and interactions. We used the 'emmeans' function in the package 'emmeans' to calculate estimated marginal means, confidence intervals, and conduct post hoc contrasts (version 1.10.3; Lenth, 2024). For the significant main effects of experiment we conducted pairwise comparisons between Experiments 1 and 2 (clay versus dye) and Experiments 2 and 3 (dye with stirring versus dye without stirring) without directly comparing experiments 1 and 3. For the significant interactions

(treatment x time of recording and experiment x time of recording) we evaluated the pairwise comparisons of the predictor variables of primary interest (treatment and experiment, respectively) at each time of recording (midday and evening).

Results

Overall, zebrafish moved greater distances when exposed to dye compared to when exposed to suspended sediment, with their mean speed being 26% faster, their maximum speed being 15% higher, and their mean activity level being 8% higher in dye compared to when in suspended sediments. In contrast, the fish performed more pursuit behaviors in suspended sediments compared to dye, performing 24% more aggressive contacts, 126% more chases, and 116% more follows. The zebrafish also moved more in the fluctuating visibility treatments compared to in the clear treatments, with their mean speed being 25% faster, their maximum speed 6% higher, and their mean activity level being 11% higher in the flux treatment compared to the clear water treatment. In contrast, there was no significant difference between fish movement in the stable and clear water treatments.

Social cohesion (Principal Component 1)

Across all three treatments and experiments the mean shoal area was 40.6 ± 1.1 cm² (mean \pm standard error) and mean neighbor distance was on average of 11.2 ± 0.1 cm. We detected a significant interaction between treatment and time (Wald $\chi^2 = 6.27$, $df = 2$, $P = 0.04$; Table 1), which would suggest the effect of treatment varied between the midday and evening recordings. However, this significant interaction did not remain in the post hoc analysis (Online Resource Table 7) and therefore is not a robust result. We detected no differences in the amount of social cohesion between the clay and stirred dye experiments or between the two dye experiments (Table 1, Online Resource Table 6, and Online Resource Fig. 2). We also detected no differences across our treatments (clear, stable, and flux).

Movement (Principal Component 2)

Across all three treatments and experiments the mean fish speed was 7.3 ± 0.1 cm/s, the maximum speed was on average 19.8 ± 0.3 cm/s, and the proportion of time fish were active (range 0–1) was an average of 0.9 ± 0.01 , meaning that the fish were active for ~90% of the trial time. Again, we detected a significant interaction between treatment and time (Wald $\chi^2 = 10.60$, $df = 2$, $P = 0.005$; Table 1, Fig. 3, and Online Resource Fig. 3), indicating that the effect of treatment varied by the time of day of the recording. Post hoc analyses of this result showed that fish movement was higher in the flux condition compared to the clear condition across both the midday and evening recordings (midday, t -ratio = 6.16, $P < 0.001$; evening, t -ratio = 3.00, $P = 0.01$; Table 1, Fig. 3, and Online Resource Tables 6 and 7) with

mean fish speed being on average 25% higher, maximum fish speed being 6% higher, and activity being on average 11% higher in the flux condition than in the clear water condition. At midday fish movement was also greater in the flux condition compared to the stable condition (t -ratio = 4.01, $P < 0.001$; Tables 1 and Online Resource Table 7); however, we did not observe these effects in the evening (Online Resource Table 7), meaning there was no clear difference in fish movement between the flux and stable conditions when visibility was equivalent between the two conditions. We also observed more movement in the stirred dye experiment than in the clay experiment (t -ratio = -6.46, $P < 0.001$; Table 1, Fig. 4a Online Resource Fig. 3, and Online Resource Tables 6 and 7). The mean fish speed was on average 26% higher, the maximum fish speed was 15% higher, and the fish were 8% more active in dye compared to clay. There

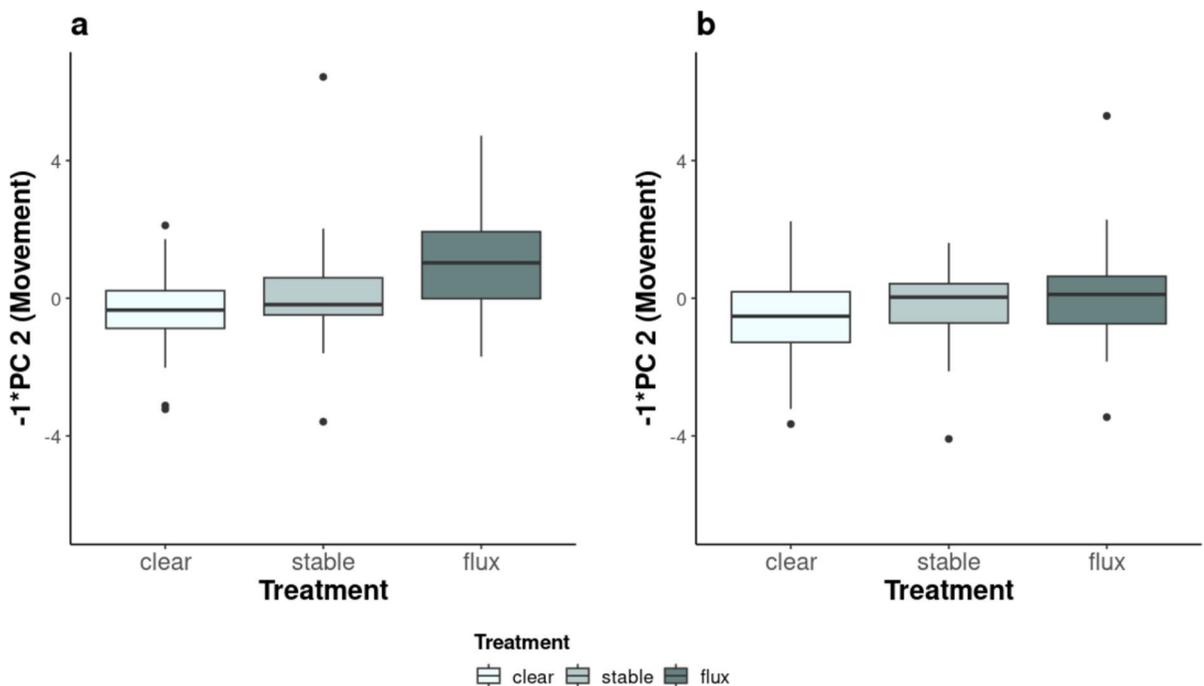


Fig. 3 Fish movement (as captured by PC 2) across time of recording and dye treatment condition. Movement is presented here as $-1 \times \text{PC 2}$ so that increasing values on the y-axis are indicative of increasing levels of movement. **a** represents the midday recordings and **b** represents the evening recordings. The clear, stable, and flux dye treatments are represented by increasingly dark shades of blue starting with the palest for clear, the middle shade for stable, and the darkest for flux. Both figures show data centered around zero. In the midday record-

ings, fish movement is higher in the flux treatment than both the clear and stable treatments, while in the evening recordings fish movement is only higher in the flux treatment compared to the clear treatment. The middle line of each box plot represents the median, the lower and upper hinges respectively represent the first and third inter-quartiles, and the upper and lower whiskers respectively extend to the largest and smallest datapoint with $1.5 \times$ the interquartile range of the corresponding hinge

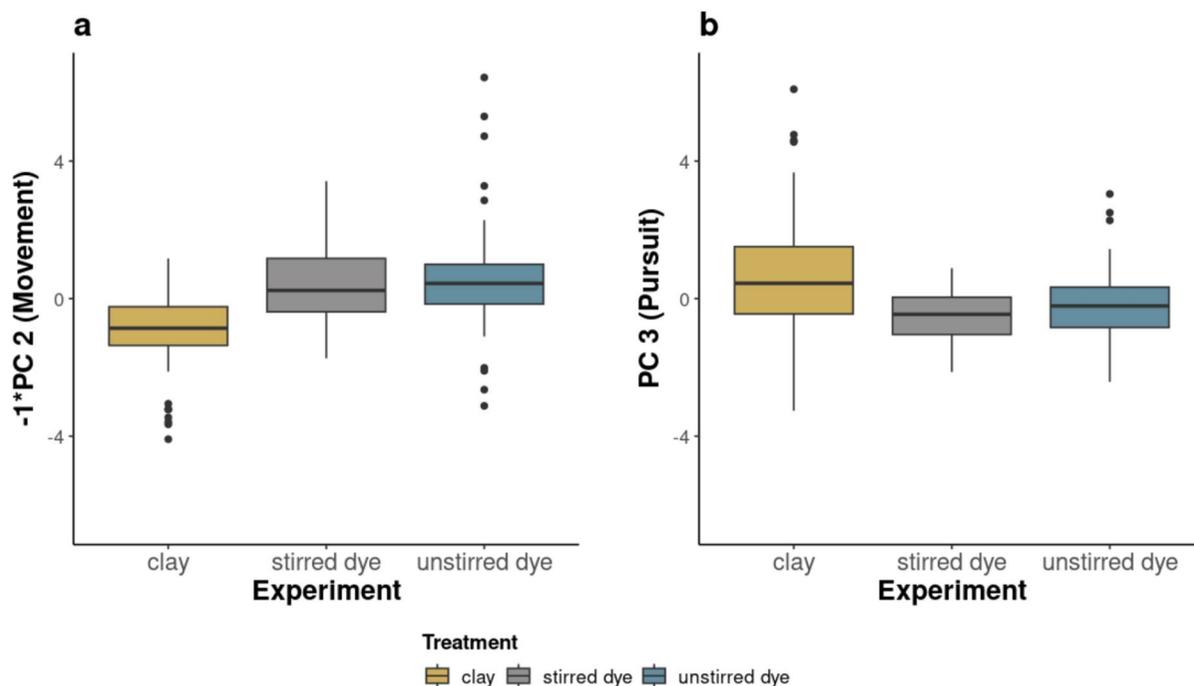


Fig. 4 Fish movement (as captured by PC 2) and pursuit behaviors (PC 3) across all three experiments. In both sub-figures the clay experiment is represented by tan, the stirred dye experiment by grey, and the unstirred dye experiment by blue. The middle line of each box plot represents the median, the lower and upper hinges respectively represent the first and third inter-quartiles, and the upper and lower whiskers respectively extend to the largest and smallest datapoint with $1.5 \times$ the interquartile range of the corresponding hinge. **a** represents fish movement, with movement presented as $-1 \times \text{PC 2}$ so that increasing values on the y-axis are indicative of increas-

ing levels of movement. **a** shows data centered around zero, with movement values lowest in the clay experiment and similar across the stirred and unstirred dye experiments. **b** represents fish pursuit behaviors, with increasing values on the y-axis being indicative of increasing levels of pursuit behaviors. **b** shows data clustered around zero, with higher numbers of pursuit behaviors performed in the clay experiment compared to the dye experiments, and similar numbers of pursuit behaviors performed between the stirred and unstirred dye experiments

was no clear difference in movement between the two dye experiments (Fig. 4a, Online Resource Fig. 3, and Online Resource Table 7), suggesting that the stirring regime had minimal effect on fish movement.

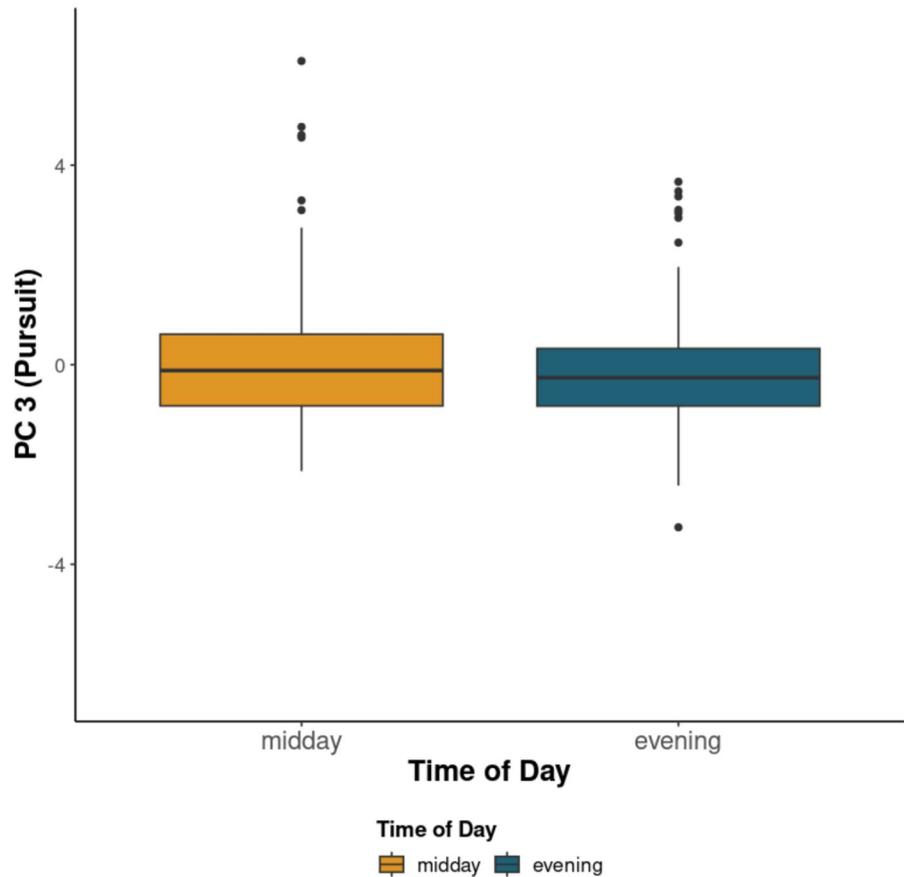
Pursuit (Principal Component 3)

Across all three treatments and experiments there were an average of 26.8 ± 1.4 aggressive contacts 33.4 ± 1.7 chases, and 14.9 ± 0.9 follows. Pursuit behavior was higher in the clay experiment than in the stirred dye experiment ($t\text{-ratio} = -5.15$, $P < 0.001$; Table 1, Fig. 4b, Online Resource Fig. 4, and Online Resource Table 6 and 7). The number of aggressive contacts were on average 24% higher,

chases were on average 126% higher, and follows were on average 116% higher in clay compared to dye. There was no clear difference between the two dye experiments (Online Resource Table 7), again suggesting that the stirring regime had minimal effect on pursuit behaviors.

Our visibility treatments had no effect on fish pursuit behaviors (Table 1, Online Resource Fig. 4, and Online Resource Table 6). Pursuit was overall higher at midday compared to the evening (Wald $\chi^2 = 5.65$, $df = 1$, $P = 0.02$; Fig. 5). There were on average of 19% more aggressive contacts, on average of 15% more chases, and on average of 8% more follows in our midday recordings compared to the evening recordings.

Fig. 5 The frequency of pursuit behaviors (as captured by PC 3) across time of day. Increasing values of PC 3 are indicative of increasing levels of pursuit behaviors. The midday recordings are represented by orange and the evening recordings by dark blue. The middle line of each box plot represents the median, the lower and upper hinges respectively represent the first and third interquartiles, and the upper and lower whiskers respectively extend to the largest and smallest datapoint with $1.5 \times$ the interquartile range of the corresponding hinge. The figure shows data clustered around zero, with higher numbers of pursuit behaviors performed in the clay experiment compared to the dye experiments, and similar numbers of pursuit behaviors performed between the stirred and unstirred dye experiments



Hierarchy establishment (Principal Component 4)

Across all treatments and experiments there were an average of 2.8 ± 0.2 lateral displays and polarity was an average of 0.48 ± 0.002 (range 0 to 1). We detected a significant interaction between experiment and time (Wald $\chi^2 = 8.71$, $df = 2$, $P = 0.01$). Post hoc analysis of this interaction revealed a non-significant trend toward more hierarchy establishment behaviors in the unstirred dye compared to the stirred dye experiments at midday (t-ratio = 2.16, $P = 0.06$; Table 1, Online Resource Fig. 5, and Online Resource Table 7), with lateral displays being 58% higher when we did not stir compared to the stirred dye experiment. We did not observe this trend in the evening (Online Resource Table 7). We detected no difference in the degree of hierarchy establishment between the clay and stirred dye experiments (Table 1, Online Resource Fig. 5, and Online Resource Table 6). The fish did not show

differences in the degree of hierarchy establishment across the three visibility conditions (Table 1).

Discussion

Suspended particulate matter can alter social interactions (Kimbell & Morrell, 2016; Aspbury et al., 2019), reduce prey capture rates (Salonen & Engström-Öst, 2013; Cano-Rocabayera et al., 2020; Ortega et al., 2020), and inflict long-term impacts on visual development in aquatic animals (Ehlman et al., 2015; Goerger et al., 2021). Precisely controlling suspended particulate levels under lab conditions requires additional equipment and/or experimenter effort (Sutherland, 2006), which has caused some researchers to “simulate” turbidity with low light conditions (Sundin et al., 2010; Swanbrow et al., 2012). Here, we tested if low light levels produced by dye (via light absorption) and restricted visual range by

kaolin clay (via light scattering) would cause contrasting behavioral responses in zebrafish. As predicted, we found that the effects of black pond dye were not equivalent to the effects of kaolin clay, with fish being faster and more active (PC 2) and performing fewer pursuit behaviors (PC 3) under dye conditions compared to clay. Furthermore, even a short two day exposure to fluctuating visibility was sufficient to cause differences in fish movement (PC 2) compared to consistently poor visibility.

Differences between clay and dye

Fish moved faster and spent more time active under dye conditions compared to clay (PC 2) but performed more pursuit behaviors under clay conditions compared to dye (PC 3), suggesting black pond dye does not effectively simulate suspended sediment conditions. Fish movement in particular (PC 2) appeared to be affected more strongly by dye than by clay (Fig. 4a). Our results contrast with earlier research that found zebrafish showed similar capacity to detect moving visual stimuli under both suspended sediments versus low light conditions (Suriyampola et al., 2018, 2020). This suggests that tests of relative sensory capacity alone may not be sufficient to predict actual behavior. Instead, a combination of both sensory and behavioral testing is necessary to fully understand the interactions between the broader visual environment and adaptation at the sensory level.

For example, imagine a hypothetical environment where low light conditions are primarily caused by trees casting shadows, which the fish can easily swim away from, while suspended sediments are primarily caused by rain stirring up sediment, which may be harder for fish to escape from until the sediment settles down naturally. In this hypothetical scenario, fish may evolve to move faster when in low light but not change their movement in suspended sediments even if the relative visibility was the same under both conditions, which would produce a result where measured sensory ability did not predict behavior.

In the case of our fish, the low light conditions caused by pond dye may have interfered with the fish's circadian cycles, extending the "dawn" period of the light cycle when zebrafish are most active (Blanco-Vives & Sánchez-Vázquez, 2009; Ben-Moshe Livne et al., 2016; Doldur-Balli et al., 2024). Exactly why zebrafish are most active in the morning

is unknown, though it may be driven by their preference for feeding just before dawn and spawning just after dawn (Krylov et al., 2021). Alternatively, the dye itself could also have altered fish behavior in a way that the dim lighting used in previous research did not, though previous research on similar dyes suggest even long term use of dye has minimal effect on body composition (Ludwig et al., 2010; Tucker & Mischke, 2020) or on aggression (Bristow et al., 1996).

Dye logistical difficulties

Dye also poses its own unique methodological challenges primarily affecting recording. Water and glass both possess reflective surfaces which can cause glare or reflections that obscure aquatic subjects during filming, an effect that black pond dye exacerbates. Black dye also absorbs light, which reduces light penetration into the water and consequently reduces the visual contrast of the fish against their background. This reduced contrast increases the difficulty of direct observation of the fish by human observers (primarily on film, but in person as well) and can confound the automated tracking of fish when using a program such as TRex. Though we did not test any alternative tracking programs to TRex, we expect this issue to be similar across different tracking programs. The combination of increased glare and reflections and the reduced contrast ultimately determined the upper limit of dye concentrations we could use for our experiments.

We found glare could be minimized by placing a transparent matte film over the recorded surface and using more diffuse lighting, though the matte film blurred the subjects and the diffuse lighting reduced contrast. We minimized reflections by creating a uniform, unpatterned backdrop across from the reflecting surface. Glare and contrast may also be improved by filming aquaria from underneath with diffuse top-down lighting which we have used in other studies to increase usable dye concentrations (Anderson et al., in review).

The effects of fluctuating visibility on fish behavior

Overall, fluctuating visibility had a stronger impact on fish movement (PC 2) than did consistent but poor visibility: fish movement was consistently greater in the flux condition compared to the clear condition

while there was never a distinct difference in fish movement between the stable poor visibility and clear conditions. While water clarity was on average lower in the flux condition than the stable condition, the difference in fish movement seems to be primarily driven by experience with *fluctuating* visibility rather than *current* visibility. During the evening recordings, fish movement was higher in the flux condition compared to the clear condition while there was no notable difference between fish movement in the stable and clear conditions. During these evening recordings the flux and stable conditions had the same approximate water clarity (as estimated by turbidity meter and turbidity tube), so the difference in movement at this time could not have been caused by differences in visibility.

Fish movement (PC 2) was also greater in the flux condition compared to the stable condition during the midday recordings but not the evening recordings, reinforcing the conclusion that fluctuating visibility had a stronger effect on fish movement than stable visibility. Though the difference in fish movement between the flux and stable conditions could have been caused by the relatively lower water clarity in the flux condition during the midday recording, this is unlikely considering the similar observed difference between these treatments during the evening recordings (when water clarity was the same between both treatments). Zebrafish also tend to be more active during the morning compared to the afternoon (Hurd et al., 1998; MacPhail et al., 2009; Krylov et al., 2021), which could have increased the movement effects observed during the less active evening recordings. Higher zebrafish activity in the morning would similarly explain the greater number of pursuit behaviors performed during the midday recordings compared to the evening recordings.

The fact that the fish moved more during the flux treatment suggests that the fluctuating visual conditions were more stressful than stable visual conditions. Fish often increase their movement rates when stressed, likely as an attempt to escape the stressful environment and move to a less stressful environment (McDonnell & Chapman, 2015). Similarly, other fluctuating environmental conditions such as temperature have been shown to increase stress levels in fish compared to stable conditions (Morash et al., 2018), suggesting that fluctuating environmental conditions may be generally more stressful than stable conditions.

We propose that fluctuation should be considered as a factor separate, though not independent, from water clarity. Many studies investigate the effects of fluctuating visibility without incorporating a stable visibility control (Goerger et al., 2021; Heubel & Schlupp, 2006; Lowe et al., 2015; Van de Meutter et al., 2005) which, as seen in our results here, may result in a stronger effect than poor visibility alone. Furthermore, the fluctuation within natural environments itself should be studied further. With this research we tested one pattern of fluctuation: low visibility in the morning and high visibility in the afternoon. Higher suspended sediment levels in the morning followed by lower sediment levels in the afternoon is a pattern observed in many natural aquatic ecosystems (Cooper et al., 2016) but is by no means representative of all natural suspended sediment fluctuation patterns, particularly those that occur over longer timescales (Gillain, 2005; Loperfido et al., 2010).

Conclusions

Suspended sediment levels in aquatic habitats are changing rapidly at a global scale (Dethier et al., 2022) so further research into the behavioral responses of fish species to understand and mitigate the effects of suspended sediments is urgently needed. Exploring the effects of restricted visibility across a wide range of systems and species will identify those most vulnerable to changes in visual conditions and requiring conservation effort. In our experiments with zebrafish we compared the effects of visual range reduced by kaolin clay and lighting reduced by dye. We predicted that dye, which primarily absorbs light, would affect fish behavior differently than kaolin clay, which primarily scatters light. As predicted, fish responses to dye contrasted with responses to kaolin clay, resulting in more movement but less pursuit behavior in dye compared to clay, suggesting that dye cannot be used as a substitute for suspended sediments or other forms of suspended particulate matter in research. We also identified fluctuating visibility as another aspect of water clarity in need of further research in our rapidly changing world.

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Data availability All data is available at <https://doi.org/10.5281/zenodo.16739177> and all code is available at <https://doi.org/10.5281/zenodo.18378650>.

Declarations

Competing Interests The authors declare no competing or financial interests.

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