Research report

Quantification of shoaling behaviour in zebrafish (*Danio rerio*)

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Abstract

Zebrafish has been a favourite of developmental biologists and numerous genetic tools have been developed for this species. In recent years, zebrafish has become an increasingly popular subject of neuroscientists and behavioural scientists. One of the typical characteristics of zebrafish is shoaling, individuals forming a tight group in which fish swim together. The biological mechanisms of social behaviours are complex and not well understood in vertebrates, and zebrafish, due to its highly social nature and the genetic tools developed for it, may represent an excellent animal model with which these mechanisms may be studied. Improvement of behavioural quantification methods would facilitate research in this area. We describe a custom software application that allows the precise quantification of several parameters of group cohesion in zebrafish. We also present three experimental examples to illuminate the use of our methodology, and show how group cohesion changes in response to manipulations of the environment.
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1. Introduction

Zebrafish have been frequently utilized in developmental biology and genetics in the past three decades. However, interest in the behaviour and brain function of this species has recently increased [3,18]. Given the prolific nature, the ease of maintenance, and the numerous genetic tools developed for this species, a promising future is forecast for zebrafish in behavioural neuroscience and behaviour genetics [35,3].

An interesting behavioural feature of zebrafish is their tendency to form tight groups, or shoals. Several aspects of what characteristics of shoal mates are preferred by zebrafish have been studied. Characteristics including shoal size and gender [31], body size [28], fin length [15], stripe pattern [37], and overall pigmentation [8,36] have been investigated. Moretz et al. [19] and Engeszer et al. [8] have examined the effects of early social experience on shoaling preference. Wright et al. [39] have demonstrated that fish from different populations show differences in inter- and intra-population shoaling, and argued that there is a genetic component underlying the tendency of zebrafish to shoal. For example, quantitative trait loci

(QTL's) associated with shoaling as well as other behavioural traits including antipredatory behaviour have been identified in zebrafish [40,41].

Often, shoaling behaviour is quantified by measuring the preference of a test fish placed in a central compartment of a test tank flanked by two adjacent compartments which contain a shoal of conspecifics or are empty [38]. While this method may shed light on shoaling preferences and on the overall tendency of a test subject to shoal, it does not allow the analysis of the internal dynamics of association among fish in free-swimming shoals. This test set up also suffers from the artificial nature of the situation: the test fish has access only to visual stimuli and is otherwise isolated from its shoal. Olfactory, auditory and lateral line perceived information is not available to the test fish and it is unable to join and swim together, i.e. interact, with its shoal mates. As a result, the behaviour of the test fish may not reflect what members of a zebrafish shoal would normally do under naturalistic conditions. A method that enables the analysis of dynamic behaviour of fish while in a freely moving shoal would, therefore, be of use.

Two distinct methods exist with which the 3D dynamics of shoaling in free-swimming groups of fish have been investigated. The stereo technique [6,14] utilizes two or more cameras to triangulate the position of each fish. The shadow technique [21,17] uses two light sources to project shadows of the fish onto the

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apparatus' substrate. Even the most computerized applications of these methods, such as the Galatea system [26,27], require specialized hardware and the positions of the fish to be entered manually.

Currently, no methodology exists that would allow automatic location of every member of a shoal of freely swimming fish from a video source. Zebrafish are small (3–4 cm) and when tested in a large arena that allows the investigation of shoaling tendencies a single subject may occupy as few as 10–20 pixels on the video image. This is far too small for individually marked fish to be identified, or for two shadows cast by one fish to be differentiated. In addition, fish are often so close together that they appear as one target in the video image. Furthermore, although fluorescent dyes have been successfully used to label fish [10], the number of different colours that a tracking or image analysis system could reliably distinguish is limited by software (e.g. Noldus's Ethovision Color-Pro, version 3.1, can track up to 16 distinctly coloured subjects), by the availability of distinguishable fluorescent dye colours, and by the short length of dye retention [7]. In addition, dye injections may damage fish or otherwise modify both their own behaviour and the reactions they elicit from shoal mates. For instance, it has been shown that zebrafish shoaling preferences are influenced by conspecific coloration [8,23].

Here, we present a custom software application designed to explore dynamic changes in shoal density over time and to overcome the issues presented above. The software allows any number of fish to be tracked at any temporal resolution, in two dimensions. It does not require the fish to be individually recognizable (although it does allow for tracking of individual fish if they are identifiable). The locations of fish in a video image are coded manually, as in other applications of this sort, but all other aspects of the process are automated to increase the efficiency and speed of coding. The software is capable of utilizing most common video file formats and requires no special hardware. The software does not support 3D tracking of fish, and this may be a crucial limitation for studying some aspects of shoaling. This disadvantage is balanced by the ease of use and speed of coding of data with the current program. In the current paper, we describe this custom application and present supporting experimental examples to demonstrate the utility of the system.

In order to exemplify the utility of our technique, we manipulated shoaling behaviour using different environmental stimuli which have been shown to have an effect on shoal density [13,2]. We exposed zebrafish to a scattered food source or to the appearance of an aerial predator model.

The advantages of shoaling include protection from predation, via risk dilution and the 'confusion effect' [16,20,42] and greater access to food resources [25]. However, shoaling fish may also compete for food [25] or forage less efficiently when in a tight shoal [34]. Thus, it has been suggested that shoaling density represents a trade-off between two opposing forces [24]: protection from predation, which is assumed to increase with increasing shoal density [34,32], and the need to forage efficiently, which is assumed to vary in inverse relationship to shoal density [33]. As a result, we expected the presence of

scattered food to reduce shoal density [33,34] and the presentation of an aerial predator model to increase shoal density, at least during the time the model was present [34,24]. We further hypothesized that these manipulations would affect measures of shoaling cohesion (such as the distance between individual fish) as compared to a control condition (e.g. [32]).

2. Methods

2.1. Subjects

Zebrafish (adult, 4–6-month-old, wild-type short fin variety) were obtained from a local pet supplier (Big Al's Aquarium Services Warehouse Outlet, Mississauga, Ontario, Canada). The subjects were housed in 40 litre tanks for two months before the start of the experiments. Each tank contained 16 fish forming an experimental group whose shoaling behaviour was later tested together. The tanks contained "system" water that was previously reverse osmosis purified and mixed with sea salt ('Instant Ocean' sea salt, Aquarium Systems Inc., OH) so that conductivity of the water was 800 μSiemens. The water of the holding tanks was filtered using external canister filters (EHEIM Classic, Model 2213, Deizisau, Germany) and was aerated and maintained at a temperature of 26 ± 2 °C. Fluorescent light fixtures placed above the tanks provided illumination (light on at 7:00 h and off at 20:00 h). Fish were fed a mixture of ground freeze-dried krill and flake food (Tetramin Tropical Flakes, Tetra, USA). Subjects that were tested as a group were housed together (i.e. each 40 l tank contained a group of 16 fish). Each group was made up of approximately equal numbers of males and females. Fish were tested between 11:00 and 15:00 h each day.

2.2. Apparatus

Fish were tested in a circular white plastic tank with a diameter of 91 cm. The tank was filled with system water to a depth of 30 cm. The tank was centered in a testing room that contained fish tanks and a large one-way mirror window. The room was lit by four ceiling spot-lights (50 W each), equidistant from the center of the arena. A digital video camera (Sony DCR-HC20, Sony Corporation, Japan) was mounted on the ceiling above the center of the tank, such that the entire tank was visible in the videoframe. The distance of the camera from the water surface was 170 cm for the predator model task and 140 cm for the foraging task.

For the predator study, a hawk model, 80 cm in length and with a wing span of 110 cm, was constructed of black corrugated plastic sheets. The model was attached to a transparent nylon fishing line that was run through a pulley system attached to the ceiling of the testing room, so that the model could be pulled across the room by an operator outside the testing room. A small weight (500 g) was attached to the other end of the line, to pull the model back to its original position. The hawk model was suspended approximately 140 cm above the surface of the water so that it would not obstruct the view of the camera in any position.

2.3. Procedure

2.3.1. Experiment 1: Habituation

The goal of this experiment was to study whether shoaling behaviour changes in response to repeated exposure to the same tank, i.e. whether habituation to the novel test tank may be detected in shoaling. One might expect that fish initially swim closer to each other in a novel environment but with time this increased shoal cohesion diminishes [4]. It was also expected that repeated exposures to the same test tank would decrease shoal cohesion. First, fish were removed from their home tank and placed in a transparent Plexiglas start box with a footprint of 21 cm² (which did not have a top or bottom) in the center of the testing tank. When all the fish in the group were in the start box, the box was lifted out of the water, releasing the fish into the circular observation tank. Fish were filmed in this tank for 30 min and then returned to their home tank. Eight groups of fish were tested. Each group was exposed to the arena six times, i.e. once a day on days 1, 4, 8, 11, 15 and 18. Shoal cohesion (the average distance between

members of the shoal) was quantified at a sampling rate of once every 10 s throughout the session.

2.3.2. Experiment 2: Effect of distributed food

The goal of this experiment was to study how scattered food affects shoaling behaviour in zebrafish. It was expected that shoal cohesion would decrease due to competition for food [33,34]. The same eight groups of fish used in the first experiment were retested once, 60 days after their last exposure to the arena. The fish were deprived of food for 24 h prior to testing. This is a very mild food deprivation, given that zebrafish can stay healthy without food for several days. Ninety floating Betta Bio-Gold Baby pellets (Hikari Sales U.S.A., Inc., CA) were randomly scattered on the surface of the water of the circular observation tank before the fish were placed in the tank. It has previously been demonstrated that the presentation of food leads to a decrease in shoaling density, and that scattered food has a greater effect than food that is spatially clumped [34]. Fish were filmed for 10 min in the tank before being returned to their home tanks. Five control groups of identically treated fish were tested similarly, but with no food present. The order of filming the control and the foraging groups was random. Shoal cohesion (the average distance between members of the shoal) was quantified at a sampling rate of once every 10 s throughout the session.

2.3.3. Experiment 3: Effect of an aerial predator model

The goal of this experiment was to investigate how an aerial predator, the hawk model, influences shoaling behaviour in zebrafish. It is believed that shoaling behaviour provides effective protection as it divides the attention of predators [16,20]. Thus, we might expect that shoal cohesion should increase in the presence of a predator [34,24]. It has been demonstrated [24] that the appearance of an aerial predator decreases the willingness of minnows to forage in exposed areas of an enclosure. Eight groups of 16 naïve zebrafish each were monitored in the circular observation tank for 20 min. What species of birds prey upon zebrafish in nature has not been documented. It is also not known whether zebrafish selectively respond to a particular silhouette resembling sympatric birds of prey. The hawk model simply served as a large enough object that moved overhead. The "predator" was passed forth and back over the arena three times during the 20 min session, at the beginning of the 5th, 10th, and 15th minute of the session. Each predator exposure lasted for 2 s (both directions). All other experimental details were as described above. Shoal cohesion (the average distance between members of the shoal) was quantified for 60 s preceding and 30 s subsequent to the appearance of the predator with a sampling rate of 1 image/s.

2.4. Statistical analysis

Video recordings of the sessions were converted to Windows Media Player files (WMV). The files were coded using the custom software described below. Groups of fish were tracked at a sampling rate of once per 10 s over the entire 10, 20, or 30 min session. For the predator study, the 30 s period following each appearance of the predator was also coded at 1 s intervals. The data were exported into SPSS (Version 14 for the PC) for further analysis. The unit of statistical analysis was the shoal (consisting of 16 fish) and $n = 8$ shoals were tested in each experiment. Alpha was set at .05 throughout for the determination of significant effects. Repeated Measures Variance Analyses were performed and, where appropriate, the effect of treatment (e.g. presence or absence of food, a between subject factor) was also investigated. Where significant effects were observed, post hoc Tukey HSD multiple comparison tests or, when required, pair-wise comparisons (t-tests) were conducted using the Holm type-1 error correction method [1]. A portion of the data was coded independently by two observers and the results were compared to examine inter-observer reliability using a paired two tailed t-test.

2.5. Software design

The software described here relies on a human observer to locate the fish on the image window on the computer screen. It then extracts the coordinates of the locations identified by the observer, calculates the distances between them, and also provides other relevant measures, as described below. The software outputs the calculated parameters to a text file. The software at this point does not allow automated localization of the subjects, but given the ability of the observer to pinpoint the subjects on the screen, it allows recording of shoaling behaviour beyond the precision of current publicly available video image based tracking systems.

The user must select a video file to be tracked, which is loaded and displayed over the entire screen. A set of controls is overlaid on one corner of the video. The controls allow the user to navigate the video by either playing or pausing it, rewinding or fast-forwarding any amount of time, or skipping directly to any point in the file. All references to points in the file are by time-code in milliseconds from the start of the file. Thus, for example, the user can type "10000" in the appropriate box to skip to a point 10 s from the start of the file.

Before tracking a file, the user must first specify the experimental parameters: the shape of the enclosure, the number of fish in the group, and the required sampling rate in milliseconds (e.g. "1000" for once per second), and specify a name for the output file. The user may select which measures will be output to the file from a list of available measures, and may also specify custom measures (see below). Next, the user specifies the physical parameters of the tracking arena by clicking on the key points of the experimental tank. In a circular tank, for example, the user clicks the center and circumference. The user can modify or move the arena thus defined until it precisely matches the edges of the experimental tank. The coordinates and distance (in screen units) of the arena thus defined are used in all calculations and are output to the data file, for reference. The user also specifies the size (diameter or width) of the tank in cm which, divided by the size in screen units, is used by the program to scale all measurements, so that all the distance measures output by the program are in cm. Thus, for example, if the diameter of a circular tank is 100 cm and is 5000 screen units in the video image, two fish that are measured to be 200 screen units apart will be registered as being 4 cm apart in the output file. Note that the software assumes that there is no distortion (caused, for instance, by a wide-angle lens on the camera) in the image.

To begin tracking, the video is paused at the first frame to be tracked. The user clicks with the mouse on the snout (where identifiable) or body of each fish in the group. The software places a small red circle at each click point, so that the user can keep track of which fish have already been identified in the current frame. When the number of fish identified reaches the previously specified size of the group, the software calculates all requested distances, writes the results to the output file, and skips forward to the next frame to be tracked (as defined by the user input sampling rate). All red circles are cleared from the screen. The process repeats until the user terminates the program or the video file ends.

The number of fish tracked so far in the current frame and the current location in the file in milliseconds are displayed beside the video controls at all times. In addition, the user may navigate the video at any point during the tracking without losing any data tracked so far. Thus, for example, if some fish are not clearly visible on the screen, the user can skip forward or backward a few frames. If the fish move slightly during this time, this becomes apparent on the previous or following screens and thus mislabelled or missing subjects may be avoided. This feature is extremely useful as fish often pass under or above each other, or swim so close to each other that they appear as one fish on the video image. The user can return to the frame being tracked by rewinding or typing in the time-code.

The software calculates a number of measures of interest, any of which may be output to the results file. Most measures may be calculated either for each fish in each frame or as an average for each frame. Some measures – those that depend on the enclosure – are only available if an enclosure has been specified (which is not required). The measures calculated are listed below.

First, the polar coordinates of the fish are found (Fig. 1). A horizontal line extending from the center of the enclosure to its right edge is taken as the horizontal axis, corresponding to $0°$. Angle increases in a counter-clockwise direction. Angles greater than $180°$ are expressed as negative values (e.g. $270° = −90°$). The polar coordinates of a fish are expressed as its angle in degrees from the horizontal axis and its distance in cm from the center of the enclosure.

The program then calculates, for each fish, its average (and the variance) and median distances from all the other fish in the group, its distance from the center of the enclosure, and its distance from its nearest neighbour (the latter is a measure commonly used to index shoal cohesion [21]). For each frame tracked, the software also gives the average and median distances between any two fish in the group (and the variance), the average nearest neighbour distance (NND) [21], the average distance of the group from the center of the tank, and the spread of the group (the smallest wedge, centered at the center of the tank and extending to its edges, that captures within it all the fish; see Fig. 1).
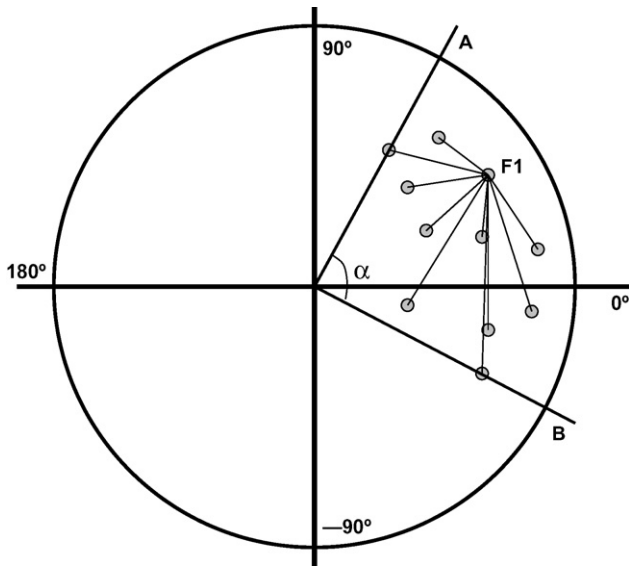
Fig. 1. Schematic of some of the measures extracted by the software. The large circle represents the tank, upon which axes have been overlaid. The axes' labels show the angle assigned to fish on each of the axes (0, 90, 180, and −90°). The small filled circles represent fish. Here, the distances between the fish marked F1 and the other fish are shown, as an example. The two lines labelled A and B delimit the smallest arc of the circle that encloses all the fish, which defines the angle α, the spread of the group.

The program can also calculate the distance of each fish (and an average) from a user-defined line, e.g. the edge of the tank, or from any fixed point. The distances between two distinct groups of fish (e.g. two different species) in the same tank can also be quantified. The distance from the first fish tracked in each frame may also be output (for cases in which there is a leader to the shoal, this would give the distance from the leader for each fish, and an average), or from a moving point (which needs to be re-specified in each frame tracked).

The program also calculates a measure of the area taken up by the group of fish. This is defined as the smallest n-sided convex polygon (i.e. containing no vertices with angles larger than 180°), which contains within it all the fish. For each frame, the software outputs the screen coordinates of all the vertices of this polygon and calculates its area (in cm^2).

Finally, the software also incorporates an expression evaluator that allows the user to specify his/her own expressions to be output to the results file. Up to four additional measures may be specified using any of the above measures as parameters. In addition, the software outputs to file the screen coordinates of each fish in each tracked frame (and the calibration information necessary to turn this into distances in cm), allowing the user to calculate numerous measures of interest post hoc.

Below we focus on the results obtained using one of the measures extracted by the software, the average distance between all fish.

The software was written in Visual Basic (Version 5.0, Microsoft Corporation). The entire application, when compiled, takes up only 85 kb of memory. Whilst the measures extracted by the program are written into the software (i.e. not accessible at run-time) for simplicity of use, they are separated in the code so as to be easily modified and augmented, if desired. Application files and source code are available from the corresponding author.

Although the identification of the locations of the fish requires a human observer, the automation of all other aspects of coding greatly increases the speed with which tracking proceeds. The flexibility of the software also allows tracking at any temporal resolution (up to the temporal resolution of the video, usually 30 frames/s).

There are several advantages to the present system over other systems used for similar purposes. First, the user can control and easily change the temporal resolution of quantification. Second, the precision of the system is not influenced by noise in the image, a problem often encountered with video-tracking systems that automatically track subjects (such as Ethovision). In other words, as the fish are tracked manually, they do not need to appear large in the picture, and this

allows the testing of shoals of small fish (such as zebrafish) in large tanks. As a result, the entire experimental tank, even if very large, may be viewed at all times and thus both the location of each fish relative to the other fish and to the tank may be quantified, an advantage compared to other applications where the camera had to follow the shoal [22]. Other applications that automatically track subjects, such as Ethovision, require that the subjects be clearly differentiated from their surroundings (i.e. high contrast with the substrate), that the subjects be easily identifiable by being of different colours (the problems this can raise are discussed above), or that the subjects are placed in separate compartments within the observed field of view. In addition, automated tracking approaches often have trouble dealing with frames in which two fish are so close together that they appear as one target. The current software application does not suffer from these limitations. For example, the user may move backwards and forwards in the video in arbitrarily small steps without affecting the tracking of the current frame. This use of future or past frames allows the user to identify where the 'missing' fish are located.

3. Results

To test the reliability of the system, two different observers independently coded the same data. Thirty-five non-consecutive randomly chosen video frames from Experiment 1 were coded by two of the observers that later coded all the remaining data. Differences in the average distance between fish registered by the software for each frame were calculated. The average difference between the results of the two coders was 0.60 cm (S.E.M. ±0.0997) and the greatest discrepancy for any frame was 2.11 cm. The differences were not significant (matched-sample t-test, $t(34) = 1.32$, $p = 0.195$). These levels of error in determining the position of the fish are comparable to those of similar systems [14,17].

3.1. Experiment 1

For the analysis of the time-series data from Experiment 1, the roughly 180 data points from each session (once every 10 s for 30 min) were reduced to 61 data points by averaging together the values of every three consecutive data points. Thus, for the 6 days of the experiment, we obtained a 61 (interval) × 6 (day) two-factorial repeated measures design requiring a repeated measures ANOVA with 366 levels. Unfortunately, our statistical software can only analyze the effect of an independent variable (factor) with a maximum of 99 levels. Thus, we decided first to investigate whether shoal density changes across days (see Fig. 2). We averaged the distance values obtained for each day and conducted the ANOVA with day as the repeated measure factor (with six levels: day 1, 4, 8, 11, 15, and 18). The results showed no significant day effect ($F(5, 35) = 0.536$, $p > 0.05$). Given the high temporal resolution we could achieve with our recording method, we subsequently investigated how shoal cohesion may change within a session (time interval, the repeated measure factor with 61 levels) separately for each day. The results are presented in Fig. 3. We assumed that shoal cohesion should decrease with time, as fish get used to the test tank throughout the session. However, the analysis showed that the effect of time was only significant on day 1 (ANOVA $F(60, 420) = 1.817$, $p < 0.05$). The significant ANOVA term was due to the fact that during the very first interval the distance between fish was larger than during several following intervals (Tukey
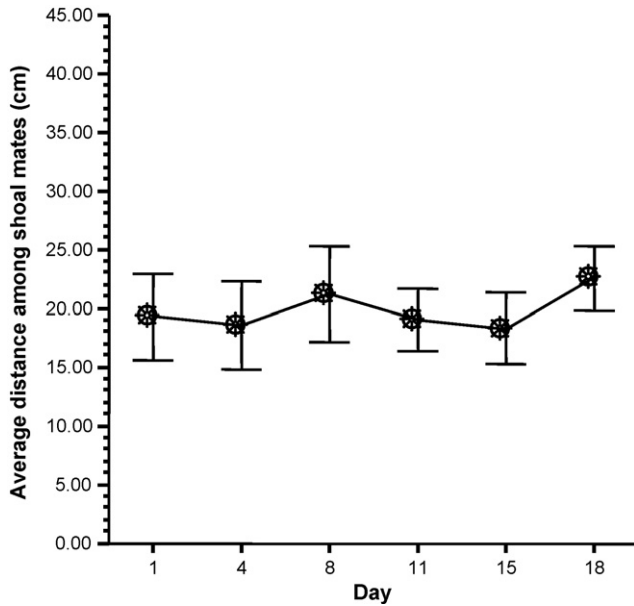
Fig. 2. Average distance between all fish of the shoal does not change across multiple days. Data are averaged over all time points for each session and over $n = 8$ shoals (each shoal containing 16 fish) to illustrate shoaling behaviour across days. Distances were calculated every 30 s over the entire 30 min session. Means ± S.E.M. are shown.

HSD multiple post hoc comparison test, $p < 0.05$). However, no significant time effect was found on day 4 ($F(60, 420) = 0.694$, $p > 0.05$), on day 8 ($F(60, 360) = 0.835$, $p > 0.05$), on day 11 ($F(60, 420) = 1.060$, $p > 0.05$), on day 15 ($F(60, 420) = 1.120$, $p > 0.05$), or on day 18 ($F(60, 420) = 0.685$, $p > 0.05$). In summary, these results show that after the very first 30 s interval shoal cohesion remains constant throughout the session and across days. Surprisingly, repeated exposure to the same open tank resulted in no significant change in shoal cohesion, a result that may be due to inability of zebrafish to remember the prior exposure event after the 3-day intertrial intervals or to the recurrent fear inducing aspect of the open field, possibilities that we intend to investigate in the future and discuss below.

3.2. Experiment 2

We predicted that food-deprived fish tested in an arena that contained scattered food would stay more distant from each other than fish tested in the absence of food, and this prediction was confirmed by our results (Fig. 4). The presence of food in the arena significantly increased the average distance between the fish ($F(1, 11) = 11.056$, $p < 0.01$). Furthermore, there was no significant change in shoaling density over time during the session ($F(60, 660) = 0.697$, $p > 0.90$) and the interaction between food treatment and time was also non-significant ($F(60, 660) = 0.519$, $p > 0.99$).
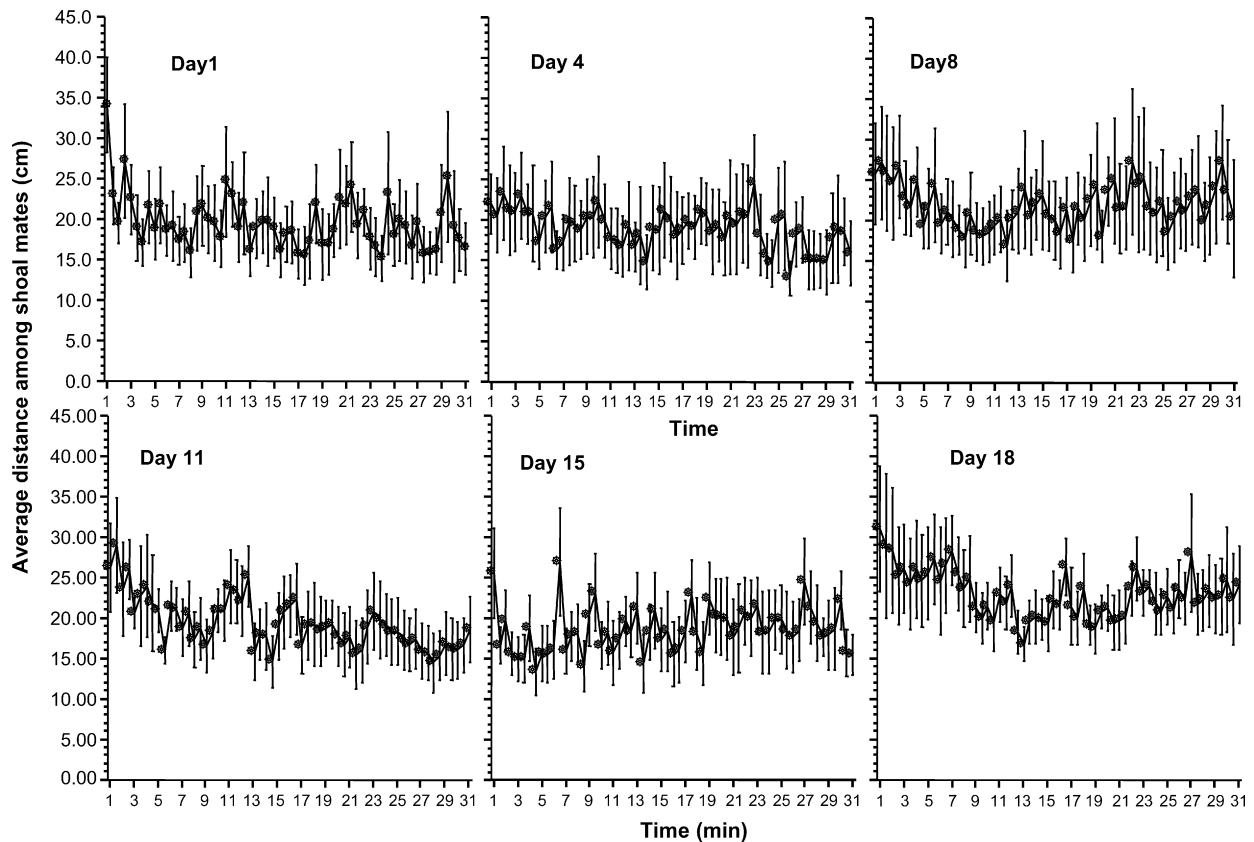

Fig. 3. Average distance between all fish of the shoal (shoal cohesion) does not change within sessions. Data are averaged over eight shoals of fish (each shoal containing 16 fish) and are shown separately for each day of testing (i.e. for days 1, 4, 8, 11, 15 and 18). Distances were calculated every 30 s over the entire 30 min session. Means ± S.E.M. are shown.
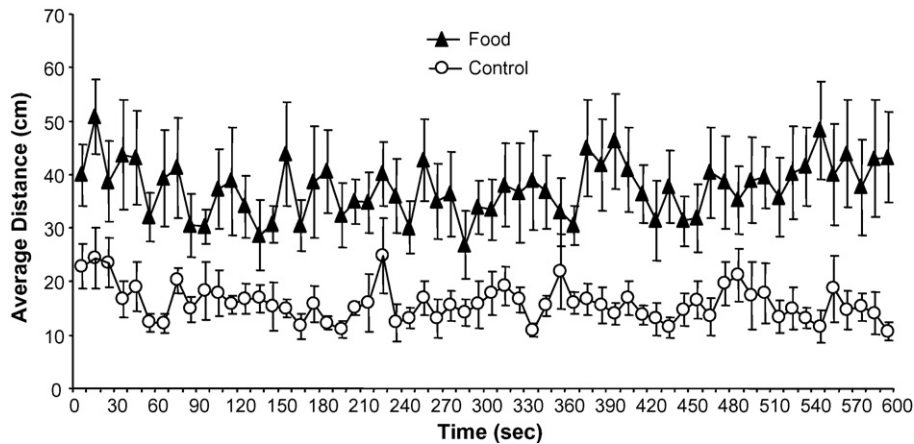
Fig. 4. Dispersed food increases distance between fish of the shoal. Average distance between all fish of the shoal over the course of a session is shown. Data are averaged over shoals (eight experimental groups and five control groups). Distances were calculated every 10 s over the entire 10 min session. Means ± S.E.M. are shown.

3.3. Experiment 3

Based on previously published results [20,34,24] we expected shoal density to increase in response to a model predator flown over the arena. The results shown in Fig. 5, however, demonstrate that this is not exactly what happened. The temporal trajectories of shoal density, quantified as the average distance among shoal mates, suggest a decrease of shoal density (increase of distance among shoal mates) in response to the appearance of the predator model. It is possible that the fish also displayed changes in swimming depth as a result of the appearance of the aerial predator. The current software is not equipped to detect such changes.

The predator model was flown over the arena three times during the 20 min session. The distances between the fish were coded at 1 s intervals for 60 s before and 30 s after each appearance of the predator. Thus, the experimental design of this study was a two factorial nested repeated measures design with Time (1 s intervals of the observation sessions, predator absent 60 levels, predator present 30 levels) and Repetition (three predator exposures). Given that SPSS can analyze the effects of factors with a cumulative maximum total of 99 levels, the above 270 level design had to be analyzed in multiple steps.

First, we analyzed whether there was any temporal change in shoal density during the 60 s before each of the three predator exposures, using a repeated measures ANOVA for each of these periods separately. This analysis revealed that shoal density did not change significantly during any of the three periods (effect of time first period $F(59, 413) = 0.239$, $p > 0.05$; second period $F(59, 413) = 0.670$, $p > 0.05$; third period $F(59, 413) = 0.465$, $p > 0.05$).

Next, we analyzed whether the appearance of the predator model led to changes in shoal density. This analysis was also run separately for each of the three periods during and immediately following the predator model presentations. ANOVA demonstrated that the appearance of the predator model elicited a significant change in shoal density during each of these periods (effect of time first period $F(29, 203) = 2.17$, $p = 0.001$; second period $F(29, 203) = 3.06$, $p < 0.001$; third period $F(29, 203) = 6.08$, $p < 0.001$). Perusal of Fig. 5 suggests that the significant temporal change in shoal density was due to a quick increase of distance among shoal mates in response to the appearance of the predator model, followed by a robust decrease of the distance among shoal mates (i.e. the shoal spread out when the predator model appeared, and then clumped together). This decrease reached below the previous distance, i.e. the zebrafish shoal tightened more than during baseline recording.

Although the temporal trajectories of shoal distance changes are fairly similar for the first, second and third predator model exposure, the decrease of shoaling distance that follows the exposure appears to become more pronounced for later exposures. These observations were confirmed by the analysis of separate time points. For this we compared the 30 post-predator time points with the 60 s pre-predator average. The analysis showed that zebrafish shoals responded with a significant increase of distance among shoal mates to the appearance of the predator model. For the first predator presentation the increase of distance was significant at time points 1, 2, 3, 4, 5, 6, 13, and 14 s post-predator (one sample t-test, $t(7) > 2.4$, $p < 0.05$; Holm correction employed as recommended by Aickin and Gensler [1]), and this increase dissipated by the end of the 30 s period. In response to the second predator presentation, the increase

Fig. 5. The appearance of the model of an aerial predator elicits significant changes in shoal cohesion in zebrafish (average distance among members of the shoal). The first panel of each row (A, C, E) shows the baseline response during a 60 s period immediately before predator model exposure ((A) before first, (C) before second, (E) before the third presentation). The second panel of each row (B, D, F) shows the response to the predator model during and immediately after its appearance for a 30 s period ((B) first, (D) second, (F) third predator presentation). Distances for each shoal were calculated once every second. Means ± S.E.M. are shown. Sample sizes, i.e. number of shoals tested, $n = 8$. Each shoal contained 16 zebrafish. The thin horizontal line shows the mean distance of shoal mates during the 60 s period preceding the predator model exposure. To aid comparison, the grey zone around the thin line shows the S.E.M. of the mean distance during the baseline period. The short thick black line on panels (B), (D), and (F), indicates the presence of the moving predator model.
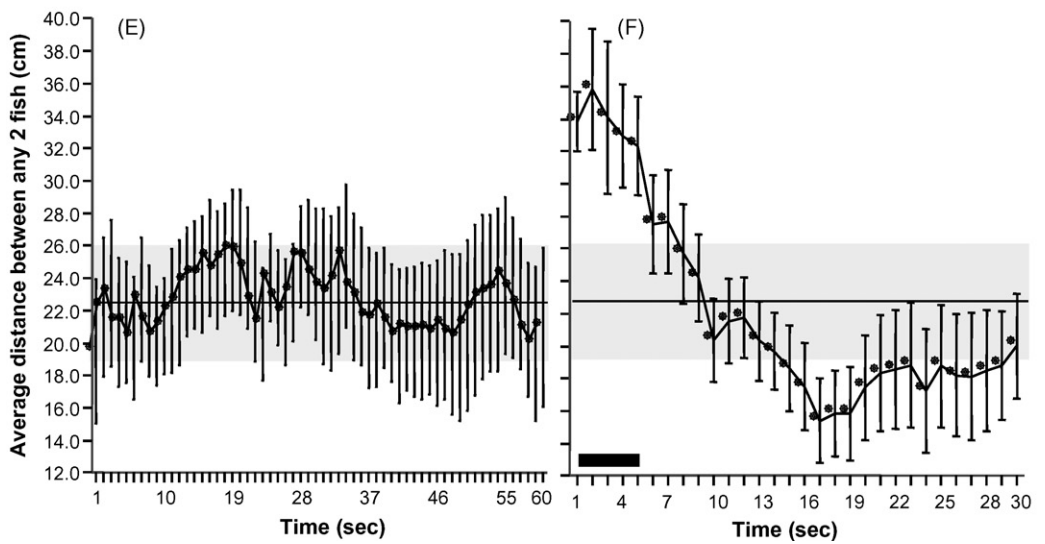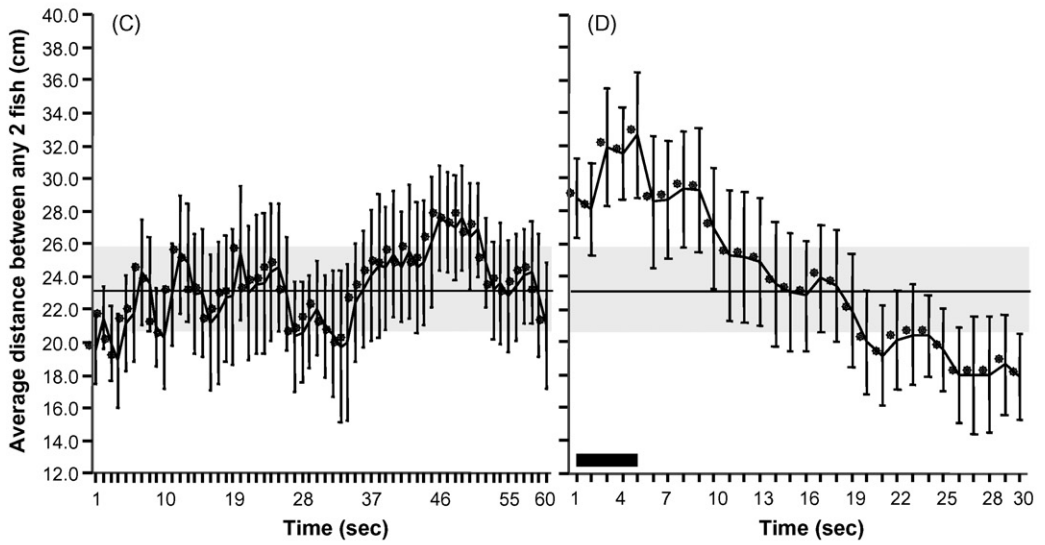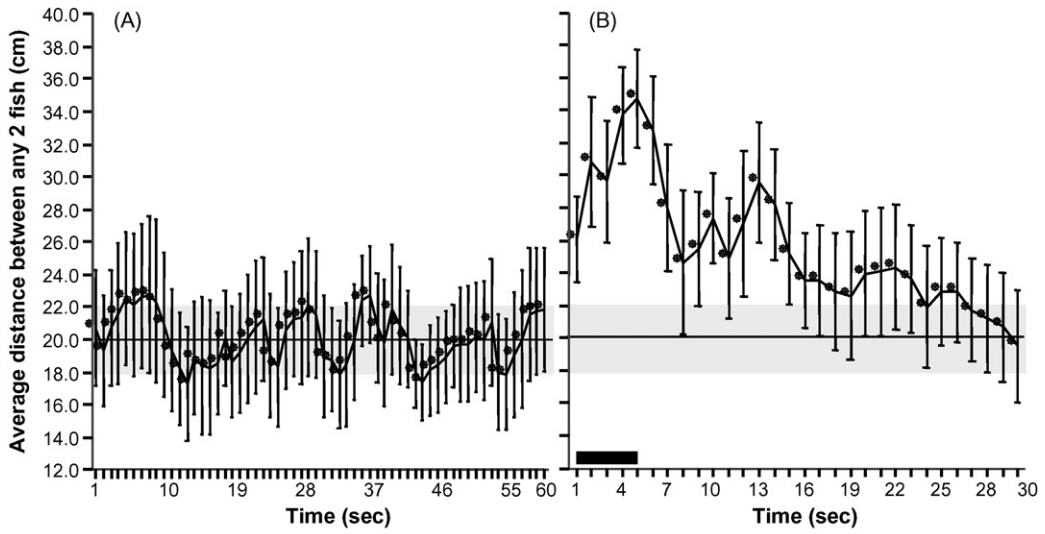
of distance among shoal mates was significant for the 3rd, 4th, and 5th second post-predator ($t(7) > 2.4$, $p < 0.05$; Holm correction employed) but the subsequent shoal distance decrease now appeared to go below the level of the pre-predator value. In response to the third predator exposure the pattern of temporal change of shoal density was again similar. The appearance of the predator elicited a significant increase of distance during the time points 1, 2, 3, 4, and 5 s post-predator (one sample t-test, $t(7) > 2.5$, $p < 0.05$; Holm correction employed) and now the decrease of distance during the latter part of the 30 s post-predator reached significance, i.e. shoaling distance was significantly below the mean value recorded for the pre-predator exposure period (time points 17, 18, and 19 s, one sample t-test, $t(7) > 2.4$, $p < 0.05$; Holm correction employed). These results agree with previous findings [24] that also show no habituation over repeated exposures to an aerial predator. Briefly, the above suggests that the appearance of an aerial predator model elicits an immediate escape reaction, i.e. scattering, which is followed by a fast reassembly of the shoal. Furthermore, repeated exposure to the predator leads to a transient increase in shoal cohesion immediately after the predator model disappears, i.e. a temporary tightening of the group.

4. Discussion

The software system developed by us and utilized here is a fast, flexible, and simple way to track groups of animals from video recordings. The system recognizes most common formats of video file and requires nothing more than a personal computer to run on. We have found that an observer with some practice can code a single frame of video (containing 16 fish) in under half a minute. The software can deal with observation arenas of any shape. The current list of measures the system quantifies is extensive and additional measures may easily be added as specific applications require.

The current system does not provide 3D information about the location of the fish, as it is primarily designed to work in large observation arenas in which the movement and distribution of small subjects are observed. In the current example, the zebrafish, which reach a length of about 4 cm at maturity, were in a 91 cm diameter, 30 cm deep, circular tank. It is notable that the mouth structure and foraging behaviour of zebrafish suggest the species lives near the water surface. We also observed that zebrafish tend to stay near the surface of the water [3]. Thus not recording the third dimension of shoal distribution, i.e. depth, may have led to minimal loss of information in the current study. It is also notable that in this setup individual fish may occupy no more than 10–20 pixels on the screen, far too few for an accurate 3D position to be determined by either of the two most commonly used methods discussed above. Additionally, our system allows easy motion forward and backward in the video in small intervals during tracking, which facilitates identification of fish that move very close to or overlap with each other at certain time points.

The newly developed system is particularly useful for high-resolution temporal analysis of shoaling. To the best of our knowledge, no studies to date have examined temporal changes in shoal density with the current resolution. Previous studies that tracked fish in free-swimming shoals only presented behavioural parameters including shoal cohesion (density) for the entire session [2,13,17]. With our high-resolution tracking we found not only that shoal cohesion remained unaltered across days of observation but also that it did not significantly change with time within a session. This was an unexpected finding. It is possible that the ideal distance between members of a shoal is insensitive to novelty and thus habituation to novelty does not alter its manifestation. Alternatively, it is possible that in the current experimental set up longer periods of exposure to the same environment would have been needed to detect shoal cohesion changes. Delaney and colleagues [4] found that zebrafish shoaled for 2–3 h before dispersing in the tank. The arena these authors used contained artificial plants and it is thus possible that zebrafish did not habituate to the novel environment in our studies because our observation tank with its white background and absence of hiding places remained aversive throughout the repeated trials. It is also possible that the experimental fish did not remember the arena from trial to trial separated by the 3-day intertrial intervals. At this point we do not know which of these explanations is correct. Systematic analysis of the mnemonic characteristics of zebrafish as well as of the environmental conditions (e.g. [4,9]) under which fear reactions diminish or increase will clarify these questions.

Traditionally, fish that are within four body-lengths of each other (about 16 cm for zebrafish) are considered part of the same shoal [12]. The average distance between shoal mates reported here appears greater than this figure. Importantly, however, the traditional measure refers to the distance between the nearest neighbours (NND) within the shoal. The measure we report is the average distance among all members of the shoal, which includes distances between fish that are not nearest neighbours. Although these two measures (NND and average distance among all shoal members) correlate with each other, we opted for the use of the latter because we felt it more completely describes the distribution of fish within the shoal. Importantly, using this measure we found that shoal cohesion in zebrafish was fairly stable (about 20–25 cm for a shoal of 16 zebrafish) for the intervals of 30 min trials and across multiple trials separated by days of intertrial interval. Naturally, this stability does not mean that shoal cohesion is unchangeable. Our experiment with the distributed food source clearly showed that when competition among shoal mates was increased, so was the distance among the members of the shoal: the presence of food scattered in the tank doubled the distance among shoal mates compared to the baseline value. This finding, which was expected based on previous results on competition among members of animal groups including reptiles [43], birds [5], mammals [11], and other species of fish [32,34], is now documented in zebrafish for the first time. This result confirms the notion that shoaling may be maladaptive when it comes to foraging as it increases competition for food [33,25]. This argument may be valid when food is not localized and identification of a food source does not require cooperation, a situation likely hold true for zebrafish [34]. However, it may be premature to conclude on this issue, as the ecology, types of food sources, and the foraging strategies of zebrafish in nature have

not been fully characterized, nor have their food preferences been studied under laboratory conditions.

Although the function of shoaling in foraging may be controversial, there is strong consensus with regard to its role in predator avoidance [29 and references therein]. Aggregation of prey may be an effective antipredatory response via either the confusion effect, i.e. by making the predator less able to focus on an individual target [29,16] or by making the predator get satiated quickly, thereby reducing the probability of catching a particular prey individual within the group [30]. The current study did not investigate the precise role of shoaling in predator–prey interaction, whether it is effective, and whether it is effective against particular types of predators. It simply provided the first detailed description of the temporal changes that occur in shoal cohesion in response to a brief appearance of an aerial predator model. The detailed description was made possible by our newly developed custom software, which allows shoal cohesion to be measured at any temporal resolution. The results revealed an abrupt and transient increase of distance among shoal mates in response to the appearance of the predator followed by a fast regrouping to a cohesive shoal in which the distance among shoal mates temporarily decreased below baseline. It is also possible that swimming depth increased as a result of the appearance of the predator, but we did not measure depth information in the current study. Others have suggested that the appearance of predators may affect shoaling in just such a manner [20,16] but this response has not previously been experimentally quantified.

In summary, the current study provides a new method for easily and flexibly quantifying shoaling behaviour and its fine-scale temporal changes. The results are preliminary in nature but they suggest that, with the use of our new method, the characterization of shoaling behaviour may answer a spectrum of questions ranging from the ecology of fish species to the analysis of fear reducing pharmaceutical substances including anxiolytics.

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