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The effects of flow on schooling *Devario aequipinnatus*: school structure, startle response and information transmission

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Abstract

To assess how flow affects school structure and threat detection, startle response rates of solitary and small groups of giant danio *Devario aequipinnatus* were compared to visual looming stimuli in flow and no-flow conditions. The instantaneous position and heading of each *D. aequipinnatus* were extracted from high-speed videos. Behavioural results indicate that (1) school structure is altered in flow such that *D. aequipinnatus* orient upstream while spanning out in a crosswise direction, (2) the probability of at least one *D. aequipinnatus* detecting the visual looming stimulus is higher in flow than no flow for both solitary *D. aequipinnatus* and groups of eight *D. aequipinnatus*, however, (3) the probability of three or more individuals responding is higher in no flow than flow. Taken together, these results indicate a higher probability of stimulus detection in flow but a higher probability of internal transmission of information in no flow. Finally, results were well predicted by a computational model of collective fright response that included the probability of direct detection (based on signal detection theory) and indirect detection (*i.e. via* interactions between group members) of threatening stimuli. This model provides a new theoretical framework for analysing the collective transfer of information among groups of fishes and other organisms.

Keywords

collective behaviour; information transmission; predator avoidance; rheotaxis

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INTRODUCTION

In schooling fishes, local interactions benefit the school by improving predator detection and avoidance. A group of animals is capable of transferring directional information about discrete sources in the external environment (Breder, 1959; Radakov, 1973; Magurran & Higham, 1988; Ryer & Olla, 1991; Couzin *et al.*, 2006; Ward *et al.*, 2008). Rapid predator evasion and waves of information transmission have been observed in schooling fishes, in the laboratory (Radakov, 1973) and in the field (Axelson *et al.*, 2001; Gerlotto *et al.*, 2006), with model or natural predators. This evasion tactic, also known as the Trafalgar effect (Treherne & Foster, 1981; Godin & Morgan, 1985), transmits information within the school *via* waves of response that can spread faster than the speed of an approaching predator (Radakov, 1973). Thus, social information transmission is likely to play a role in the collective response to a predator. The information content (*e.g.* whether it conveys general alarm, the direction and speed of the approaching predator, or the escape direction of nearby fishes), however, is not well understood, including the sensory mechanisms by which information is transmitted through the school.

Fishes use a variety of sensory modalities to receive information from a predator or other fish in the school, including vision (Dill, 1974; Guthrie & Muntz 1993), olfaction (Ferrari & Chivers, 2006; Mathuru *et al.*, 2012) and lateral line sensing (McHenry *et al.*, 2009; Mirjany *et al.*, 2011). Although the extent to which ambient currents might affect the ability of fishes to detect predators, either directly or indirectly, is unknown, currents would be expected to have a large effect on the flow-sensing lateral line system. For example, local hydrodynamic disturbances produced from the C-start escape manoeuvres of fishes in response to a predator are capable of stimulating the lateral line of nearby fish in a school (Tytell & Lauder, 2008), but the ability of fishes to detect these local flows may be masked by the presence of ambient water motions, such as those in a stream (Coombs *et al.*, 2001; Engelmann *et al.*, 2002). On the other hand, the fact that flow elicits rheotactic behaviour means that fishes are more likely to be aligned in a common direction, which is typically upstream. Rheotacting fishes should thus have less directional noise, meaning that deviations from the polarized direction such as startle responses may be more easily detected *via* vision or the lateral line. Additionally, increased alignment has the effect of minimizing relative motion between adjacent fish, thus reducing hydrodynamic and optical-flow noise generated by the movements of nearby fish.

In teleosts, the neural basis of startle-escape behaviour has been studied in much detail (Eaton *et al.*, 2001; Korn & Faber, 2005). The behaviour is controlled by a system of reticulospinal neurons, including a pair of large Mauthner neurons on which multimodal (auditory, visual and lateral line) inputs converge (Fukami *et al.*, 1965; Eaton & Emberley, 1991; Zottoli *et al.*, 1995; Preuss *et al.*, 2006). A single action potential in one Mauthner cell reliably activates contralateral spinal motor neurons causing a fast, startle response away from a potential threat (Eaton *et al.*, 1977; Preuss & Faber, 2003; Preuss *et al.*, 2006; Weiss *et al.*, 2009). Startle probability is a quantifiable behavioural measure that reflects the excitability of the Mauthner cell system remarkably well (Preuss & Faber 2003; Neumeister *et al.* 2008, 2010). In fact, Mauthner cells are regarded as decision-making neurons for the

execution of the startle response and there is evidence for both ecological and social regulation of the decision to startle at the level of the Mauthner cell (Neumeister *et al.*, 2010).

While quantification of startle probability has been investigated frequently in individual fish, the evaluation of startle probability of groups of fishes, as well as how the startle probability varies under different flow conditions, has been rarely studied. Godin *et al.* (1988) measured the probability as a function of school size of at least one individual startling in a school of glowlight tetra *Hemigrammus erythrozonus* Durbin 1909 subjected to an artificial threat stimulus (a brief flash of light); this study provides evidence that the probability of detection increases with group size. Domenici & Batty (1997) compared the kinematics of startle responses of solitary herring *Clupea harengus* L. 1758 to schooling *C. harengus* and found that when responding to a sound stimulus, schooling *C. harengus* had longer latencies but improved directionality. Additionally, other studies of startle response behaviour have been conducted in various ecological conditions including turbid (Meager *et al.*, 2006) and hypoxic conditions (Lefrancois *et al.*, 2005).

In the current study, startle response rates to an external threat (a visual looming stimulus) were quantified for solitary giant danio *Devario aequipinnatus* (McClelland 1839) and groups of eight *D. aequipinnatus* in flow and no flow conditions. A further aim of this study was to determine the effects of flow on the collective transmission of threat information within groups of fish, as measured by the number of *D. aequipinnatus* responding to an external threat. Godin *et al.* (1988) used classical theories of signal detection to predict the number of fish responding. These predictions underestimate the experimentally observed number of *H. erythrozonus* responding. One explanation for the disagreement between theory and experiment is that the independent probability signal detection model used by Godin *et al.* (1988) fails to take into account socially transmitted information. It could thus be hypothesized that the probability of an individual fish responding in a group may depend on the behaviour of other shoal mates. In this study, a probabilistic model that includes socially transmitted information predicts the number of fish responding. The aims of this study were thus to quantify the role of bulk flow on school structure and startle response probability in groups of fish, as well as to investigate the effect of flow on information transmission.

MATERIALS & METHODS

FISH CARE

Devario aequipinnatus, 3–4 cm in standard body length (L_S), were obtained from commercial aquarium suppliers and housed in 76 l tanks with *c.* 5–6 *D. aequipinnatus* per tank. *Devario aequipinnatus* are a suitable model system to investigate the role of environmental and social inputs in the decision to escape from a predator, because they inhabit streams and rivers, are a facultative schooling species and exhibit characteristic startle response behaviour. *Devario aequipinnatus* were maintained at 21–23°C on a 12L:12D cycle. Experimental protocols for the care and use of animals were approved by the Institutional Animal Care and Use Committee at Bowling Green State University, where the experiments were conducted (protocol #10-018).

EXPERIMENTAL SET-UP

Flow-tank experiments were carried out in a rectangular, plexiglass channel ($154 \times 28 \times 35$ cm) filled to a depth of 25 cm (Fig. 1) and similar in design to that described by Vogel & Labarbera (1978). *Devario aequipinnatus* were tested in the centre of the flow tank in a removable compartment with dimensions of equal length ($25 \times 25 \times 25$ cm) to avoid possible directional bias. Water in the flow tank was circulated through a 20.32 cm (8") polyvinyl chloride return pipe via a 12.7 cm (5") aluminum impeller blade attached to a chem-stirrer (IKA Labor Technik RW 20DZM; www.ika.com). Water circulation was facilitated with a baffle placed at the downstream end of the tank. Turbulence created by the chem-stirrers was reduced with two coarse collimators consisting of plastic egg crates (3 cm thick with 1 cm pores) placed at the upstream and downstream end of the flow tank. Additionally, a fine collimator composed of drinking straws (3 cm in length) was located between the upstream coarse collimator and the working compartment. The flow tank was calibrated using dye to create a calibration curve for converting impeller speed (rpm) to flow velocity (cm s^{-1}). The flow speed used ($c.7 \text{ cm s}^{-1}$ or 468 rpm) corresponds to $2 L_S \text{ s}^{-1}$. Although flow speeds normally encountered by *D. aequipinnatus* in the wild are not well characterized, this speed was sufficient to evoke rheotactic behaviour and was probably well within the range of flow speeds encountered in their natural habitats (McClure *et al.*, 2006).

Two 43 cm (17") liquid crystal display (LCD) monitors (Dell P170S, 60 Hz; www.dell.com) were placed on each side of the flow tank's working area for stimulus display. An additional 20 cm monitor (Accele Electronics, Inc. Accelelevision 20 cm LCD module LCD3LVGA; www.iaft.com) was placed outside the working area, for verification of the stimulus display. A dorsal view of *D. aequipinnatus* was recorded at 400 fps using a high-speed camera (Itronx DRS Data and Lightning RDT high-speed camera; www.itronx.com), with a spatial resolution of 1280×1024 pixels. The camera was mounted 140 cm above the experimental set-up and calibrated before each trial. The working compartment was backlit with a 34 W bulb diffused quarter stop by an optical diffuser to provide uniform, upwelling light within the tank.

STIMULUS DESIGN

Looming stimuli consisted of a computer-generated series of black-on-white disks that increased in size to give the appearance of a constant approach velocity (Dill, 1974) until they reached a maximum size of 15 cm. Even though the centre of the stimuli was randomly positioned on the computer screen, the entire stimulus was always present on the monitor. Five stimuli with various approach speeds (20, 40, 80, 160 and 320 cm s^{-1}) were generated in Matlab (2007, R2007a Mathworks; www.mathworks.com). Stimulus speeds were selected to fall within the range of swimming speeds of the natural predators of *D. aequipinnatus* (Shahnawaz *et al.*, 2010). These looming stimuli were projected in random screen locations onto either one of two monitors on opposing sides of the flow-tank working area. Random screen locations minimized the effect of habituation or place preferences resulting from predictability of the stimuli. The size (43 cm) of the LCD monitors relative to the tank size (25×25 cm), randomizing the stimulus locations provided a variable angle of approach, even to the rheotacting *D. aequipinnatus*. Given the robust startle responses of *D. aequipinnatus* to the computer stimuli and the refresh rate of the computer monitors (60 Hz), which is well

above the range of flicker fusion frequencies (39–45 Hz) measured for other cyprinids [*e.g.* goldfish *Carassius auratus* (L. 1758) and zebrafish *Danio rerio* (Hamilton 1822)] (Makhankov, 2005), monitor refresh rate is unlikely to have been a significant factor in these studies.

EXPERIMENTAL DESIGN

The experimental design consisted of a multi-way design with two within-subjects variables (flow condition and looming speed) and one between-subjects variable (social context). Both flow condition and social context had two levels: flow present (7 cm s^{-1}) or flow absent (0 cm s^{-1}) and solitary (one individual) or social (group of eight *D. aequipinnatus*). To assess the probability of a startle response with or without a looming stimulus, *D. aequipinnatus* were exposed to blank trials (no stimulus) and five different looming speeds (20, 40, 80, 160 and 320 cm s^{-1}). Different loom speeds were used so that stimulus-response functions could be generated and the best looming speeds identified for generating startle responses under the conditions of these experiments. Blank trials and stimulus trials of varying loom speeds were presented randomly with inter-stimulus intervals ranging from 3 to 10 min. Eight replicates of solitary and grouped *D. aequipinnatus* were presented with two sets of stimuli in each of the flow and no flow conditions in a repeated-measures, counter-balanced blocked design, so that half of the replicates were exposed to the flow condition first and the other half were exposed to the no-flow condition first. Before the start of each trial, water temperature of the experimental tank was measured to ensure that the temperatures did not deviate from the home tank or change over time by $> 2^\circ\text{C}$. *Devario aequipinnatus* were transferred from the holding tank to the experimental tank using a plastic-lined net, which provided a cushion of water around the *D. aequipinnatus*, in order to minimize stress and possible damage to the lateral line system.

EXPERIMENTAL PROTOCOL

Solitary and groups of eight *D. aequipinnatus* in two flow conditions (0 and $2 L_S \text{ s}^{-1}$) were filmed in response to blank and stimulus trials in a repeated-measure block design. Trials were conducted during the day between 0900 and 1700 hours on two consecutive days with one day of tests in flow and the other in no flow. Each replicate was tested at the same time of day to help avoid potential confounds from order and time-of-day effects.

Trials were automated using custom scripts in Linux (OpenSuse version 11.2; www.opensuse.org) and Matlab to create movies of the stimulus presentation and a 30 min acclimation period. During habituation and inter-trial intervals, the monitors displayed blank white screens. The stimulus movie was played on VLC media player (videolan.org) at 60 fps from a single Linux computer with an extended desktop display. The display was split three ways by a multi-monitor adapter (Dell Multi-monitor hub adapter, DP-to-DVI, 64XHK; www.Dell.com) to the left and right side monitors, as well as to a small verification screen. The verification screen displayed movies being shown on either screen, with a black square in either the upper-left or upper-right corner corresponding to the active monitor.

At the start of each acclimation period, the experimenter left the experimental room and monitored trials from outside of the experimental room on another desktop computer, which

collected and saved the data from the overhead camera. To co-ordinate data capture with the timing of stimulus presentation, the camera was triggered through a data acquisition board (National Instruments USB-6525; www.ni.com), which sent a 5 V signal to a video-capture module connected to the camera (Midas 2.0 AD2M Module for auto-download/auto-reset/auto-triggering; <http://xcitex.com>).

Behavioural trials were recorded using a single, overhead video camera, and stored on hard drives for later analysis. Behaviour was recorded for 8 s, which included the entire presentation of blank or looming stimuli, as well as several seconds before and after. Although 8 s was the maximum recording time of the camera hardware, this was enough time to capture startle responses, even taking into consideration the variability in latency and duration (Meager *et al.*, 2006).

FISH TRACKING

Fright response kinematics and latency were analysed using a custom, multi-fish tracking system developed in Matlab (Butail & Paley, 2012). The tracking system automatically reconstructs the full-body trajectories of schooling fishes using two-dimensional silhouettes. The shape of each fish is modelled as a series of elliptical cross-sections along a flexible midline. The size of each ellipse is estimated using an iterated extended Kalman filter (note that for a single overhead view, only one of the elliptical axes is estimated and the other is assumed to be constant). The shape model informs an optimization algorithm that locates the fish midline in each frame. The tracking system was used to monitor the head position (the point midway between the eyes) and head orientation of each *D. aequipinnatus* throughout each trial. Verification and correction of tracking error was performed with a custom Matlab interface by manually selecting points on the midline overlaid on the raw video frames.

CHARACTERIZING THE STARTLE RESPONSE

The startle response of an individual *D. aequipinnatus* was assessed using the angular rate of rotation of its head orientation (Domenici & Blake, 1997), which was estimated by the tracking system (Butail & Paley, 2012). The rotation rate of the head orientation was calculated by a numerical (two-point) differencing method over time and compared to a threshold to determine whether a startle response occurred. An appropriate threshold would include fast turning responses, possibly mediated by the Mauthner cell, and exclude slower turns of longer duration that may be caused by normal swimming motion or the downstream displacement by the flow. A threshold of $3000^{\circ}\text{s}^{-1}$ was selected by evaluating the rotation rate of head orientation during independent observations by the authors of known startle responses. The rotation rate and latency of startle responses vary, depending on the neuronal control (Meager *et al.*, 2006), and thus some slower startle responses may have been excluded. Latency values were measured by calculating the time from the onset of the stimulus to the first turning of the head.

RELATIVE POSITIONS

For grouped *D. aequipinnatus*, standard quantitative assessments of shoal properties were measured, including nearest neighbour distance (NND) and the ratio of distance between the first and second nearest neighbour ($\text{NND1}/\text{NND2}^{-1}$) (Delcourt & Poncin, 2012). Distances

were calculated along the line between *D. aequipinnatus* head positions. The spatial probability of neighbour position was calculated by considering each *D. aequipinnatus* in the school as a focal individual; the relative position and relative orientation of every other *D. aequipinnatus* in the school were considered over all time points of the undisturbed school (*i.e.* before the *D. aequipinnatus* were startled). Therefore, at each time the neighbour was in a particular position, one count was added to the corresponding bin. The relative position of a neighbour is expressed in Cartesian co-ordinates with the focal *D. aequipinnatus* at the origin oriented along the positive y-axis. Relative positions were converted from pixels to L_S using ratios 22 cm pixel^{-1} and $4 \text{ cm } L_S^{-1}$, based on camera calibration. Data were collected from the start of the stimulus until the first reaction, if one was detected, or until the end of the stimulus presentation. No differences were observed in *D. aequipinnatus* behaviour or position even in the presence of a stimulus, unless a startle response occurred that disturbed the school. Relative positions were binned into 60×60 bins using Matlab's *hist* function. The probabilities for each bin were computed by normalizing bin counts by the bin with the highest count (*i.e.* the most visited bin). Elliptical contours of the first and second S.D. of a bivariate Gaussian distribution were fitted to these probabilities. In order to account for possible wall effects, data points within one L_S of the wall were excluded for statistics on school structure.

POLARIZATION

In the multi-agent model described by Kuramoto (1975), synchrony is quantified using the complex phase order parameter defined as

$$p_\theta \triangleq \frac{1}{N} \sum_{j=1}^N e^{i\theta_j} = \frac{1}{N} \sum_{j=1}^N (\cos\theta_j + i\sin\theta_j)$$

where θ_j is the orientation of agent j and N is the number of agents and $i = \sqrt{-1}$. The magnitude $|p_\theta|$ represents the level of alignment in orientation and ranges from 0 to 1, with values of one for fully aligned. The magnitude of p_θ is also referred to as vector strength. The orientation of p_θ indicates the direction of the average velocity, relative to 0° , which is defined to be upstream.

STATISTICAL ANALYSIS

Statistical analysis was performed using Matlab and the stats package in R (version 2.14; www.r-project.org/). General linear mixed models for binomial response assessed how well the factors of flow condition, group size and stimulus speed predicted the frequency of startle responses. For each group size, there were eight replicate groups (eight individual and eight groups of eight *D. aequipinnatus*). These groups were included in the model as a random factor. In order to compare differences in media vector strength between flow and no-flow, paired Wilcoxon rank tests were used because the data did not meet the assumptions for parametric analysis. Rayleigh tests (Batschelet, 1981) were used to determine whether the phase order parameter distributions differed significantly from random. The concentration parameter of the circular distribution of the phase order parameter and mean orientation were compared for both groups using the CircStat Toolbox

for Matlab (Berens, 2009). An equality of variance F -test was used to assess differences in school structure between flow and no flow, in both the x and y directions. This test only included data points at least one L_S (4 cm) away from any wall, in order to account for possible wall effects, although all data points were plotted for smoother visualization. A G -test was run on the frequency distributions of the number of *D. aequipinnatus* responding. Data were pooled to compare a low number of *D. aequipinnatus* responding (less than or equal to three) to higher numbers of *D. aequipinnatus* responding (half the group or more) and polynomial models were fitted to the cumulative probability distribution of the number of *D. aequipinnatus* responding using Matlab's *polyfit* function. For all statistical tests, significance level was set to $P < 0.05$.

MODEL OF INFORMATION TRANSMISSION IN SCHOOLING FISH

A new model of information transmission in schooling fish was created and fitted to the experimental data in order to investigate the role of intra-school signal propagation. Let $P_j(t)$ be the probability that agent j detects and responds to an external signal at time t , where $j = 1, \dots, N$ and N is the number of agents in the group. Assuming $P_j = P$ for all j and t , classical models of signal detection theory (Treisman, 1975; Lazarus, 1979) predict the expected probability of threat detection P_D by at least one agent in the group to be $P_D = 1 - (1 - P)^N$.

Classical theory (Treisman, 1975; Lazarus, 1979), also predicts the expected number N_D of agents responding to be $N_D = \sum_{j=1}^N P_j = NP$, which underestimates the observed number of fish responding in a school (Godin *et al.*, 1988). The discrepancy between the predicted and observed number of responding agents may be a result of social transmission in the group and intra-school interactions. Who interacts with whom can be represented and analysed by modelling the interaction network (Ballerini *et al.*, 2008; Mesbahi & Egerstedt, 2010), in which each individual receives information from a possibly limited number of neighbours according to a set of interaction rules.

Consider the following alternative model for P_j , which includes the probability of indirect detection of threatening stimuli through the interactions with other agents. An important aspect of this model is the inclusion of time t . Let $P_{j,k}$ be the probability that agent j perceives (and responds to) a cue from agent k and $P_{j,j}$ be the probability that agent j sustains the response state from one time step to the next. The notation N_j denotes the neighbour set of j , which is the set of agents that generate information received by agent j . Suppose without loss of generality that the stimulus occurs at time $t=0$. $P_j(t)$, for $t > 0$, is thus the probability at time t that agent j experiences a false alarm, sustains a response from the previous time step $t-1$, or detects and responds to a neighbouring agent that is in the response state at time $t-1$. Let $P_{j,k} = P^{int}$ for all pairs j and k be the probability of information transmission between neighbours and $P_{j,j} = P^{sus}$ be the probability of sustained response from the previous time step (assuming identical individuals). Then $P_j(t)$ becomes

$$P_j(t) = 1 - \underbrace{(1 - P^{far})}_{\text{No false alarm}} \underbrace{(1 - P^{sus} P_j(t-1))}_{\text{No sustainment}} \underbrace{\prod_{k \sim N_j(t)} (1 - P^{int} P_k(t-1) (1 - P_j(t-1)))}_{\text{No transmission from neighbours}}$$

Where $P_j(0) = P_j^{ext} = P^{ext}$ for all j is the probability that agent j directly perceives the external cue at the initial time $t=0$ and $k \sim N_j$ is each neighbour k within the neighbour set of agent j .

The probabilistic model above predicts how information flows through a network and gives insight into the experimental results, even though the interaction network and interaction probability P^{int} cannot be measured directly. Note that there are four parameters in the model (P^{far} , P^{sus} , P^{ext} , P^{int}). Two parameters, P^{far} and P^{sus} , were extracted directly from experimental results as follows: P^{far} represents the rate of false alarms, which was determined to be negligible for both flow conditions and P^{sus} represents the duration of a startle, which was set to 100 ms in the model, although startle speeds and durations often exhibit variability (Eaton *et al.*, 1977).

The interaction network used in the model to generate the neighbour sets N_j , $j = 1, \dots, N$, was also based on experimental data. While it is unclear which network topology fishes use, there is evidence for the use of nearest-neighbour networks in animal groups (Ballerini *et al.*, 2008; Herbert-Read *et al.*, 2011). Many mathematical models of fish schooling behaviour have been founded on the use of proximity-based interactions. Therefore, the results were analysed in the context of both network models using the head positions at the start of the stimulus presentation for each trial.

In order to fit the model to the data, a fast probabilistic optimization technique known as simulated annealing (Aarts *et al.*, 2003) was applied. This method mimics the physical process of gradually cooling a physical material to decrease defects, effectively minimizing the system energy (the cost function). By occasionally accepting points that raise the cost function, this algorithm avoids being trapped in local minima in early iterations and is able to explore globally for better solutions. The cost function in this case was the average mean-squared error between the model fit for the cumulative probability distribution of startle responses and the number (1–8) of *D. aequipinnatus* responding over five simulation runs. The model was optimized to find the values of P^{ext} and P^{int} that minimized the cost function. Multiple simulated annealing runs were conducted using a Boltzmann cooling schedule (Aarts *et al.*, 2003). Simulated annealing runs were terminated when the cost function did not change beyond a given tolerance.

RESULTS

DEVARIO AEQUIPINNATUS SHOW POSITIVE RHEOTAXIS IN FLOW

As a first step in the analysis, the phase order parameter p_θ , a measure of inter-fish alignment, was computed. A group of fish aligned in the same direction has a vector strength $|p_\theta|=1$ and a completely unorganized group of fish has a vector strength $|p_\theta|=0$. The

magnitude of the phase order parameter in the first time step (frame) is called the initial vector strength; the average over all time steps (video frames) of an undisturbed school is called the time-averaged vector strength [Fig. 2 (a), (b)]. A two-tailed, paired Wilcoxon signed-rank test indicated that the time-averaged vector strength was significantly higher in the flow condition (Mdn = 0.92, $n = 88$) than the no-flow condition (Mdn = 0.42, $n = 83$, $Z = -6.97$, $P < 0.001$). Moreover, fish headings in flow were not randomly distributed with respect to the upstream direction (mean vector direction = 3° ; V test, $V = -22.1$, $P > 0.05$), whereas those in no flow were randomly distributed (mean vector direction = 166° ; V test, $V = 79.9$, $P < 0.001$). Thus, the upstream rheotactic behaviour of *D. aequipinnatus* in flow enhances the degree of inter-fish alignment. Nevertheless, the distribution of orientations was significantly non-random in both the flow (Rayleigh test, $Z = 78.9$, $P < 0.001$) and no-flow group conditions (Rayleigh test, $Z = 7.25$, $P < 0.001$) suggesting that groups of *D. aequipinnatus* were partially aligned even in the absence of flow due to their inherent schooling behaviour.

Since the proclivity of *D. aequipinnatus* to head upstream may have biased the direction from which looming stimuli were received, *D. aequipinnatus* orientation with respect to the stimulus location was measured at the onset of each stimulus trial [Fig. 2 (c),(d)]. As expected, *D. aequipinnatus* heading upstream in flow were more frequently oriented at 90° or 270° with respect to the stimulus, given that they were always presented from one of two computer monitors on either side of the tank [Fig. 2(c)]. In contrast, *D. aequipinnatus* in no flow were more randomly oriented with respect to the stimulus direction [Fig. 2(d)]. The response frequency of *D. aequipinnatus* to the startle stimulus in the no-flow condition did not appear to depend on stimulus direction; the response probability was uniformly distributed (Rayleigh test; $Z = 2.01$, $P > 0.05$, binned data with bin spacing of 0.35 radians). Rayleigh tests could not be carried out on the flow trials, since the flow data had multiple modes, which violates the necessary statistical assumptions.

NEIGHBOUR POSITION AND SCHOOL STRUCTURE IS DIFFERENT IN FLOW

Two-dimensional neighbour distance in L_S was calculated for each *D. aequipinnatus* at each time step during stimulus presentation when no *D. aequipinnatus* were startle. A two-tailed, paired Wilcoxon signed-rank test indicated that the nearest-neighbour distance was larger in flow (Mdn = 0.70, $n = 88$) than in no flow (Mdn = 0.69, $n = 83$, $Z = -2.97$, $P < 0.05$). The actual size of the difference (0.01 L_S , i.e. c. 3–4 mm) was very small. There was no significant difference found in a measure of density given by the ratio of the distance of first and second nearest neighbours in flow (Mdn = 0.68, $n = 88$) or no flow (Mdn = 0.68, $n = 83$, $Z = -0.19$, $P > 0.05$). The spread of neighbour position was calculated [see Fig. 3 (a),(b)] by creating a two-dimensional histogram and fitting a bivariate Gaussian distribution to the data. The overall shape of *D. aequipinnatus* groups in flow was elongated in the crosswise direction, being more oblong [Fig. 3(a)] than circular [Fig. 3(b)]. The shape differences along the transformed x and y axes were tested with an equality of variance test (x direction: $F = 1.29$, d.f. = 2327 (flow) and d.f. = 2491 (no flow), $P < 0.001$; y direction: $F = 1.11$, $P < 0.05$). It was also observed that *D. aequipinnatus* in the flow condition tended to spend most of their time upstream [Fig. 3(c)], whereas *D. aequipinnatus* in the no-flow condition were largely located downstream [Fig. 3(d)].

EFFECTS OF FLOW, GROUP SIZE AND STIMULUS ON RESPONSE PROBABILITY

No startle responses were observed during the control (blank) trials and, across all conditions, the fastest looming speeds (80, 160 and 320 cm s⁻¹) were all significantly better at predicting startle responses than the 20 cm s⁻¹ stimuli (80 cm s⁻¹: $Z = 3.39$, $P < 0.001$; 160 cm s⁻¹: $Z = 2.93$, $P < 0.001$; 320 cm s⁻¹: $Z = 3.4$, $P < 0.001$). In general, the probability that at least one *D. aequipinnatus* responded to the looming stimulus increased as a function of stimulus speed for both solitary and grouped *D. aequipinnatus* in both flow and no-flow conditions (Fig. 4). In addition, the probability of one *D. aequipinnatus* responding was generally greater in the flow condition compared to the no-flow condition (Fig. 4).

A general linear mixed model was run in R, using flow condition, solitary v. grouped state and stimulus speed in order to predict response probability (see Table I for full results of the model). Only the three highest loom speeds (80, 160 and 320 cm s⁻¹) were used in this analysis to ensure that stimulus levels were detectable across all conditions. The effect of replicate groups was controlled for by adding it as a random factor in the model. No evidence of interaction between the two factors of flow condition and social context was found ($Z = -0.138$, $P > 0.05$, Akaike information criterion, AIC = 645.4). Flow and solitary and group states were significant predictors of response frequency (flow: $Z = 2.42$, $P < 0.05$; group size: $Z = 4.21$, $P < 0.001$), i.e. the probability of at least one *D. aequipinnatus* responding was greater for grouped compared to solitary *D. aequipinnatus* and in flow compared to no flow.

NUMBER OF *D. AEQUIPINNATUS* RESPONDING PREDICTED BY INCLUDING SOCIAL INFORMATION

Although there was a higher probability of at least one *D. aequipinnatus* responding to each of the 80, 160 or 320 cm s⁻¹ loom stimuli in the flow condition compared to the no-flow condition, the mean number of grouped *D. aequipinnatus* responding in flow (2.65, $n = 88$) and no flow (2.53, $n = 83$) did not differ (Mann-Whitney U -test, $Z = -0.38$, $P > 0.05$). Thus, while there was a higher probability of at least one *D. aequipinnatus* responding in flow compared to no flow, a greater number of *D. aequipinnatus* responded in no flow. This observation indicates higher probabilities of stimulus detection in flow but higher probability of internal information transmission in no flow, as shown in the pooled frequency distributions of the number of *D. aequipinnatus* responding in flow [Fig. 5(a)] and no-flow [Fig. 5(b)] conditions. A G -test comparing these frequency distributions in flow and no flow indicates that the two distributions are statistically different ($G = 4.44$, d.f. = 1, $P < 0.05$). The differences in the number of *D. aequipinnatus* responding can be further distinguished by fitting the frequency distributions with polynomial models to show higher probabilities of one or two *D. aequipinnatus* responding in the flow condition, but lower probabilities of three or more *D. aequipinnatus* responding as compared to the no-flow condition [Fig. 5(c)]. The flow condition does not appear to have an effect at higher numbers of *D. aequipinnatus* responding (seven to eight *D. aequipinnatus*), which may be due to very small sample sizes (in only a few trials did the entire school startle).

Next, the data were fitted to a probabilistic model of startle response that included the affect of socially transmitted information. Sample fits of the model outcomes (simulated data) to

the experimental data for the flow [Fig. 6(a)] and no-flow [Fig. 6(b)] conditions are shown. The shaded regions illustrate the range of typical outputs of the model based on one S.D. from the mean output. The parameters for the model that best fit the data were optimized using simulated annealing. The model values determined by this optimization approach are shown in Fig. 7, for both nearest-neighbour and proximity-based topologies for flow and no flow. The results indicate that, regardless of the network topology, the probability of detecting an external threat (P^{ext}) is higher in the flow condition than the no-flow condition, as was seen in the experimental results. The model predicts that the probability of detecting and responding to neighbouring *D. aequipinnatus*, P^{int} , is higher in the no-flow condition than in the flow condition, which could not be observed directly from the experiments. Additionally, the results of the model suggest that P^{ext} does not depend on the interaction topology, whereas P^{int} decreases exponentially as the presumed number of interacting neighbours increases.

DISCUSSION

As far as is known, this study is the first to examine school structure, startle response probability and information transmission in the environmental context of a flow field. For many species, social interaction and predator avoidance are important parts of behavioural ecology and several studies have attempted to model and understand how schooling fish might handle predator avoidance (Zheng *et al.*, 2005; Wood & Ackland, 2007; Lemasson *et al.*, 2009). These studies lack a key ecological factor (flow) that fishes often experience in their natural habitats. Flow brings with it benefits (*e.g.* drifting prey) as well as challenges, such as hydrodynamic noise and disruption of positional stability. It was demonstrated that (1) the polarity and two-dimensional shape of grouped *D. aequipinnatus* in the flow condition are altered relative to the no-flow condition such that *D. aequipinnatus* orient upstream while spanning out in a cross-stream direction, (2) the probability that at least one *D. aequipinnatus* responds to a looming visual stimulus is higher in flow than in no flow and (3) the number of *D. aequipinnatus* responding to the looming stimulus is higher in no flow than flow. Furthermore, a computational model of collective startle response was developed that predicted the results; the model indicated that, relative to no flow, flow impairs the transmission of social information in the form of propagated startle responses.

THE EFFECT OF FLOW ON GROUP FORMATION AND POLARITY

It is well-known that fishes exhibit rheotactic behaviours in the presence of flow (Lyon, 1904; Arnold, 1974). Thus, the finding that the flow condition caused solitary *D. aequipinnatus* and grouped *D. aequipinnatus* to orient predominantly upstream is not new. The change in the two-dimensional shape of the group in the flow condition, however, is a new and surprising finding and may have implications for threat detection in flow. Given that *D. aequipinnatus* in flow tended to be located further upstream than those in no flow (Fig. 3), it is possible that the observed cross-stream distribution in flow was the result of a wall effect. That is, the upstream barrier might have prevented *D. aequipinnatus* from moving further upstream, causing them to span out in a cross-stream direction. Although wall effects cannot be ruled out, they seem less likely given that the data analysis excluded all instances in which *D. aequipinnatus* were less than 1 L_S away from the wall.

Alternatively, *D. aequipinnatus* may position themselves in order to increase rheotactic performance. Rheotaxis is a robust behaviour with many potential benefits, including enhanced detection of prey and energy conservation (Arnold, 1974; Montgomery *et al.*, 1997). Visual (optical-flow) cues appear to be sufficient for rheotaxis under many conditions (Arnold, 1974). By positioning themselves laterally to each other, fish may be obtaining enhanced visual cues (about other fish) that would otherwise be absent.

Interestingly, Partridge & Pitcher (1980) showed that ablation of the lateral line affects the structure of saithe *Pollachius virens* (L. 1758) schools in a manner that appears to be similar to the effects of flow observed in this study on group structure of *D. aequipinnatus*. That is, lateral-line-impaired *P. virens* showed an increase in the frequency of neighbours directly alongside at *c.* 90° compared to control groups (all senses intact) or visually deprived groups of *P. virens*. Thus, it is conceivable that the flow conditions of this experiment may have had similar disabling effects on the lateral line. That is, the surrounding ambient flow may have masked the ability of *D. aequipinnatus* to detect the flow signals generated by neighbouring fish. In this regard, there is both behavioural (Coombs *et al.*, 2001) and physiological evidence (Engelmann *et al.*, 2002; Chagnaud *et al.*, 2007) that unidirectional (DC) flow does indeed compromise the ability of the lateral line to detect oscillatory (AC) flows, such as those generated by a swimming fish (Kalmijn, 1988). This general scenario is consistent with the finding that social transmission of information may be greater in no flow than in flow (Fig. 7).

STARTLE RESPONSE PROBABILITY AND SOCIAL INFORMATION TRANSMISSION

For both individual and groups of eight *D. aequipinnatus*, the probability of at least one *D. aequipinnatus* responding was higher in flow. This result was somewhat unexpected, given that *D. aequipinnatus* in the flow condition were expending much more energy by swimming into the flow. The simplest explanation is that because the orientations of *D. aequipinnatus* in no-flow were random over time, the stimulus may have been positioned outside the visual field of the *D. aequipinnatus* (*i.e.* in the blind zone), thus reducing the overall probability of detection. This is in contrast to the flow condition, where rheotaxis induced by the flow field allowed the stimuli to largely lie within the visual field [Fig. 2 (c), (d)].

Although there was a higher probability of at least one *D. aequipinnatus* responding in the flow condition, results showed lower probabilities of four or more *D. aequipinnatus* responding in flow as compared to the no-flow condition. Explanations as to why *D. aequipinnatus* in the flow condition did not appear to be responding may deal with both detection abilities and response abilities or proclivities. *Devario aequipinnatus* in the flow condition were expending more energy than they would in no flow to swim against the flow and to maintain position so that they were not displaced downstream. The decision to respond to the looming stimulus was thus more costly in flow than in no flow. It may be possible that *D. aequipinnatus* in flow were detecting signals from neighbouring *D. aequipinnatus* regarding a threat, but that their threshold criterion for responding was higher. Given that the individual probability of response was higher in flow for solitary *D. aequipinnatus*, however, an increased threshold for response is an unlikely explanation.

Another explanation is that flow signals produced by the movements of neighbouring *D. aequipinnatus* were weaker (because *D. aequipinnatus* were further apart and flow signals attenuate very steeply with distance) and masked by the ambient flow, making the transmission of hydrodynamic information from one *D. aequipinnatus* to the next more difficult. Finally, the crosswise distribution of *D. aequipinnatus* into side-by-side positions may have prevented those in the interior of the group from having a direct line of sight to the looming stimuli, partially occluding the vision of *D. aequipinnatus* on the interior of the school.

Fishes use a variety of sensory modalities to receive information regarding the presence of a threat, including vision (Dill, 1974; Guthrie & Muntz, 1993), olfaction (Ferrari & Chivers, 2006; Mathuru *et al.*, 2012) and lateral line sensing (McHenry *et al.*, 2009; Mirjany *et al.*, 2011). The predatory stimulus used in these experiments was purely visual. While the presence of any unimodal cue may be strong enough to elicit a startle response, the use of multi-modal cues has been shown to improve accuracy in predator detection in the mosquitofish *Gambusia holbrooki* Girard 1859 (Ward & Mehner, 2010). Fish in a school may gain additional visual, hydrodynamic, and olfactory cues from their neighbours that support the presence of a threat, or be in conflict with it (*e.g.* neighbours not responding to the looming stimulus). Thus, if hydrodynamic and visual cues from neighbouring individuals are masked or occluded in the flow condition, fish may have a lower probability to respond. This may be true even for *D. aequipinnatus* detecting the looming stimulus if they additionally receive conflicting information from other *D. aequipinnatus* that do not respond.

VARIABILITY OF STARTLE RESPONSES

Experimental analysis of the response latencies of startle responses in fishes have provided evidence that certain fishes are more likely to respond to a potential threat than others (Marras & Domenici, 2013). The wide range of response latencies (207 – 907 ms) measured in this study is indicative of substantial variability in responsiveness. Whether or not this variability can be attributed to individual performance differences or stimulus differences (*e.g.* the orientation of the *D. aequipinnatus* with regard to the looming stimulus) is uncertain. The tracking software used in this study does not allow the maintenance of individual identities to be preserved between trials, so it was not possible to determine whether some individuals were more likely to respond than others. Moreover, the latency measures cannot distinguish between responses elicited by the looming stimulus itself *v.* those that may have been elicited by the startle responses of other *D. aequipinnatus*. Indeed, several startle responses occurred after the looming stimulus ended, suggesting the possibility of the latter.

From a mechanistic perspective, it is interesting to note that the range of response latencies measured in this study is very similar to that measured from freely swimming *C. auratus* in response to looming stimuli shown to be effective at eliciting Mauthner cell-mediated escape responses (Preuss *et al.*, 2006). A site of multisensory integration, the Mauthner cell relies on the sum of all of its sensory inputs in order to reach the threshold level for firing and initiating the escape response (Korn & Faber, 2005). Whereas the visual input is indirectly relayed to the Mauthner cell from a visual processing region of the midbrain, both

lateral line and auditory inputs are direct from the sense organs themselves (Zottoli *et al.*, 1995). Thus, the transmission of information from the visual system is slower and delayed relative to that from mechanosensory systems. Moreover, different senses in different species have different influences on the probability of M cell firing (Canfield & Rose, 1996). In *C. auratus*, for example, visual or auditory inputs alone are capable of exciting the M cell, whereas lateral line inputs may bias the probability of a response but only in combination with inputs from other senses. Thus, at least some of the variability in startle response latency may be explained in terms of the relative contributions of different senses, which in this study involves vision (*via* the looming stimulus), as well as the potential for auditory and lateral line senses (*via* the movements of nearby startled fish).

MODELLING INFORMATION TRANSMISSION

While social information cannot be measured directly from the experimental data, it can be hypothesized that the increased number of *D. aequipinnatus* responding in the no-flow condition may be a result of increased information transmission. To test this hypothesis, a probabilistic model to predict the number of *D. aequipinnatus* startling was developed. Fitting the model to the data indicated increased internal information transmission (P^{int}) in the no-flow case for all topologies. The results of the model agree with the prediction that social information transmission is higher in the no-flow condition. It is not well understood how fishes transmit information between individuals, or what interaction networks fishes are using. For the analysis here, both neighbour-based and proximity-based topologies were used to determine when *D. aequipinnatus* might be interacting with each other. Fishes may also use a sensory-based topology (Strandburg-Peshkin *et al.*, 2013), however the results of the model presented are robust to network topology, in that for all network topologies simulated, the same pattern of values (P^{int} higher in no flow and P^{ext} higher in flow) was observed (Fig. 7).

While the model of information transmission proposed here was used specifically to fit the experimental data in this study with *D. aequipinnatus*, the probabilistic model can be generalized to generate testable hypotheses and predictions regarding questions of information transmission in other collective groups of animals, including the roles of density, polarization, and interaction topologies.

This study is the first to compare startle response probabilities in the ecologically relevant conditions of flow and no flow. Here it is shown that startle behaviour and intra-school information transmission is different in flow and no-flow conditions and thus may have ecological consequences for fish schools avoiding predators in different environments, since the individual likelihood of escaping a predator attack depends on both the individual and the group. In this study spatially-uniform flow was used at a speed that is probably within the range of those experienced by *D. aequipinnatus* in their natural environment (McClure *et al.*, 2006). It would additionally be of interest to investigate the effects of different flow speeds or flow characteristics, such as turbulence.

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FIG. 1.

Schematic overview of the flow tank and the experimental set-up. (a) Schematic of the flow tank (adapted from Vogel & LaBarbera 1978). An impeller drives water through a return tube. The collimators reduce turbulence and fish are tested in the 25.4×25.4 cm working area. The working area is backlit with evenly distributed fluorescent lighting. (b) Three-dimensional illustration of the flow tank with overhead mounted camera, connected to a computer outside the experimental room. Stimuli were presented randomly to either side of the tank by the computer monitors attached to a Linux computer that generated the stimulus movies. (c) Top view of the working area during a flow trial. Arrows, the direction of flow.

FIG. 2.

Time-averaged vector strength and direction associated with groups of eight *Devario aequipinnatus* in flow (a) and no flow (b), where the orientation 0° is in the upstream direction. Vector strength ranges from 0 to 1, with 1 being the highest degree of alignment. Probability of individual *D. aequipinnatus* in solitary trials responding to a looming stimulus according to orientation with respect to the stimulus location at stimulus onset in flow (c) and no flow (d), where the orientation 0° is in the stimulus direction. Length of rose histogram indicates the frequency with which *D. aequipinnatus* are oriented in any given direction at the time of stimulus onset, whereas the colour indicates the probability of a response, computed from the total number of solitary trials.

FIG. 3.

(a) and (b) Probability density functions of relative position. Colour corresponds to the number of points in each bin, normalized by the bin with the most points ($\odot$ red, are 1 S.D. away from the mean; $\circ$, the average nearest-neighbour distance). (a) Flow and (b) no flow. (c) and (d) Scatterplots and corresponding histograms of *Devario aequipinnatus* position in the tank (L_S , standard length).

FIG. 4.

Response probabilities of at least one *Devario aequipinnatus* startling at various stimulus speeds in individual (■, □) and groups of eight *D. aequipinnatus* (●, ○). Values are means $\pm$ S.E. There were eight blocks for each condition and, within each of the blocks, each stimulus was shown twice (16 measurements per point) (■, ●, flow; □, ○, no flow).

FIG. 5.

Frequency distributions of the number of *Devario aequipinnatus* responding in (a) flow and (b) no flow. (c) Cumulative probability distribution of the number of *D. aequipinnatus* responding (■, flow trials; □, no-flow trials). line with filled black circles, polynomial model fit to the flow distribution; line of open white circles, polynomial fit to the no flow distribution.

FIG. 6.

Example of an optimal fit of a probabilistic model ($\square$, simulated data) to the cumulative probability distributions of a certain number of *Devario aequipinnatus* startling in (a) flow and (b) no flow ($\blacksquare$, experimental data; shaded area, mean fit of the model $\pm$ S.D.).

FIG. 7.

Semi-log plots of optimized values of P^{ext} (the probability of directly responding to a threat) and P^{int} (the probability of responding to cues from other individuals) in flow (■) and no flow (□) for (a) and (c) n -neighbour and (b) and (d) metric distance topologies. In the n -neighbour topology, fish receive signals from the closest n number of neighbours, whereas in the metric distance topology networks, fish receive signals from all neighbouring fish within a given radial distance.

Linear mixed-effects model investigating the effect of stimulus speed, group size and flow condition on response. Replicate groups were controlled for as a random effect

TABLE I

Fixed effect	Level	Value	S.E.	z-value	P-value	95% C.I.	
						Lower bound	Upper bound
Intercept		-2.2533	0.4102	-5.493	< 0.001	-3.0573	-1.4492
Stimulus speed	40	0.63420	0.4089	1.524	> 0.05	0.1677	1.5771
	80	1.4171	0.4169	3.399	< 0.001	-0.1784	1.4246
	160	1.2065	0.4121	2.928	< 0.005	0.5999	2.2343
	320	1.6823	0.4209	3.996	< 0.001	0.3988	2.0142
Flow		0.8724	0.3595	2.426	< 0.05	0.8574	2.5073
Group size n=8		1.5981	0.3769	4.241	< 0.001	0.8595	2.3368
Group:flow		0.0619	0.5129	-0.138	> 0.05	-0.09433	1.0671