



Effects of multiple stressors on fish shoal collective motion are independent and vary with shoaling metric

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Collective movement is critical to the survival of some animals. Despite substantial progress in understanding animal collectives such as fish shoals and bird flocks, it is unknown how collective behaviour is affected by changes in multiple environmental conditions that can interact as stressors. Using a fully factorial repeated-measures design, we tested the independent and combined effects of darkness and acoustic noise on the collective motion of three-spined sticklebacks, *Gasterosteus aculeatus*, quantified using high-resolution tracking data from video. Corresponding to the importance of vision in shoaling behaviour, darkness increased nearest-neighbour distances and reduced coordination, measured as speed correlations and differences in directional heading between nearest neighbours. Although individual swimming speeds were not impacted by darkness, the group's centre of mass was slower, an emergent effect of reduced polarisation (i.e. greater group disorder) in darkness. While additional acoustic noise had no detectable effect on these variables, it altered group structure, with fish being more likely to be found side by side one another. Fish were also further from the arena wall (i.e. showed reduced wall following) when there was additional acoustic noise. There was only weak evidence for additive or interactive effects of the two stressors. Across the different environmental contexts, there were consistent, repeatable differences between groups (i.e. group personality variation) in the speed, turning angle and distance from the arena wall of individuals, but the only collective behaviour that was repeatable was group polarization. Our study demonstrates that multiple stressors can have independent effects that impact different aspects of behaviour and highlights the need for empirical studies on multiple stressors as their effects can be unpredictable.

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Collective behaviour can provide substantial benefits in finding resources (Garnier, Guérécheau, Combe, Fourcassié, & Theraulaz, 2009) and avoiding predators (Ioannou, 2017a). These benefits often depend on maintaining group cohesion through coordination of where and when to move (Conradt & Roper, 2005), the structure of how individuals are organized within groups (Calovi et al., 2015; Herbert-Read, Wade, Ramnarine, & Ioannou, 2019) and effective information transfer between individuals (Ioannou, 2017b; Lei, Escobedo, Sire, & Theraulaz, 2020). In turn, responding to group-mates depends on the sensory and cognitive abilities of individuals; for example in fish, both vision (Kowalko et al., 2013) and the lateral line (Faucher, Parmentier, Becco, Vandewalle, & Vandewalle, 2010)

are used during schooling behaviour. While great strides have been made in understanding collective motion in animals (Hemelrijk & Hildenbrandt, 2012; Herbert-Read, 2016), only a handful of studies have tested how group cohesion, structure and dynamics change under environmental conditions that potentially interfere with the ability of individuals to behave collectively.

In animal groups that move collectively such as fish shoals and bird flocks, individuals are generally understood to use cues from the position and movement of other individuals, rather than intentional signals, to coordinate movement (Ioannou, Couzin, James, Croft, & Krause, 2011; Lemasson et al., 2018). Sudden accelerations or changes of direction may indicate that a neighbour has detected a predator or food, while more gradual changes in speed and direction are likely to indicate the future locations of neighbours (Ioannou, Singh, & Couzin, 2015). Studies of collective motion are increasingly considering the underlying sensory mechanisms explicitly (Lemasson, Anderson, & Goodwin, 2013;

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Pita, Moore, Tyrrell, & Fernández-Juricic, 2015; Strandburg-Peshkin et al., 2013). Vision and the lateral line are the primary sensory inputs used in shoaling by fish (Ioannou et al., 2011), with vision generally accepted to be important for the attraction between neighbours (Partridge & Pitcher, 1980), while the lateral line modulates repulsion to avoid collisions (Faucher et al., 2010).

To investigate the importance of different sensory modalities in the shoaling of fish, empirical work has manipulated the sensory ability of individuals using chemical treatments (Faucher et al., 2010) or by cutting nerves (Partridge & Pitcher, 1980; Pitcher, Partridge, & Wardle, 1976) to test for the role of the lateral line, and using blindfolds to test for the role of vision (Partridge & Pitcher, 1980; Pitcher et al., 1976). An alternative approach is to examine how shoaling changes under variable environmental conditions such as darkness (McCartt, Lynch Jr., & Johnson, 1997; Miyazaki et al., 2000; Pitcher & Turner, 1986; Ryer & Olla, 1998) and turbidity (Chamberlain & Ioannou, 2019; Ohata, Masuda, Takahashi, & Yamashita, 2014). Changes in environmental conditions (broadly defined as 'stressors'; Orr et al., 2020) can disrupt shoal cohesion, structure and coordination through masking, where the transfer of sensory information between neighbours is reduced (McNett, Luan, & Cocroft, 2010). For example, reduced cohesion in dark or turbid water can be explained by a lack of available visual information regarding groupmates (Chamberlain & Ioannou, 2019; Pitcher & Turner, 1986). Attention can also shift away from the behaviour of other individuals in the group when environmental conditions require more attention to be focused towards other tasks (Chan, Giraldo-Perez, Smith, & Blumstein, 2010), such as detecting predators when visibility is constrained (Sohel & Lindström, 2015). Moreover, changes in environmental conditions can cause direct stress (Wingfield, 2005); for example, as measured in cortisol secretion (Wysocki, Dittami, & Ladich, 2006). These masking, distraction and stress effects of such environmental 'stressors' are not mutually exclusive, and one effect can cause another: for example, not being able to detect predators (a masking effect) may be the reason stress can increase in turbid water (Sutherland, Maki, & Vaughan, 2008). Some environmental stressors are unlikely to mask visual cues, however, so that any effects they have on collective behaviour can be attributed to distraction and/or stress. These variables can include acoustic noise (Purser & Radford, 2011; Wysocki et al., 2006), dissolved carbon dioxide (Duteil et al., 2016), dissolved oxygen (Domenici, Silvana, Steffensen, & Batty, 2002; Moss & McFarland, 1970) and temperature (Bartolini, Butail, & Porfiri, 2015).

Animals are rarely exposed to variation in one environmental parameter alone due to the multiple effects that natural or anthropogenic environmental changes have (Davis et al., 2018; Pistevo, Nagelkerken, Rossi, & Connell, 2017). Multiple stressors can combine in a variety of different ways in terms of their effects (Côté, Darling, & Brown, 2016; Harding, Gordon, Eastcott, Simpson, & Radford, 2019; Orr et al., 2020). There may be no obvious additional effect of a second stressor if a ceiling in response is reached. Alternatively, two stressors may produce an additive effect (the combined responses of single stressors) or an interactive effect, which may be synergistic (greater than an additive response) or antagonistic (less than an additive response). Most studies of how environmental variables affect behaviour have focused on single stressors (Harding et al., 2019; McMahon, Rohr, & Bernal, 2017). However, predicting responses to multiple stressors from their effects in isolation is difficult, and instead controlled experiments are required to test how multiple stressors interact (Darling & Côté, 2008).

Here, we tested how altering abiotic conditions impacts collective motion in fish shoals, focusing on the influence of multiple

stressors (Halfwerk & Slabbekoorn, 2015; Orr et al., 2020). Three-spined sticklebacks, *Gasterosteus aculeatus*, acclimate well to laboratory conditions and visual communication forms a critical component of stickleback social behaviour (Huntingford & Ruiz-Gomez, 2009). The EDA gene, located on the stickleback chromosome 4, significantly contributes to the evolution of schooling ability in three-spined sticklebacks, measured as spatial position within the school (Greenwood, Mills, Wark, Archambeault, & Peichel, 2016). This region is also linked to the patterning of sensory neuromasts in the lateral line (Wark et al., 2012), and evidence ties the evolution of the lateral line to the evolution of schooling behaviour in sticklebacks (Greenwood, Wark, Yoshida, & Peichel, 2013). While both vision and the lateral line have been implicated in the acquisition of social information needed for schooling, there has been no direct assessment of their relative roles, and it is likely that they are used complementarily (Partridge & Pitcher, 1980). As acknowledged in most literature exploring the sensory biology of stickleback, it is also likely that the relative roles of these modalities will vary between populations as sticklebacks occupy a broad range of habitats. For instance, the number of neuromasts is found to differ depending on the habitat and ecology of stickleback populations (Wark & Peichel, 2010). Visual disturbance, previously tested by changing water turbidity and vegetation density, affects stickleback mate choice, cue reliance and sexual selection of visual traits (Candolin, Salesto, & Evers, 2007; Heuschele, Mannerla, Gienapp, & Candolin, 2009). Previous research has shown effects of acoustic noise exposure on foraging ability (Purser & Radford, 2011; Voellmy, Purser, Flynn, et al., 2014) and antipredator responses (Voellmy, Purser, Simpson, & Radford, 2014). As such, *G. aculeatus* represents a good model species for testing the interaction of visual and acoustic disturbance.

Shoals of three-spined sticklebacks were tested in either darkness, additional acoustic noise, both darkness and additional noise in combination or neither (the control) in a fully factorial repeated-measures design to explore whether the effects of darkness and additional noise are independent of one another or interact. Darkness and acoustic noise were chosen to minimize the overlap in their potential effects. Light levels are detected through vision and will directly impact the ability of fish to see one another because visual information will be masked in low light conditions. By contrast, fish detect sound through otoliths and the lateral line (Popper & Schilt, 2008); the frequency of acoustic noise presented to the fish in this study (>100 Hz) is unlikely to interfere with the lateral line and mask sensory information during shoaling (which occurs at <10 Hz; Coombs, Bleckmann, Fay, & Popper, 2014). Although sound production and hearing are important in antagonistic and mating behaviours of some fish (Ladich & Winkler, 2017), there is a lack of evidence demonstrating that hearing is important in the collective movement of fish. Thus, there is unlikely to be a masking effect of additional noise on collective movement, but it may distract (Purser & Radford, 2011) and/or induce stress (Wysocki et al., 2006), which are also potential effects of darkness. As well as metrics of collective behaviour, we also measured individual level behaviours of speed (i.e. activity), turning angle and the distance to the arena wall to determine whether our treatments induced stress. As we used a repeated-measures design where groups were tested repeatedly over multiple days, we could also test whether groups differed consistently in their behaviour across the different environmental conditions. This group level personality variation is well documented, particularly in insects (Planas-Sitjà, Deneubourg, Gibon, & Sempo, 2015; Salazar, Planas-Sitjà, Deneubourg, & Sempo, 2015), but has also been observed in the collective behaviour of fish shoals (Jolles, Laskowski, Boogert, & Manica, 2018).

METHODS

Experimental Subjects and Housing

Fish were caught under licence from the Environment Agency in November 2017 from the River Cary, Somerset, U.K. (grid ref: ST 469 303), before being transported to the aquarium facilities at the University of Bristol. They were held in river water in covered plastic containers and transported by car for 1 h. They were then housed in groups of 50–100 individuals in glass tanks (70 × 40 cm and 34 cm high, with water depth ca. 30 cm), enriched with artificial vegetation and shelter. The air-supply stone was kept at low pressure to minimize vibration and acoustic noise from air bubbles (Purser & Radford, 2011). Water temperature was maintained at 14.2 ± 0.5 °C (mean \pm SD) and the light regime was 11:13 h light:dark. Under these conditions, three-spined sticklebacks are not in a breeding condition. Fish were fed once a day with defrosted bloodworm (chironomid) larvae, and not fed until after being tested on test days. Experimental trials were conducted January to March 2019 using 68 mixed-sex subjects (mean standard body length \pm SD = 53.6 \pm 4.8 mm) in 17 groups of four fish. Each group of four fish was moved to a glass holding tank (70 × 20 cm and 34 cm high) 72 h before the first treatment and remained there throughout the test period, except during experimental trials.

Experimental Treatments

Sound treatments were playbacks of either ambient sound (control) or ambient sound with additional noise. To control for any differences in the sounds present in the different holding tanks, and so that the ambient-control playback in the arena created a similar acoustic environment as in their holding tank, the ambient-sound track for a group was made using a recording from its holding tank. Thus, corresponding to each of the 10 holding tanks, 10 ambient tracks were constructed. An HTI-96-MIN omnidirectional hydrophone with inbuilt preamplifier (manufacturer-calibrated sensitivity: -164.3 dB re 1 V/ μ Pa; frequency range 20–30 000 Hz, High Tech Inc., Gulfport, MS, U.S.A.) and a digital recorder (PCM-M10, 44.1 kHz sampling rate, Sony Corporation, Tokyo, Japan) was used to make a 5 min ambient-sound recording in each holding tank at half water depth. Ambient tracks of 20 min were generated by repeating the relevant recording in each case. For the additional-noise tracks, acoustic square tones (no waveform alias) were overlaid onto the second half (i.e. the last 10 min) of the ambient-sound tracks; all playback tracks included a 10 min pretreatment acclimation period. The additional noise consisted of a 1 s tone, with no ramp up, played 30 times/min, with the times at which these tones occurred randomized. The frequencies of these tones were 100 Hz, 200 Hz, 400 Hz and 800 Hz (Fig. 1), played

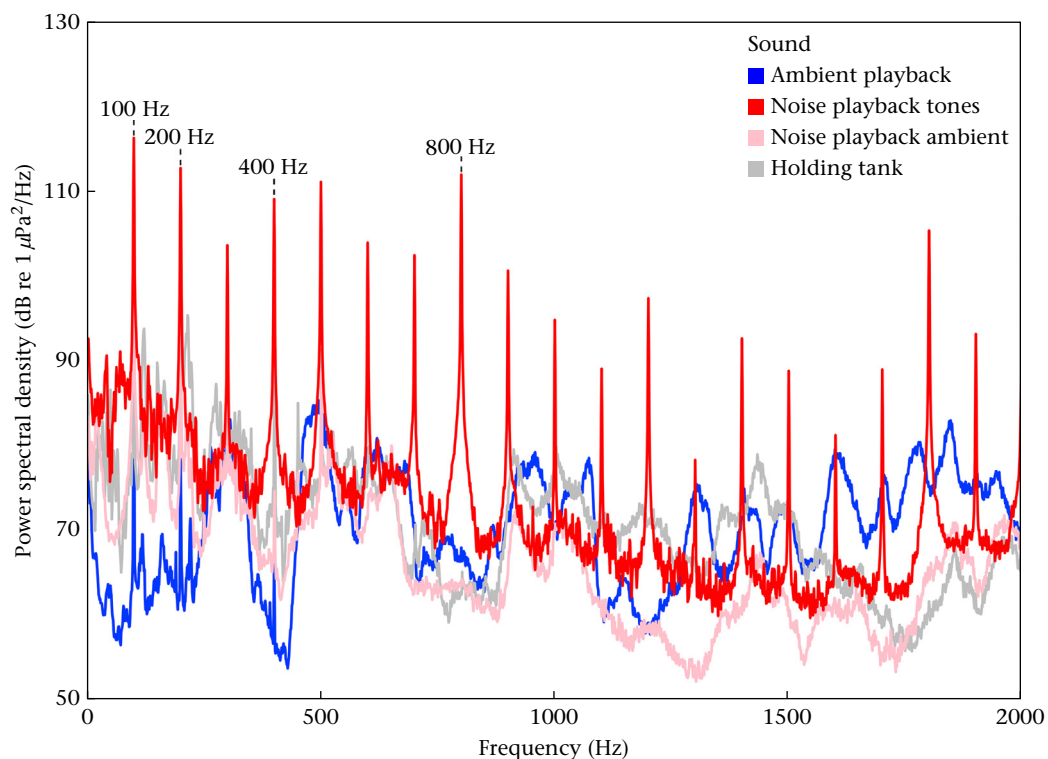


Figure 1. Illustrative sound pressure levels (SPL) during playback of experimental treatments and holding tank conditions. All recordings were analysed using the MATLAB acoustics analysis package paPAM (Mathworks, Natick, MA, U.S.A.), with a sampling frequency of 48 kHz, Hamming window, 50% window overlap, FFT length = sampling frequency. The square tone frequencies (100, 200, 400 and 800 Hz) are labelled; harmonics as a result of the multifrequency square tone playback are evident at 100 Hz intervals. All recordings are averaged across 30 s examples from two tank locations, 17 cm and 33.5 cm from the centre of the tank, which bounded where the fish spent most of their time during the trials; with the exception of the holding tank where only one 30 s recording is presented. Noise playback tones: the experimental conditions when the multifrequency square tones were played inside the tank (the 30 tones presented in a 1 min recording were spliced out and appended together); noise playback ambient: the noise conditions inside the tank between each 1 s tone (30 s presented as the time in between the tones during a 1 min recording, having been appended together). Fish in holding tanks were exposed to a root-mean-square (RMS) sound pressure level (SPL) in the frequency band 20–2000 Hz of 114.59 dB re 1 μ Pa, averaged over 30 s. In the control treatment, and during quiet periods during the noise treatment, RMS SPL in the same frequency band over 30 s (averaged over two tank locations bounding where the fish spent most of their time during trials) was 108.03 and 106.71 dB re 1 μ Pa, respectively. RMS SPL during multifrequency square tone playback, over 30 s and averaged from the two tank positions, was 122.69 dB re 1 μ Pa (20–2000 Hz). An individual illustrative square tone from each tank location had a 0 peak level of 132.53 and 127.95 dB re 1 μ Pa, respectively.

simultaneously to ensure that the tones were in the hearing range of the three-spined stickleback (Purser & Radford, 2011). Using multiple tracks for both sound treatments reduced issues associated with pseudoreplication (Kroodsmma, Byers, Goodale, Johnson, & Liu, 2001). Sound files were constructed using Audacity 2.3.0 (<https://www.audacityteam.org/>). They were played in the experimental tank via an Apple Inc. iPod Nano (3rd Generation, frequency response: 20–20 000 Hz), connected to an amplifier (Kemo Electronic GmbH; 18 W; frequency response: 40–20 000 Hz) and underwater loudspeaker (UW-30, frequency response: 100–10 000 Hz, University Sound).

We used a white-noise playback as this has been shown to have minimal effects on fear-related stress behaviours in sticklebacks (Purser & Radford, 2011); thus, the main effect expected from our noise stimulus was distraction. Animals are habituated to an acoustic baseline, or soundscape, formed within their natural or captive environment. The addition of noise (such as pure tones or motorboat noise playback) to this soundscape represents a novel disruption to their respective acoustic baseline. Such additional noise has been demonstrated to affect fish physiology and behaviour (Kunc, McLaughlin, & Schmidt, 2016). We used a synthetic noise playback that enabled design of a carefully controlled

acoustic stimulus that represented a departure from the ambient control by being significantly louder, with the peak of tones likely to be within the hearing range of the study species, and with an impulsive nature uncharacteristic of what the fish had experienced before. Similarly, our darkness treatment was a significant departure from what the fish in our experiment would normally experience as the tests were conducted during the light phase of their normal circadian rhythm; thus, was an unexpected drop in light level.

Light treatments were either ambient lighting conditions like those in the holding tanks (control) or darkness. Light levels in each treatment were measured using an underwater light data logger (HOBO Pendant MX, range 0–167 731 lx (15 582 lm/ft²), accuracy $\pm 10\%$). In the darkness treatment, light measured 0 lm/ft² and in ambient conditions 16 lm/ft². In the 10 min acclimation period at the start of each trial, ambient lighting was provided; in the subsequent 10 min period, lighting was either left as ambient or, in the darkness treatment, the overhead fluorescent lights were switched off. All trials were lit throughout by two infrared lamps (VK-B15ID12, wavelength: 850 nm, power 15 W, voltage 12 V) mounted ca. 150 cm above the water surface. This infrared light is not perceived by sticklebacks as their visual range does not include

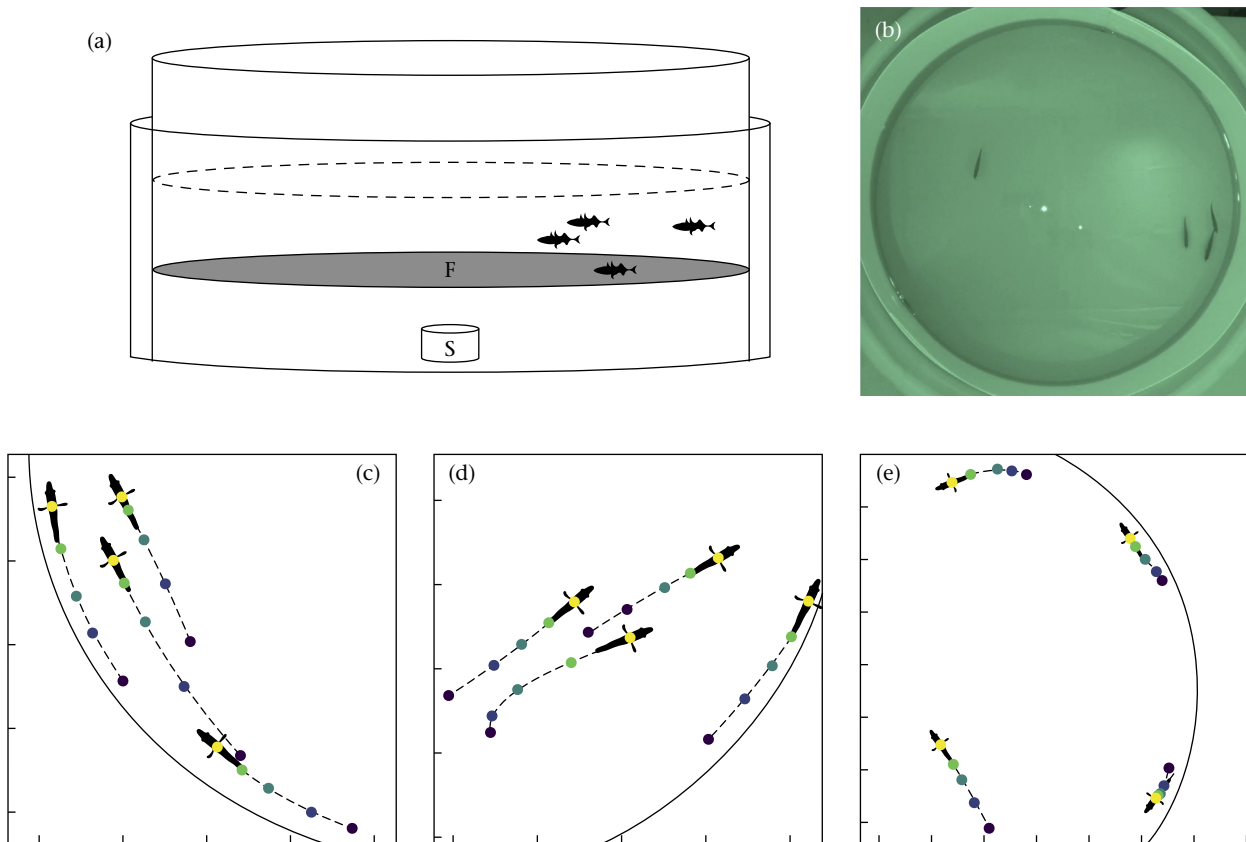


Figure 2. The experimental design and example fish trajectories. (a) The experimental set-up was positioned within a cylindrical tank measuring 70 × 33 cm. The underwater loudspeaker (S) is concealed beneath the 6 mm thick white acrylic arena floor (F, coloured grey for illustration only). The floor was mounted 12 cm above the base of the tank, with the underwater loudspeaker beneath this within a square plastic container 21.5 cm wide and 11 cm high. Elastic cord was used to suspend the loudspeaker to reduce vibrations. The fish were constrained to the water volume above the arena floor by an additional 1 mm thick white-foamed PVC wall within the tank. The dashed line indicates the water level during trials. White curtains surrounded the tank to minimize visual disturbance and diffuse light to minimize reflections on the water surface. (b) The arena was illuminated and filmed in infrared. (c, d, e) Illustrative examples of fish trajectories from (c) a control trial, (d) a trial with additional noise and (e) a trial in darkness. For each fish's trajectory, five consecutive sampled points are shown, each separated by 10 frames. Fish positions are shown by the coloured filled circles and consecutive points are joined with a dashed line. Circle colour represents fish positions at the same sample time point, with the most recent coloured yellow and the colours becoming darker further back in time. The curved solid line shows the arena wall. Each axis tick represents 100 pixels; the image zooms out as the panels progress from (c) to (e). The examples have been selected to illustrate the effects of the experimental treatments, where additional noise causes fish to be further from the arena wall and nearest neighbours to be more side by side (d), and darkness causes increased spacing between individuals and reduced coordination in direction of travel (e).

wavelengths above 700 nm, based on recent spectral sensitivity models (Rennison, Owens, Heckman, Schluter, & Veen, 2016). By only allowing infrared light into the camera, the captured footage did not differ between light treatments (ambient and darkness), thus avoiding any bias in the tracking of fish trajectories (Berdahl, Torney, Ioannou, Faria, & Couzin, 2013).

Experimental Protocol

Groups were tested once in each of the four treatments in a repeated-measures design with darkness and additional noise in isolation and in combination, in addition to a control treatment with ambient light and ambient sound. Each group was tested in one of the four treatments per day for 4 consecutive days. Treatment order was randomized for each group to reduce order effects. Testing took place in a round 67 cm diameter arena with a water depth of 8 cm (Fig. 2). The underwater loudspeaker was positioned under the centre of the arena floor, facing upwards, so out of view of the fish above. Although fish can be affected by noise pollution from terrestrial sources such as road traffic (Crovo, Mendonça, Holt, & Johnston, 2015), the transmission of sound across the air–water interface is poor (Erbe, Parsons, Duncan, Osterrieder, & Allen, 2017). As such, we chose to use a sound source within the water as our stimulus to replicate as much as possible the acoustic conditions generated by underwater sources. We would expect that an external noise source would have a reduced effect on behaviour.

A randomly selected fish group was transferred from its holding tank to the experimental tank. At approximately 30 s from introduction of the fish into the tank, the ambient-sound playback began. After a 10 min acclimation period, other than in the control treatment (ambient sound and light), darkness was induced by turning the overhead fluorescent lighting off and/or an additional-noise playback track was started. Trials were filmed from above throughout using a Sony FDR-AX53 digital camcorder positioned 149 cm above the tank centre, fitted with an optical glass infrared light filter (Hoya Y1IR72055 55 mm, range 760–860 nm, light transmission 95%). Trials were recorded in 4K (3840 × 2160 pixels) using the infrared sensitive NIGHTSHOT setting at a frame rate of 25 frames/s. Data from one trial (in the control treatment of ambient sound and light) could not be analysed and included because the video file was corrupted.

As we filmed from above using a single camera, our data collection and analyses were limited to two dimensions, effectively ignoring how fish changed their position in the depth plane (Macrì et al., 2017). For zebrafish, *Danio rerio*, tested alone, this has recently been shown to lose information and require larger sample sizes than using trajectory data from all three dimensions (Macrì et al., 2017). In studying collective movement of freely moving animals, 3D tracking of the motion of individuals is considerably more difficult (Puckett, Kelley, & Ouellette, 2014), and laboratory studies of fish collective motion typically use shallow water to minimize possible movement in the depth plane and only use 2D tracking (Berdahl et al., 2013; Herbert-Read et al., 2019; Herbert-Read, Kremer, Brintjes, Radford, & Ioannou, 2017; Perez-Escudero, Vicente-Page, Hinz, Arganda, & de Polavieja, 2014). In a rare example that 3D trajectories for individuals in shoals were obtained, it was found that identifying leaders could be done reliably using only 2D data (Watts, Nagy, Holbrook, Biro, & Burt de Perera, 2017). Similarly, targeting by fish predators of free-swimming fish shoals appears to yield similar results if only 2D views are considered, rather than all three dimensions (Romenskyy et al., 2019). Thus, 2D trajectories are likely to provide adequate information about how our treatments altered the individual and collective behaviour of fish in our experiment.

Data Processing and Behavioural Parameters

Videos were converted to MPEG-4 HD (1920 × 1080 pixels) in Handbrake Version 1.2.0 (<https://handbrake.fr/>) and fish trajectories were tracked in idTracker version 2.1 (Perez-Escudero et al., 2014). Trajectories were then processed in R version 3.5.1 (R Core Team, 2017). In cases where there were any missing coordinates for a frame or the two frames preceding it (which were used to calculate the direction and turning angle of each fish), all data were removed from that frame as social parameters could not be reliably calculated if not all fish positions were known. Trajectories were smoothed using a Savitsky–Golay filter in the trajr package version 1.3.0 (McLean & Skowron Volponi, 2018) with polynomial order of 3 and a filter length of 31 frames. Fish coordinates at every 10th frame, paired with the two preceding frames, were then saved to calculate the behavioural parameters. In this down-sampled data

Table 1

Structure of models and results of model comparisons explaining variance in behavioural parameters of individuals and groups during the experimental treatments

Response variable	Explanatory variables	ΔAICc	df
Speed	Sound + Darkness	0.0	7
	Sound * Darkness	0.1	8
	Sound	0.2	6
	Test order only	0.7	5
	Darkness	0.8	6
Turn angle	Test order only	0.0	5
	Darkness	0.1	6
	Sound + Darkness	3.9	7
	Sound	4.2	6
Distance from arena wall	Sound * Darkness	10.2	8
	Sound	0.0	6
	Sound + Darkness	5.2	7
	Test order only	9.7	5
Nearest-neighbour distance	Sound * Darkness	10.2	8
	Darkness	14.7	6
	Darkness	0.0	6
	Sound + Darkness	2.1	7
	Sound * Darkness	4.2	8
Maximum speed	Test order only	46.2	5
	Sound	48.3	6
	Darkness	0.0	6
	Darkness	0.0	6
cross correlation with nearest neighbour	Sound + Darkness	8.0	7
	Test order only	9.1	5
	Sound * Darkness	13.2	8
Heading to nearest neighbour	Sound	16.8	6
	Darkness	0.0	6
	Sound + Darkness	6.1	7
	Sound * Darkness	10.9	8
Bearing of nearest neighbour	Test order only	44.8	5
	Sound	50.1	6
	Sound * Darkness	0.0	8
	Sound + Darkness	1.0	7
	Sound	1.8	6
Group polarization	Darkness	12.0	6
	Test order only	12.6	5
	Darkness	0.0	5
	Sound + Darkness	5.2	6
	Sound * Darkness	12.5	7
Group speed	Test order only	38.8	4
	Sound	44.9	5
	Darkness	0.0	5
	Sound + Darkness	2.2	6
	Sound * Darkness	4.2	7
	Test order only	22.7	4
	Sound	25.5	5

Each row shows the result from a different model, which differ based on the response variable and the explanatory variables included in that model. All models included trial order (1–4) as a main effect. ΔAICc: the difference in the corrected Akaike information criterion between the model and the most likely model. An asterisk indicates an interaction term between the sound and darkness treatments, while a plus sign indicates the main effects only.

set, if the speed of any individual in the group exceeded a threshold of 25 pixels (ca. 17 mm) per frame, that frame was removed as fast speeds are likely to be due to any remaining tracking errors. However, smoothing the trajectories and removing frames where high speeds were likely to be due to tracking errors may also have excluded genuinely very fast fish movements that are characteristic of startle responses (Chicoli et al., 2014). We thus did not attempt to quantify startle responses.

Data from the first 8 min of the treatment period (i.e. after the 10 min acclimation period) were used in the analysis; the full 10 min treatment period was not used due to lost data in the final 2 min in some trials. The following behavioural parameters were calculated for each fish: its median speed (pixels/frame), turning angle (degrees) and distance to the arena wall (pixels), and the median distance to (pixels), angular bearing of (degrees) and angular difference in heading with (degrees) the nearest neighbour at each frame (Herbert-Read et al., 2017). The maximum cross correlation in speed with the nearest neighbour was also calculated; this could range from 0 (no correlation) to 1 (maximum correlation). The bearing of the nearest neighbour could range between 0° (the neighbour directly ahead or behind) and 90° (the neighbour directly alongside the focal fish). The median polarization of the group (also ranging from no polarization (0) to a maximum possible value of 1) and the speed of the group's centre of mass (pixels/frame) were calculated for each group (MacGregor, Herbert-Read, & Ioannou, 2020). Medians were used as summary statistics as the distributions of the behavioural parameters were skewed.

Statistical Analysis

Each behavioural variable was analysed as a response variable in separate linear mixed models (LMM) or generalized linear mixed models (GLMM). Darkness treatment (darkness or ambient light) and sound treatment (additional noise or ambient) were fixed terms. For behavioural parameters measured at an individual level, trial identity nested within group identity was included as the random term as there were four fish per trial and groups were tested multiple times, while for group level parameters (group centroid speed and group polarization), the random term was group identity as there was only one value for each group per trial. For each response variable, five models were constructed that included either the sound*darkness interaction, both explanatory variables as main effects only, darkness only, sound only or neither of these variables; all models included trial order (1–4) as a main effect (Table 1). The likelihood of each model given the data was compared using the corrected Akaike information criterion (AICc) with the *bbmle* package version 1.0.20 (Bolker & R Development Core Team, 2017). If the difference in the AICc between two models is greater than 2 units, it provides strong evidence that the model with the lower AICc is more likely given the data (Burnham & Anderson, 2002). By comparing models with and without explanatory variables of interest, it can be inferred which explanatory variables are important in explaining variation in the response variable (Table 1).

We also tested whether there was consistent behavioural variation between groups across the different environmental conditions. The full model for each behavioural response variable, which included the sound*darkness interaction, was compared using the AICc to the same model with the group identity random effect term removed. For behavioural parameters measured at an individual level, trial identity remained in the models as a random effect, and for the group level parameters (group centroid speed and group polarization), the models with group identity were compared to linear models without any random terms. The identities of

individual fish within groups could not be reliably known between different trials of the same group; thus, we did not test for repeatability between individuals, only groups.

Median individual speed, natural log-transformed median turn angle, natural log-transformed distance to the arena wall, maximum speed cross correlation with the nearest neighbour, natural log-transformed median heading difference with the nearest neighbour, median bearing of the nearest neighbour, median group polarization and median group speed were analysed using LMMs. The distribution of the residuals was confirmed to be normal using QQ plots, and the residuals plotted against the fitted values to ensure homoscedasticity. The median nearest-neighbour distance was analysed using negative binomial GLMMs, with dispersion tested using the *DHARMA* package in R version 0.2.4 (Hartig, 2019). All statistical models used the *lme4* package version 1.1–21 (Bates, Mächler, Bolker, & Walker, 2015) in R version 3.5.1 (R Core Team, 2017).

Ethical Note

All methods complied with ASAB/ABS Guidelines for the treatment of animals in behavioural research and teaching and were approved by the University of Bristol Animal Services Ethical Committee (UB/17/060 and UIN/17/075). Exposing live animals to the treatments was essential to understand their effects on behaviour, but future work can replace the use of live animals with simulation modelling of fish collective motion to explore the mechanisms and implications of our results. Exposure to the environmental treatments was limited to 10 min to minimize stress and long-term negative effects of the treatments; our study was thus aimed at short-term, rather than chronic, exposure. The results examining individual level behaviours of speed, turning angle and thigmotaxis showed no evidence that the treatments induced stress. After use in the laboratory, fish were rehomed in a pond unconnected to other water bodies, in accordance with the U.K.'s Environment Agency regulations.

RESULTS

There was no evidence from the model comparisons that the median speed and turning angle of individuals were affected by

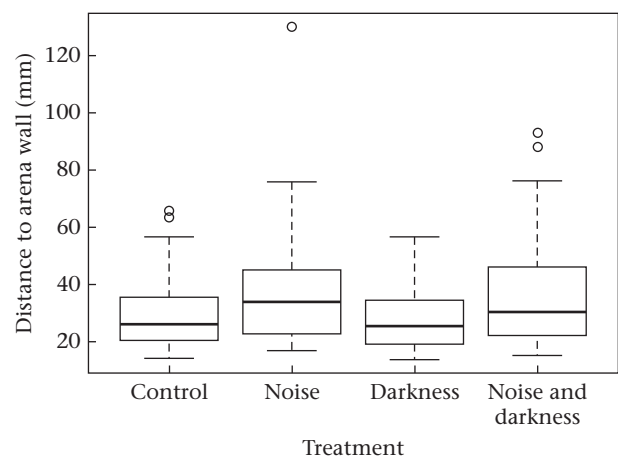


Figure 3. The effect of darkness and additional noise ('Noise') on the median distance individual fish were from the wall of the arena. Seventeen shoals of four fish were each tested once under each of the four treatments. The median across individual fish is shown by the solid line, the interquartile range is enclosed within the box, the whiskers extend to the most extreme data point within 1.5 × the interquartile range and circles show data points outside the range of the whiskers.

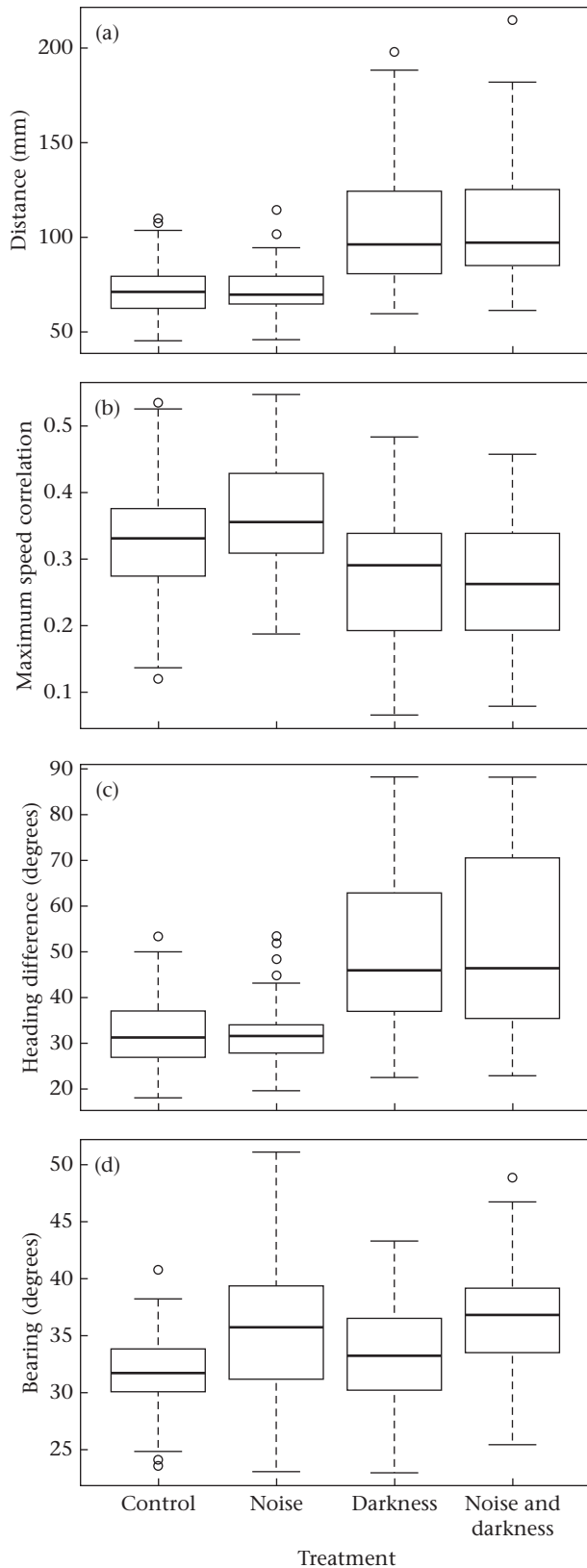


Figure 4. The effect of darkness and additional noise ('Noise') on individual level parameters of collective motion in stickleback shoals. (a) Nearest-neighbour distance, (b) maximum speed correlation, (c) heading difference and (d) bearing. Data are median values across frames for individual fish, except for the maximum speed correlation. Seventeen shoals of four fish were each tested once under each of the four treatments. All variables are calculated relative to the nearest neighbour of each

either darkness or additional noise (Table 1). For speed, although the main effects model had the lowest AICc, the model with only treatment order had a ΔAICc of 0.7, indicating that this model was comparatively well supported by the data, and with fewer parameters (Table 1). The model with only treatment order had the lowest AICc when the turning angle was the response variable (Table 1). By contrast, fish in treatments with additional noise were on average further from the arena wall than the control treatment or the treatment with darkness only (Fig. 3); that is, the fish showed reduced thigmotaxis (wall following). Both models that included sound as an explanatory variable had AICc scores >2 AICc lower than the test order only model, and the model with only sound was the most likely model, with the model with both main effects having a ΔAICc of 5.2 (Table 1).

For each of the collective behaviour response variables nearest-neighbour distance, speed correlation, heading difference, group polarization and group speed, the models with the best support (lowest AICc, i.e. $\Delta\text{AICc} = 0$) were the models with darkness as the only main effect (Table 1). The next most likely models had a ΔAICc of >2 compared to these most likely models, indicating strong support for the models with only darkness as a main effect relative to the other models. Adding the sound treatment as an explanatory variable did not make these statistical models explaining cohesion and coordination more likely (Table 1). Thus, there was no evidence that additional noise and darkness had an additive or interactive (synergistic or antagonistic) effect on these behavioural responses, as neither the models with both main effects nor that with the interaction term were within 2 ΔAICc of the most likely model.

Spacing between individuals, measured as the nearest-neighbour distance, increased in the trials with darkness, so that group cohesion declined (Fig. 4a). The maximum speed cross correlation was also affected by the darkness treatment, decreasing in darkness and indicating a decline in coordination in movement (Fig. 4b). The heading difference between individuals increased in darkness, also indicating a decline in coordination as individuals were less aligned in their direction of travel (Fig. 4c). Corresponding to this effect on the difference in heading between nearest neighbours, the group polarization was lower in darkness than in ambient light (Fig. 5a). Although individual speed was not affected by darkness, the speed of the group's centre was slower in the darkness treatment (Fig. 5b). This can be explained by the decline in group polarization because the centre of mass of groups with low polarization is slower than in more polarized groups, even if individuals' swimming speeds are constant.

By contrast, the angular bearing of the nearest neighbour relative to a fish's direction of travel was greater when there was additional noise compared to ambient sound (Fig. 4d). Larger angles correspond to neighbours being found side by side (i.e. at 90°); thus, the spatial arrangement of individuals shifted with additional noise. The three models including the sound treatment were all within 2 ΔAICc units of the most likely model, while the model with darkness treatment and treatment order had a ΔAICc of 12, 0.6 ΔAICc units less than the model with only treatment order (Table 1). The mostly likely model was that with the sound*darkness interaction and the second most likely ($\Delta\text{AICc} = 1.0$) was the model with both main effects, providing some evidence that both treatments were important in their effect on the bearing of the nearest neighbour. However, the effect of additional noise was considerably greater than darkness, and the model lacking the

individual fish (for example, the distance is the distance of the nearest neighbour). The median across individual fish is shown by the solid line, the interquartile range is enclosed within the box, the whiskers extend to the most extreme data point within $1.5 \times$ the interquartile range and circles show data points outside the range of the whiskers.

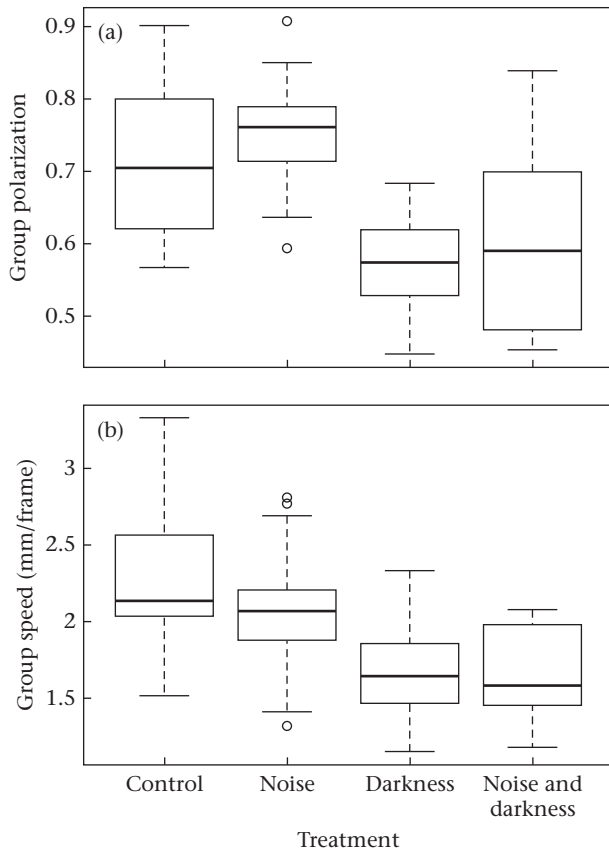


Figure 5. The effect of darkness and additional noise ('Noise') on group level parameters of collective motion in stickleback shoals. (a) Group polarization and (b) group speed. Data are median values across frames for each group. Seventeen shoals of four fish were each tested once under each of the four treatments. The median across individual fish is shown by the solid line, the interquartile range is enclosed within the box, the whiskers extend to the most extreme data point within $1.5 \times$ the interquartile range and circles show data points outside the range of the whiskers.

darkness treatment had an ΔAICc of 1.8. While the estimated effect of additional noise was to increase the median bearing by 3.45 degrees, the effect of darkness increased it by only 1.30 degrees. The interaction effect revealed an antagonistic effect of the two stressors; however, this effect was small with the bearing being 0.57 degrees less than expected from the additive effect of additional noise and darkness.

The results on whether behavioural parameters were repeatable, that is, differed consistently between groups across the different environmental conditions, were mixed. The model for individuals' speeds with group identity as a random effect was more likely than the same model lacking group identity ($\Delta\text{AICc} = 8.3$) and, similarly, removing the group identity random effect also made the model for turning angles ($\Delta\text{AICc} = 11.5$), the distance to the arena wall ($\Delta\text{AICc} = 3.0$) and group polarization ($\Delta\text{AICc} = 5.3$) less likely. The importance of the group identity random effect for these variables suggests that the different groups differed consistently across the treatments. By contrast, the models for nearest-neighbour distance, maximum speed correlation between nearest neighbours and bearing to the nearest neighbour were more likely when the group identity term was removed (distance: $\Delta\text{AICc} = 1.6$; speed correlation: $\Delta\text{AICc} = 2.1$; bearing: $\Delta\text{AICc} = 1.4$). The models for heading difference with the nearest neighbour and group speed were more likely when group identity was included, but the models with this term removed were within 2 AICc units (heading difference: $\Delta\text{AICc} = 0.1$; group speed:

$\Delta\text{AICc} = 1.2$), also suggesting groups were not consistently different in these behavioural parameters.

DISCUSSION

Previous studies have tested for the effects on shoaling behaviour of individual environmental conditions in isolation, including darkness (McCartt, Lynch, & Johnson, 1997; Miyazaki et al., 2000; Pitcher & Turner, 1986; Ryer & Olla, 1998), acoustic noise (Currie, White, Leighton, & Kemp, 2020; Herbert-Read et al., 2017; Sarà et al., 2007), turbidity (Chamberlain & Ioannou, 2019; Ohata et al., 2014), dissolved carbon dioxide (Duteil et al., 2016), dissolved oxygen (Domenici et al., 2002; Moss & McFarland, 1970) and temperature (Bartolini et al., 2015). Our study is the first to test the combined effect of multiple potential stressors on collective motion, and we found that the effects of darkness and additional acoustic noise were largely independent. In general, the two stressors influenced different aspects of collective movement. Darkness reduced cohesion and coordination, with fish being further apart, less aligned in their direction of movement and less correlated in their speed of movement. Additional acoustic noise instead affected shoal structure, with nearest neighbours being more likely to be found side by side than in front or behind. There was some evidence of an interactive effect between additional noise and darkness on one response variable (the bearing of the nearest neighbour), but based on the estimated effects, this effect was weak relative to the effect of additional noise. Evidence that groups differed consistently from one another was limited to only one of the measures of collective behaviour, group polarization. The other measures that varied consistently between groups were individual speed, turning angle and the distance to the arena wall, which are all individual rather than social behavioural parameters (where social parameters can only be measured when there is at least one other individual present). Thus, there was little evidence for group level personality variation in collective behaviour, unlike previous studies (Jolles et al., 2018; Planas-Sitjà et al., 2015; Salazar et al., 2015).

With reduced cohesion, reduced coordination in the direction of travel and reduced coordination in speed, it is likely that the position, direction and movement of nearest neighbours were masked under darkness, which is an example of unimodal interference of visual information (Halfwerk & Slabbekoorn, 2015). This is consistent with the role that vision plays in collective behaviour in fish (Partridge & Pitcher, 1980). In addition, the lack of visual information regarding both other fish and potential threats could have induced stress, reducing the ability of individuals to coordinate shoaling (Pitcher et al., 1976). Such an effect would depend on the typical light levels, and hence the visual information, fish are used to. In deep or turbid water where vision is limited, fish adapt over short (Ehlman, Martinez, & Sih, 2018), developmental (Ehlman, Sandkam, Breden, & Sih, 2015) and evolutionary (Kowalko et al., 2013) timescales. Thus, it would be key in further work to test whether sticklebacks adapted to darker environments, either through experimental manipulation or by testing fish from different habitats, can maintain shoal coordination in our experimental set-up. A reduction in the groups' centroid speed in darkness could be explained by the reduced coordination in the direction of travel of individuals, measured as group polarization (MacGregor et al., 2020). In general, coordinated collective behaviour has evolved to maximize resource use (Garnier et al., 2009; Ioannou & Dall, 2016) and minimize the risk of being eaten (Bazazi et al., 2010; Ioannou, Rocque, Herbert-Read, Duffield, & Firth, 2019). Any disruption to collective behaviour is therefore expected to have impacts on survival and knock-on ecological effects. This is particularly true for fish, with a large proportion of fish species

showing shoaling behaviour for at least part of their life cycle (Shaw, 1978).

It is unlikely that darkness was being perceived as a threat as it did not cause an increase in fear-related behaviours. The median swimming speed of individuals was not affected by darkness; speed is a measure of activity, which typically declines when perceived risk increases (Anholt, Werner, & Skelly, 2000). Similarly, animals such as sticklebacks that use refuges (Bevan, Gosetto, Jenkins, Barnes, & Ioannou, 2018) respond to increased perceived risk by swimming close to structures such as walls (Gréacias, Valentin, & Aubin-Horth, 2018), and there was no evidence that the darkness treatment affected the fish's distance to the arena wall. While there is no explicit documentation of higher stickleback predation risk in low light environments, it has been shown that when visibility is reduced as a consequence of high turbidity, they may be more vulnerable as they have weaker antipredator responses (Sohel & Lindström, 2015). Sticklebacks may compensate for this by reducing their activity in turbid water to reduce encounter rates with predators (Ajemian, Sohel, & Mattila, 2015). Based on these effects of turbidity on antipredator behaviour, it is surprising that the sticklebacks in our experiment did not respond to the darkness treatment by reducing their swimming speed and swimming closer to the arena wall.

In contrast to previous studies in sea bass, *Dicentrarchus labrax* (Herbert-Read et al., 2017), tuna, *Thunnus thynnus* (Sarà et al., 2007) and Eurasian minnow, *Phoxinus phoxinus* (Currie et al., 2020), the collective behaviour of three-spined sticklebacks in our study was relatively robust to additional noise, despite evidence that it reduces cognitive performance relating to foraging and antipredator behaviour in sticklebacks (Purser & Radford, 2011; Voellmy, Purser, Flynn, et al., 2014; Voellmy, Purser, Simpson, et al., 2014). In shoals of tuna, boat noise reduced cohesion and coordination under field conditions (Sarà et al., 2007), and similar effects of playbacks of pile driving were observed in shoals of sea bass tested under controlled laboratory conditions (Herbert-Read et al., 2017). These studies focused on anthropogenic noise and thus used different acoustic stimuli to our study, where we used white noise to minimize stress and instead to have a primarily distracting effect on the fish (Purser & Radford, 2011). Using boat noise playbacks would be more likely to have a stress-inducing effect on sticklebacks (Voellmy, Purser, Flynn, et al., 2014) and potentially show similar effects of reduced cohesion as in previous work (Herbert-Read et al., 2017; Sarà et al., 2007). These previous studies also used marine species, which are likely to have stronger shoaling and schooling tendencies than three-spined sticklebacks. By contrast, the recent study by Currie et al. (2020) showed that minnows became more cohesive and polarized when exposed to continuous or pulsed tonal sounds. Thus, the effect of acoustic noise on shoaling behaviour may vary with both the properties of the acoustic stimulus and species.

The one effect of additional noise on collective behaviour found in our study was also observed in sea bass (Herbert-Read et al., 2017), with nearest neighbours being more likely to be observed side by side in the additional-noise treatment in both studies. Although the sensory and/or cognitive mechanism(s) that explain the effect of noise on shoaling behaviour is unknown, Partridge and Pitcher (1980) found that individuals were also more likely to be found side by side in shoals of saithe, *Pollachius virens*, when the lateral line of the fish was disabled. Although the frequencies of our playback (>100 Hz) should not have interfered with the lateral line sensory system, which is sensitive to frequencies of less than 10 Hz (Coombs et al., 2014), the similarity with our results suggests a possible interaction of noise with the lateral line sensory system. Partridge and Pitcher (1980) also found that blinding fish had little effect on the bearing of the nearest neighbour, agreeing with the lack of an effect of darkness in our study. While there was no effect

of either treatment on the speed and turning angle of individuals, fish swam further from the arena wall when exposed to additional noise, indicating a reduced perception of risk. It is thus unlikely that the change in shoal structure in the additional-noise treatment was due to this treatment being perceived as threatening; Purser and Radford (2011) found only small effects of white noise on stickleback fear-related behaviours. The functional significance of the bearing of neighbours in collective behaviour is less clear than the effects caused by darkness, although the spatial arrangement of individuals within groups impacts how they contribute to deciding where to move, as groups such as fish shoals are typically led from the front (Bumann & Krause, 1993). The change in the relative position of neighbours in noisier conditions may thus be an adaptive change in behaviour which reflects reduced leader–follower tendencies and/or more frequent switching of position (Herbert-Read et al., 2019), rather than noise acting as a constraint on preferred group structure.

There is growing interest in studying the effects of multiple stressors on animal behaviour (Côté et al., 2016; Harding et al., 2019), particularly as anthropogenic activity is increasingly recognized as having effects on multiple environmental parameters (Halfwerk & Slabbekoorn, 2015). Collective behaviour is particularly important for fish (Ioannou et al., 2011) and aquatic habitats are particularly vulnerable to multiple stressors (Ormerod, Dobson, Hildrew, & Townsend, 2010). For example, climate change increases both acidification through dissolved carbon dioxide and water temperature (Pistevos et al., 2017). Multiple stressors can combine in a variety of different ways (e.g. additive, synergistic or antagonistic; Côté et al., 2016; Harding et al., 2019; Orr et al., 2020). Here, we found mostly independent effects of additional noise and darkness, and only weak evidence for a possible antagonistic effect between these two stressors. Our study was not aimed at mimicking anthropogenic effects and was conducted in the laboratory to facilitate detailed tracking of collective motion. However, the next step would be to test whether the general trend we found, that multiple environmental stressors have independent effects on different aspects of collective motion, applies to combinations of stressors found in real-world systems.

Author Contributions

All authors contributed to the design of the study. G.M.G. and I.K.D. set up the experiment with assistance from H.R.H., and G.M.G. and I.K.D. carried out the trials and tracked the videos. G.M.G. and C.C.I. conducted the statistical analysis and drafted the first version of the manuscript. All authors contributed to revising the manuscript and gave final approval for publication.

Data Availability

Data are available as Supplementary Material.

Conflict of Interest

No competing interests declared.

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Supplementary Material

Supplementary material associated with this article can be found online at <https://doi.org/10.1016/j.anbehav.2020.07.024>.

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