Collective response of fish to combined manipulations of illumination and flow

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Abstract

Collective behavior is ubiquitous among fish, yet, its how's and why's are yet to be completely elucidated. It is known that several environmental factors can dramatically influence collective behavior, by eliciting behavioral adaptations in the individuals and altering physical pathways of social interactions in the group. Yet, empirical research has mostly focused on the quantification of the role of one factor at a time, with a paucity of studies designed to explore the multi-sensory basis of collective behavior. We investigated collective behavior of zebrafish (*Danio rerio*) pairs swimming in a water channel under combined manipulations of illumination (bright and dark) and flow conditions (absence and presence). The ability of the pair to orient and school increased in the presence of the flow and when fish were allowed to visually interact under bright illumination. Shoaling, instead, was only modulated by the illumination, so that fish swam at higher relative distances in the dark, irrespective of the flow. We also found evidence in favor of a modulatory effect of flow and illumination on the formation of the pair. Specifically, in the bright illumination, fish swam more side-by-side against a flow than in placid water; likewise, in the presence of a flow, they spent more time side-by-side in the bright illumination than in the dark. These findings point at a rich interplay between flow and illumination, whose alterations have profound effects on collective behavior.

Keywords: Collective behavior, *Danio rerio*, orientation, schooling, shoaling, zebrafish

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1. Introduction

Fish collective behavior has been observed and documented across a vast range of species [1, 2, 3, 4]. The collective behavior of a group is mediated by how individuals exchange and integrate sensory cues from neighbors and the surrounding environment [5, 6]. These cues are part of a complex, multi-sensory feedback system that may involve multiple senses, such as vision, lateral line, olfaction, vestibular system, and touch [6]. Several studies [7] have identified how living in a group confers benefits to fish that may range from better predatory avoidance [8] to energy saving, in the form of hydrodynamic drafting and Karman gaiting [9, 10].

Flow is a key environmental factor that can regulate the collective response of a group, affecting its size and morphology. There have been several accounts demonstrating the role of flow on collective behavior in laboratory and semi-natural settings; yet, evidence has often been contrasting. For example, Shelton *et al.* [11] conducted a field study to investigate collective behavior of zebrafish (*Danio rerio*) in the wild, discovering that fish tend to form larger and more cohesive groups in fast-flowing currents when compared to calm waters. An equivalent, modulatory effect of the fluid flow has also been documented in chubs (*Leuciscus cephalus*) [12], which were found to aggregate more in semi-natural pools at high flow — that is, forming larger and denser shoals. Conversely, Hockley *et al.* [13] observed that guppies (*Poecilia reticulata*) form larger shoals in the absence of flow, similar to the observations made by Garner [14] on minnows (*Phoxinus phoxinus*) in different flow velocity habitats, which suggest that shoaling tendency is reduced at a high flow rate.

Beyond affecting shoaling tendency, the flow can also have a modulatory role on the swimming formation chosen by the group. In fact, different empirical studies in laboratory-scale water channels have examined swimming formation as a function of the presence and strength of a background flow. For instance, De Bie *et al.* [15] determined that pairs of minnows swim side-by-side against the current at high flow speed, while opting for an in-line swimming formation in placid water. Interestingly, under low flow, fish did not display a particular formation, so that they would be randomly positioned with respect to each other. Analogously, pairs of red nose tetra (*Hemigrammus bleheri*) were found to prefer a side-by-side configuration when swimming against a current [16]. As the number of individuals in the group increases, the tendency of swimming side-by-side is preserved, causing the emergence of phalanx formations in minnows [17]. Similarly, giant danios (*Danio aequipinnatus*) tend to span-out in the cross-stream direction, as they orient against an incoming flow [18].

While offering important insight into the role of the flow, these studies were not designed to disentangle the role of different environmental factors that could mediate collective behavior. Poor illumination and water turbidity, for example, could compromise visual cues, thereby affecting interactions within the group that underpin the collective response of several

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species [19]. Although fish may access other senses to compensate for the blockage or reduction of visual cues [6, 20, 21, 22], there is consensus regarding the consequences of poor illumination and water turbidity on collective behavior [23, 24, 25, 26, 27]. For instance, the inter-fish distance and overall shoal area have been found to increase in red shiners (*Cyprinella lutrensis*) and sand shiners (*Notropis stramineus*) [23] under increased water turbidity. Interestingly, compelling evidence in favor of a mechanistic explanation of the role of turbidity in constraining visual cues (against an alternative adaptive explanation) has been presented by Chamberlain *et al.* [24] on three-spined sticklebacks (*Gasterosteus aculeatus*).

To date, empirical evidence on the combined effect of flow and illumination on collective behavior is lacking. Here, we sought to fill this gap in knowledge through experiments on zebrafish, with varying illumination and flow conditions. We followed the reductionist approach by [15, 16], whereby we performed our experiments on fish pairs — the fundamental dyads that make up a group. Zebrafish is a tropical freshwater species native to Southeast Asia [28] that is becoming a species of choice in behavioral neuroscience and neurobiology [29, 30, 31, 32]. Several studies have demonstrated the successful use of zebrafish in detailing normal behaviors and abnormal derailments underlying social behavior [33, 34]. Likewise, there is a growing body of research that is looking at zebrafish as an ideal animal model to disentangle multi-sensory pathways and understand neural processes related to orientation against a water flow [35, 36].

We measured the behavior of fish pairs under bright illumination and in the dark (using an array of infrared lights and a camera), in the absence and in the presence of a water flow, within the experimental setup we had validated in our previous research on the orientation of zebrafish individuals [36]. Using our in-house developed multi-target tracking software [37, 38] that preserves fish identities over time, we acquired swimming trajectories of fish pairs along a five-minute experimental session. We scored several metrics of collective response, borrowing from the literature on collective behavior [1, 39] and fish orientation [36, 40]. Specifically, we studied: i) the orientation of the pair and their exploration of the test section; ii) the swimming patterns of the pair, in terms of tail beat amplitude and tail beat frequency; iii) the shoaling and schooling tendencies, in terms of the distance between the pair and their relative alignment; and iv) the swimming formation chosen by the pair (in-line versus side-by-side).

We anticipated that zebrafish pairs would orient better in the water channel in the presence of the flow, due to compelling evidence of successful orientation against a flow above a critical flow speed [40] in many species, including zebrafish. Likewise, based on our prior observations of single fish swimming in the same test section for bright and dark illuminations [36], we predicted that orientation would benefit from the access of visual cues. Grounded in evidence supporting the modulatory role of the flow on collective behavior of zebrafish [11] and the known role of vision on zebrafish social behavior [29, 30, 31, 32], we predicted that illumination and flow would also influence shoaling and schooling tendencies. Specifically, we anticipated that cohesion and coordination of the pair would benefit from the presence of a flow and bright illumination. Likewise, we predicted that flow and illumination would also influence the preference for side-by-side versus in-line swimming, with a choice towards side-by-side swimming in the presence of the flow [15, 16, 17, 18]. Given our previous research on mathematical modeling of zebrafish pairs in placid water [39], we expected that illumination would play some role on the formation choice, albeit the extent and direction of such an effect was unclear at the beginning of the experiments.

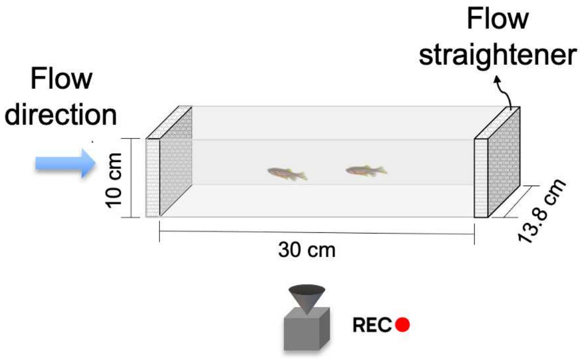


Figure 1: The test section used for experiments on fish pairs swimming against an incoming flow, in the absence or presence of light.

2. Materials and methods

2.1. Animal care and maintenance

A total of 96 wild-type adult zebrafish (*Danio rerio*) were used in this study with a 1:1 ratio of males to females. Fish were purchased from Carolina Biological Supply Co. (Burlington, NC, USA). The average fish body length (BL) was 3.6 cm. Following standard recommendations [41, 42], fish were housed in a 615 L vivarium [180 cm (length) × 60 cm (height) × 87 cm (width)] at the stocking density of approximately 3 fish/L. The vivarium tank was divided into two compartments to separate females from males, thereby easing the process of selecting sexes across experimental conditions.

Fish were kept under a 12 h light/12 h dark photo-period and fed with commercial flake food once a day, approximately at 7 pm. Temperature and pH of the water in the holding tank were maintained at 26° and 7.2, respectively. Stress coat was added to regular tap water to remove chlorine and chloramines. Fish were acclimatized in the holding facility for one month.

After an experimental session was completed, fish were transferred to a different vivarium (with the same temperature and water parameters specified above) and kept in groups to prevent social isolation.

2.2. Experimental setup

We used the same experimental setup as our previous study [36]. The setup consisted of a 151 L Blazka-type water channel (Engineering Laboratory Design Inc., Lake City, MI, USA), a video camera (Logitech C910 HD Pro Webcam without infrared filter, Logitech, Switzerland) located below the channel, an array of lights, and black curtains to minimize outside visual disturbances.

We implemented two different lighting systems to enable video-recordings while creating bright and dark illuminations for the fish. Specifically, we either used a pair of fluorescent lamps (Aqueon Full Spectrum Daylight T8, Aqueon, USA) located at the top of the channel, along with a white plexiglass sheet to dim the light intensity and provide a homogeneously lit background of 250 lx, or four infrared lights (Iluminar IRC99 Series, Iluminar, Irvine, CA) of wavelength 940 nm (which exceeds the adult zebrafish threshold of spectral sensitivity [43]).

A test section of 30 cm × 13.8 cm with a water height of 10 cm was created within the channel using flow straighteners, as shown in Figure 1. The top of the test section was kept open. We generated a parabolic fluid flow profile along the cross-stream direction by using an array of U-shaped flow straighteners of different opening sizes [36].

2.3. *Experimental conditions and procedure*

To investigate collective behavior of zebrafish swimming in the channel, we considered two binary independent variables: “current” and “illumination”. The current could either be placid (“No flow”) or follow a parabolic profile in the cross-stream direction, with a peak speed of 5.33 cm/s (“Flow”), measured 5 cm below the water surface and 15 cm streamwise through a laser Doppler velocimeter (BSA,F50, Dantec, Denmark). Illumination could be either created with infrared light not visible to the subjects (“Dark”) or at 250 lx (“Bright”).

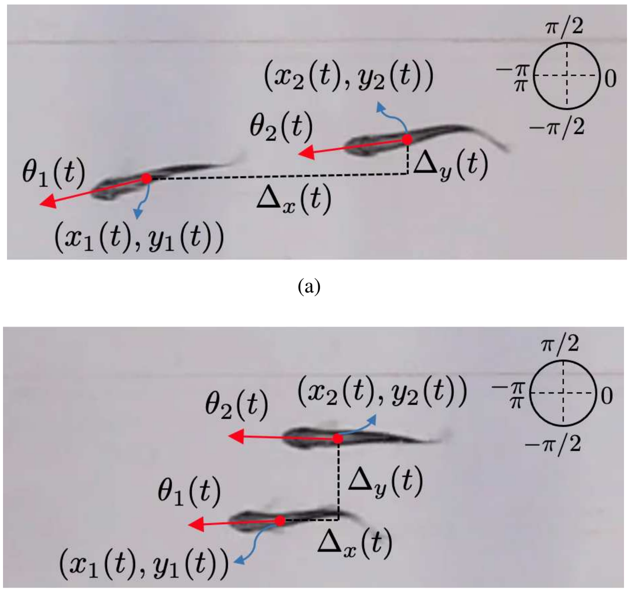
Twelve trials were conducted on fish pairs of the same sex for each condition (Bright - No flow; Bright - Flow; Dark - No flow; and Dark - Flow), yielding a total of 48 trials (six pairs of females and six pairs of males per condition). All fish were experimentally naïve, and were used only once. The experiments were performed in September. After the experiment was completed, we discovered that one trial of Dark - Flow had a corrupted video-file, which was removed from the experimental dataset.

Each trial comprised three phases. The first phase consisted of transferring two fish from the holding vivarium to the water channel and providing five minutes of habituation in placid water. Then, for the conditions in placid water (Bright - No flow and Dark - No flow), fish were simply video-recorded for five minutes. For the conditions with the flow instead (Bright - Flow and Dark - Flow), the water flow was turned on for a total of seven minutes, of which last five minutes were video-recorded.

2.4. *Fish tracking and behavioral scoring*

All videos were recorded at 30 frames per second and processed using a foreground detection algorithm in Matlab (R2019b) for highlighting the animal shape on the image and improving the tracking process [44].

The results were input to a slightly modified version of the multi-target tracking algorithm Peregrine [37, 38] that allowed for manual repairs. The pair motion was studied in the Cartesian $x - y$ plane, in which x corresponded to the streamwise direction (oriented downstream, so that orienting against the flow means swimming opposite to the x-direction) and y to the cross-stream direction. The tracking algorithm fitted a parabola on the fish blob and returned time series of swimming trajectories (that is, consecutive positions of the fish centroid) of the ith fish, $(x_i(t), y_i(t))$, along with their respective velocity and components of the heading vector, from which we calculated the heading angle of each fish, $\theta_i(t)$. An illustration of the centroid coordinates of each fish along with their heading angles are shown in Figure 2. Here, subscripts $i = 1$ and 2 are the labels of each animal in the pair.



(a)

(b)

Figure 2: Illustration of the main variables used to study fish behavior for instances of: (a) in-line and (b) side-by-side swimming. Each image corresponds to a single frame from a video-recording of a trial in Bright - Flow.

The software also extracted the shape parameters (coefficients of the fitted parabola), which we used to track the tail displacement of each fish. From the data, We classified tail beats as tail displacements exceeding a threshold of ±0.6 ,cm (outliers were filtered by capping the tail displacement to a maximum of 1.5 cm). For each fish in each trial, we counted the number of tail beats to compute the tail beat frequency (TBF) and we calculated the average amplitude of these beats to calculate the tail beat amplitude (TBA).

The output of the tracking system was used to investigate the behavior of the fish pair through the scoring of several salient metrics of collective response. In particular, we studied orientation, exploration, tail beat motion, cohesion, coordination, and time spent swimming in formation — all the metrics were adapted from previous research [36, 38, 39].

Orientation was quantified as the time average of the negative cosine of the mean heading angle $\bar{\theta}(t)$, calculated through the circular mean given by

$$\bar{\theta}(t) := \operatorname{atan2}\left(\frac{\sin(\theta_1(t)) + \sin(\theta_2(t))}{2}, \frac{\cos(\theta_1(t)) + \cos(\theta_2(t))}{2}\right), \tag{1}$$

where atan2 is the four-quadrant inverse tangent returning values in $[-\pi, \pi]$. Orientation could take values between -1 and 1, each representing the tendency of the pair to swim along the x-direction or opposite to that. In the presence of the current, this would correspond to swimming with or against the flow, respectively.

Exploration was quantified using spatial entropy. This quantity was measured by first computing the position of the centroid of the pair given by the average trajectory $(\bar{x}(t), \bar{y}(t)) := 1/2(x_1(t) + x_2(t), y_1(t) + y_2(t))$. Then, we partitioned the test section into 10×4 squares of approximately $3\,\mathrm{cm} \times 3.45\,\mathrm{cm}$ each. For each trial, we computed the probability of occupation of any of the squares as p_k by the centroid pair, with $k = 1, \ldots, 40$. Spatial entropy (SE) was computed as $SE = -\sum_{k=1}^{k=40} p_k \log(p_k)$, where the logarithm is expressed in base 2, so that entropy is measured in bits. Spatial entropy varies between 0 bits (the position of the centroid not varying for the entire trial) and $\log 40 \simeq 5.32$ bits (the position of the centroid of the pair being uniformly distributed in the test section).

Beyond scoring how fish collectively responded to the incoming flow and how they explored the test tank during trials, we also investigated their swimming patterns. Specifically, we focused on the average tail beat motion of the pair as a proxy for energy expenditure. For each trial, we obtained TBA and TBF of each fish and computed the average for the pair.

Group cohesion and coordination were measured in terms of the time average of the distance between the two fish and the alignment of their swimming directions. In particular, we computed the distance between the subjects using the swimming trajectories $\sqrt{\Delta_x(t)^2 + \Delta_y(t)^2}$, where $\Delta_x(t) := x_1(t) - x_2(t)$ is the relative streamwise distance between the fish and $\Delta_y(t) := y_1(t) - y_2(t)$ the relative cross-stream distance. To quantify the alignment between fish, we computed the time average of the instantaneous polarization, $P(t)$, given by

$$P(t) = \frac{\sqrt{[\cos\theta_1(t) + \cos\theta_2(t)]^2 + [\sin\theta_1(t) + \sin\theta_2(t)]^2}}{2}. \tag{2}$$

Polarization varies between 0 and 1, with 0 identifying the case of two fish swimming in opposite directions and 1 the case where they swim parallel.

Note that although $-\cos\bar{\theta}(t)$ and $P(t)$ are based on the swimming directions of the pair, they provide independent measures of collective behavior. The former pertains to how the pair orients with respect to the test section, the latter addresses the relative alignment of the two animals with respect to each other. For example, if the animals are swimming at a relative angle of $\pi/2$, polarization will always be equal to $\sqrt{2}/2$, but depending on the mean heading angle, the orientation can be as low as -1 (the animals swimming at $\pi/4$ and $-\pi/4$, so that the centroid is perfectly moving downstream) and as high as 1 (the animals swimming at $3\pi/4$ and $-3\pi/4$, so that the centroid is perfectly moving upstream).

We also measured the time budgeted by the pair to swim in two different formations: namely, "in-line" and "side-by-side". We only considered instances when the difference of the headings between the fish was less than 90° to exclude cases in which fish were swimming in opposite directions. We classified swimming segments as in-line or side-by-side depending on the relative distance between the fish streamwise or cross-stream. Hence, we established that the fish pair was in an "in-line" formation at time t if $|\Delta_y(t)| \leq 2\mathrm{BL}$ and $|\Delta_x(t)| > 1\mathrm{BL}$, The pair was considered to be swimming "side-by-side" if $|\Delta_y(t)| \leq 2\mathrm{BL}$ and $|\Delta_x(t)| < 1\mathrm{BL}$. Both swimming formations are illustrated in Figure 2. For each trial we obtained the time that fish spent swimming in either in-line or side-by-side formation. From these two values, we also scored a preference index for side-by-side swimming as the ratio between the time spent swimming side-by-side divided by the time spent swimming in any of the two formations.

2.5. Statistical analysis

To investigate the effect of illumination and current on orientation, exploration, TBA, TBF, relative distance between of the pair, and polarization, we conducted an independent analysis on each of these variables using a two-way ANOVA with illumination and current as between-subject factors.

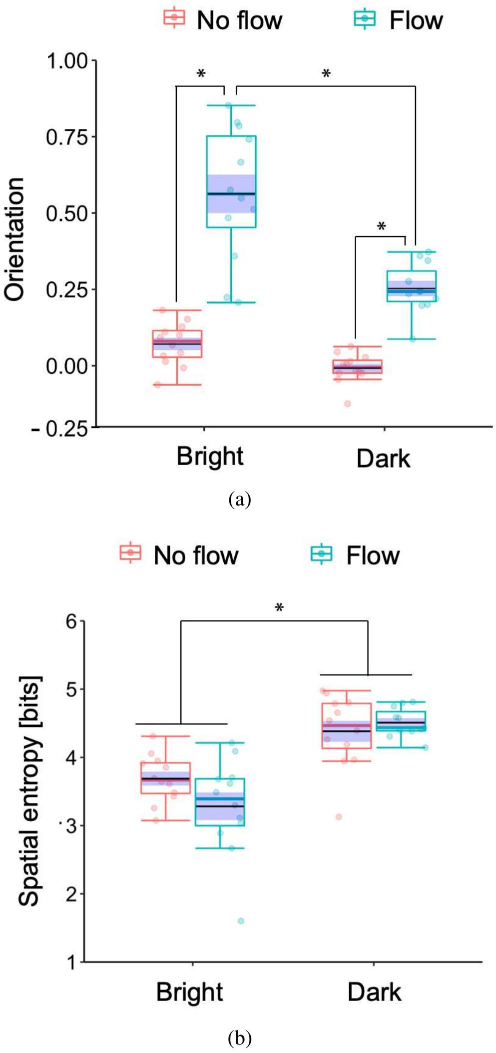
To study group preference for in-line or side-by-side swimming formations, we first conducted a two-way ANOVA for the preference index to swim side-by-side with illumination and current as between-subject factors. Then, we performed an equivalent analysis for the time spent swimming side-by-side and the time spent swimming in-line.

In the case of significant interaction in ANOVA, we performed *post-hoc* tests using the Tukey's Honest Significant Difference test. All statistical analyses were performed with the statistics software R (version 3.6.1) with a significance level of 0.050.

3. Results

3.1. Orientation and exploration

We registered that both illumination and current altered the orientation of fish pairs (main effect of illumination: $F(1, 43) = 30.080, p < 0.001$; main effect of current: $F(1, 43) = 107.760, p < 0.001$; Figure 3(a)), so that fish orientation improved in bright illumination and in the presence of the water flow. These claims were further clarified when factoring in the interaction between illumination and current (illumination x current: $F(1, 43) = 10.020, p = 0.002$; Figure 3(a)). Specifically, we observed that in the absence of flow, orientation in the dark was indistinguishable from orientation in standard illumination (n.s. in *post-hoc* comparisons). Conversely, when swimming against a flow, fish displayed higher orientation in the bright illumination than in the dark ($p < 0.001$ in *post-hoc* comparisons).



(a)

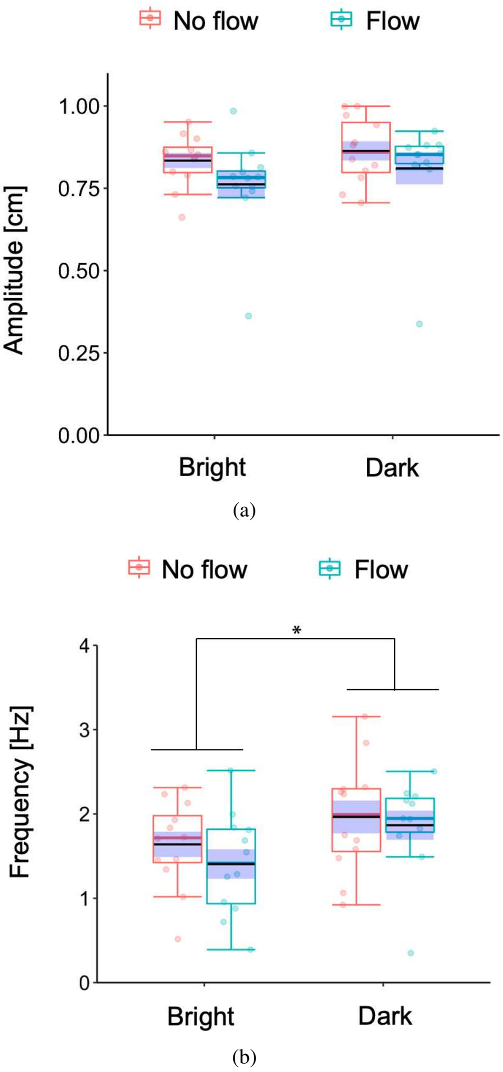
(b)

Figure 3: Effect of illumination and current on collective orientation and exploration: (a) orientation and (b) exploration. Each box plot reports: entire dataset (light blue points), mean (black line), standard error of the mean (blue shaded area), median (red/green line), first and third quartiles (red/green box), and minimum and maximum value of the dataset excluding outliers (red/green whiskers). Symbol $*$ indicates a statistical difference at $p < 0.050$.

With respect to exploration, fish exhibited a higher exploration in the dark compared to the bright illumination (main effect of illumination: $F(1,43) = 44.598, p < 0.001$; Figure 3(b)). Data analysis suggests that this effect was unrelated to the presence or absence of the water flow (main effect of current: $F(1,43) = 1.032, p = 0.315$; illumination x current: $F(1,43) = 3.465, p = 0.069$; Figure 3(b)).

3.2. Swimming patterns

While we did not register any effect of illumination and flow on TBA (main effect of current: $F(1,43) = 3.019, p = 0.089$; main effect of illumination $F(1,43) = 1.220, p = 0.275$; and illumination x current: $F(1,43) = 0.073, p = 0.788$; Figure 4(a)), we identified an effect of illumination on TBF (main effect of illumination: $F(1,43) = 5.162, p = 0.028$; Figure 4(b)) where fish increased their TBF in bright illumination.



(a)

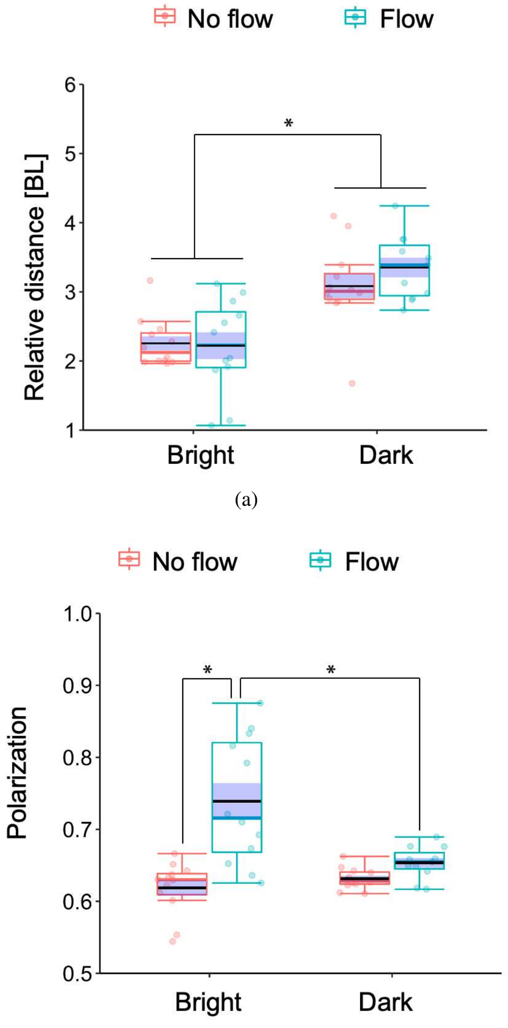
(b)

Figure 4: Effect of illumination and current on swimming patterns: (a) tail beat amplitude (TBA) and (b) tail beat frequency (TBF). TBA and TBF are the average value in the pair. Each box plot reports: entire dataset (light blue points), mean (black line), standard error of the mean (blue shaded area), median (red/green line), first and third quartiles (red/green box), and minimum and maximum value of the dataset excluding outliers (red/green whiskers). Symbol $*$ indicates a statistical difference at $p < 0.050$.

3.3. Cohesion and coordination

With respect to shoaling, fish swam closer to each other in the bright illumination (main effect of illumination: $F(1,43) = 38.147$; $p < 0.001$; Figure 5(a)). Data analysis suggests that this effect was not mediated by the current (main effect of current: $F(1,43) = 0.519$; $p = 0.475$; illumination x current: $F(1,43) = 0.919$; $p = 0.343$; Figure 5(a)).

Similar to orientation, we determined that both illumination and current altered the relative alignment of the fish pairs (main effect of illumination: $F(1,43) = 6.365$; $p = 0.015$; main effect of current: $F(1,43) = 24.554$; $p < 0.001$; Figure 5(b)), so that fish would better coordinate their swimming directions in standard illumination and in the presence of the water flow. These observations were further detailed when factoring in the interaction between illumination and current (illumination x current:



(a)

(b)

Figure 5: Effect of current and illumination on zebrafish cohesion and coordination: (a) relative distance and (b) polarization. Each box plot reports: entire dataset (light blue points), mean (black line), standard error of the mean (blue shaded area), median (red/green line), first and third quartiles (red/green box), and minimum and maximum value of the dataset excluding outliers (red/green whiskers). Symbol $*$ indicates a statistical difference after *post-hoc* analysis at $p < 0.050$.

$F(1, 43) = 11.332; p < 0.001$; Figure 5(b)). *Post-hoc* analysis revealed that fish swimming in placid water did not vary their alignment with respect to the illumination (n.s. in *post-hoc* comparisons). Conversely, in the presence of a flow, fish displayed a higher polarization in the bright illumination than in the dark ($p < 0.050$ in *post-hoc* comparisons).

3.4. Swimming formation

To gain insight into the modulatory roles of illumination and current on the swimming formation of the pair, we aggregated all the experimental data of fish swimming trajectories for each condition to create pie charts for the time budgeted by the animals to swim in formation (in-line or side-by-side) or not. Out of the five minutes of testing, fish pairs spent on average from 17% up to 42% of the time swimming in formation (Figure 6(a)) — meaning being within two BL along the cross-stream coordinate. Under bright illumination, the time spent in formation was more than 27% of the total, while in the dark, it did not exceed 18%. The largest difference between the times spent swimming in-line and side-by-side was registered in condition Bright - Flow with more than a twofold difference between them.

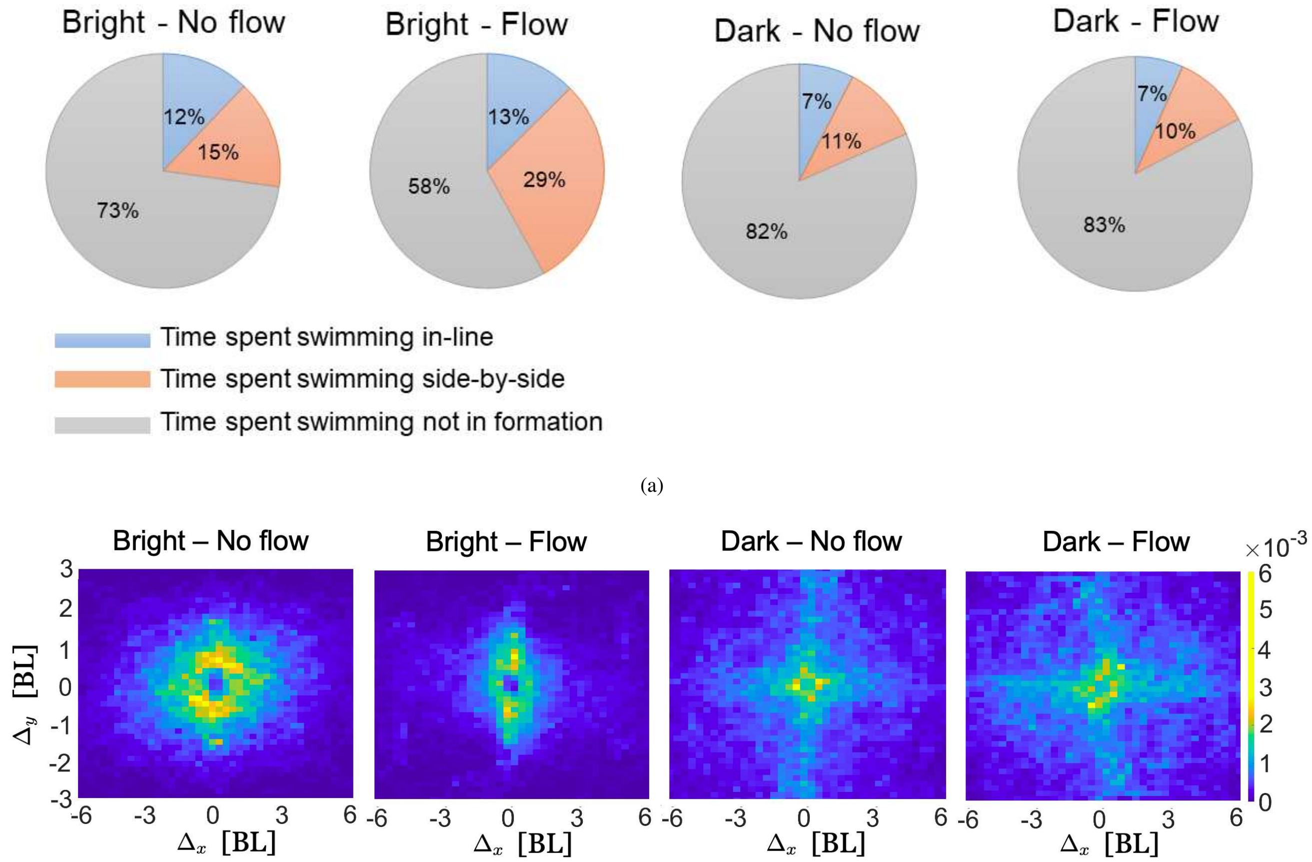
An even clearer illustration of the relative positional preference of the animals can be garnered from two-dimensional histograms of the relative cross-stream distance (Δ_y) versus the relative streamwise distance (Δ_x) (Figure 6(b)). In the dark, the position of a fish with respect to the other seems to be rather uniform. In contrast, under bright illumination we observed concentrated heat maps about the center of the plots (Δ_x in $[-2, 2]$ BL and Δ_y in $[-1, 1]$ BL). Visually comparing the heat maps for conditions Bright - No flow and Bright - No flow, we note a wider distribution streamwise in the former condition, which indicate a more even time allocation between in-line and side-by-side swimming. For condition Bright - Flow, instead, we identify two bright yellow blobs located along the vertical axis — the emergence of side-by-side swimming.

More specifically, in placid water, fish spent about the same time swimming side-by-side and in-line, so that the preference index was approximately 0.5 (Figure 7(a)). In the presence of the flow, instead, they displayed a clear preference for side-by-side swimming (main effect of current: $F(1, 43) = 4.161; p = 0.047$; Figure 7(a)), which was especially evident in bright illumination where we registered a preference index close to 0.7 — a more than two-fold difference between the times spent swimming side-by-side and in-line.

Both the current and the illumination had a modulatory effect on the time spent swimming side-by-side (current x illumination: $F(1, 43) = 6.026; p = 0.0018$; Figure 7(b)). In the bright illumination, fish swam more side-by-side against a flow than in placid water ($p = 0.006$ in *post-hoc* comparisons); such a difference was not observed when swimming in the dark (n.s. in *post-hoc* comparisons). Likewise, when swimming against a flow, fish spent more time side-by-side in bright illumination than in the dark ($p < 0.001$ in *post-hoc* comparisons); such a difference was not registered when swimming in placid water (n.s. in *post-hoc* comparisons). Finally, fish increased the time budgeted to swim in-line under bright illumination (main effect of illumination: $F(1, 43) = 16.937; p < 0.001$; Figure 7(c)).

4. Discussion

Being in a group offers several advantages to fish [7], from better predatory avoidance [8] to energy saving [9, 10]. Collective behavior is influenced by environmental factors, such as flow and illumination, which elicit behavioral adaptations and modify physical pathways of social interaction. Several studies have addressed the individual role of environmental factors, but seldom have their combined effect been examined. In this vein, it is presently difficult to disentangle the specific mechanisms that are responsible for the behavioral modulations triggered by environmental factors. For example, we know that several fish adopt side-by-side swimming when swimming against a flow [15, 16, 17, 18], but without experiments that could block



(a)

(b)

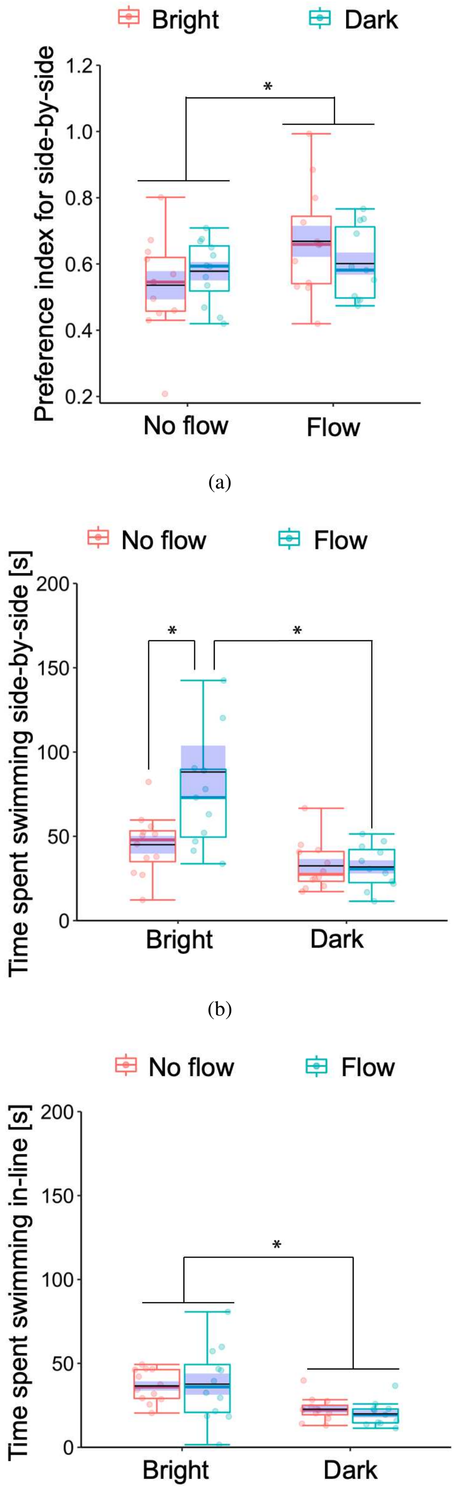
Figure 6: Analysis of aggregated data about relative positioning of the animals for all the experimental conditions: Bright - No flow, Bright - Flow, Dark - No flow, and Dark - Flow. (a) Pie charts for time spent swimming in-line, side-by-side, and not in formation. (b) Two-dimensional heat maps for the probability of a neighbor fish at a relative position. Darker blue colors and brighter yellow colors indicate low and high probabilities, respectively.

visual cues, the reliance of the formation on vision remains unclear. Will fish pursue side-by-side swimming at all if they cannot access visual cues of conspecifics, on the basis of hydrodynamic interactions? If so, will this lead to an improved cost of swimming?

Motivated by this methodological gap, we set ourselves to investigate the combined effect of illumination and flow on the collective behavior of zebrafish, a freshwater animal model with ubiquitous use in the study of the neurobiological basis of behavior. Leveraging our in-house developed multi-target tracking algorithm [37, 38], we extracted individual trajectories of a fish pair swimming in a water channel in the presence and absence of a water flow and under bright and dark illumination. To detail the behavior of the pair, we examined a number of salient metrics, including orientation, exploration, swimming patterns in terms of tail beat motion, relative distance (shoaling), polarization (schooling), and swimming formation in terms of time spent swimming in either side-by-side or in-line configurations,

In agreement with our hypotheses, we observed that zebrafish ability to orient in the water channel drastically improved in the presence of the flow. Although the biological basis of fish orientation are only partially known, there is overwhelming evidence regarding the existence of a critical flow speed, above which fish can successfully orient against the flow [40]. Our experiments support the current view that identifies sensory compensation as a cause for the persistence and robustness of fish orientation across a wide range of environmental conditions [40]. Specifically, we detected successful orientation against the flow even in the absence of visual cues; deprived of body-motion cues to vision, fish could rely on hydrodynamic signals, as well as other body-motion cues to vestibular and tactile senses [40]. This finding is in line with several other efforts, which documented successful orientation against a flow in the absence of visual cues for non-benthic species in widely different laboratory settings [35, 36, 45]. Recent work [46] has further documented an alternative pathway for fish orientation, entirely based on a passive mechanism in which the fish would respond to the incoming flow like a passive flag and the emergence of a threshold for the flow speed would be regulated by hydrodynamic interactions with the walls. It is tenable that such a mechanism could be part of the complex multisensory system that supports orientation against a flow in fish, along with vision, lateral line, vestibular system, and touch.

Predictably, under bright illumination the fish swam closer to each other, in agreement with several studies that determined



(a)

(b)

(c)

Figure 7: Effect of current and illumination on the swimming formation. (a) Preference for side-by-side versus in-line swimming. Time spent swimming (b) side-by-side and (c) in-line. Each box plot reports: entire dataset (light blue points), mean (black line), standard error of the mean (blue shaded area), median (red/green line), first and third quartiles (red/green box), and minimum and maximum value of the dataset excluding outliers (red/green whiskers). Symbol $*$ indicates a statistical difference at $p < 0.050$.

reduced shoaling tendency due to poor illumination and water turbidity [23, 24, 25, 26, 27]. Blocking visual cues challenges the ability of the animal to appraise their social environment, thereby failing to detect one another and recognize others as conspecifics, rather than objects or even threats. Another explanation for reduced shoaling tendency in the dark is in the adaptation of their behavior due to lowered perception of risk; although capable to shoal with each other in the dark by accessing information through other senses, they did not choose to do so. Such an adaptive explanation cannot be excluded by our experiments, but we consider this possibility to be less likely based on the following arguments. First, in the dark, fish explored the test section more, as seen from the high levels of spatial entropy, suggesting reduced ability to appraise the environment. Second, they displayed reduced orientation against the flow and with respect to each other, as evident from the polarization. Third, they increased their tail beat frequency — an energetically disadvantageous strategy [47] that may not be compatible with an adaptive response.

In partial disagreement with our expectations, we did not identify an effect of the flow on the shoaling tendency, whereby fish did not adjust their relative distance differently when swimming against a flow or in placid water. This finding was, however, not completely unexpected, whereby similar laboratory experiments on pairs of red nose tetra fish by Ashraf *et al.* [16] also failed to report a reduction in the relative distance between the fish as a function of the flow speed, under standard illumination. In fact, such a finding should be examined alongside other metrics to offer a more comprehensive assessment of the collective response. Ashraf *et al.* [16] offered a strong, physics-based argument regarding the hydrodynamic advantage that is bestowed by swimming in a cohesive shoal in the presence of a background flow. By showing that fish swam closer in bright illumination and that under bright illumination they reduced their tail beat frequency, we support, in fact, the proposition of a hydrodynamic advantage at close spacing by Ashraf *et al.* [16]. We also mention the possibility of a floor effect in the distance between the fish when experimenting in a relatively small water channel rather than a field setting [11] or a larger channel [15], which would prevent the detection of an improved shoaling tendency in the presence of a current. Such a possibility is indirectly supported by the improved coordination in the presence of the flow when swimming in bright illumination. It is tenable that in a small, confined, environment, schooling would be a more sensitive measure of collective behavior than shoaling.

The study of the formation chosen by the fish pair offers further support in favor of the proposition that collective behavior relies to a great extent on visual cues. Just as the relative distance between the animals increased in the dark, we registered a decrease in the time spent in any of the two formations (either in-line or side-by-side) in the dark. The time spent swimming side-by-side seemed to be more impacted by illumination than the time spent swimming in-line, likely due to the higher difficulty experienced by the animal in adjusting their swimming in a side-by-side formation as compared to an in-line formation. In fact, our classification of side-by-side versus in-line is exclusively based on the stream-wise distance, which must be less than one BL for deeming the pair to be swimming side-by-side.

In addition to a modulatory role of the illumination, our results point at a critical role of the flow, which favors side-by-side over in-line swimming. Not only did fish increase their preference for side-by-side versus in-line swimming in the presence of the flow, but also they allotted more time to swimming side-by-side. It is tenable that some form of hydrodynamic advantage might have underpinned this behavior, with each fish benefiting from the presence of the other to reduce their energy expenditure, along the lines of the arguments supporting higher shoaling tendency by de Bie *et al.* [15], Ashraf *et al.* [16, 41], and Chicoli *et al.* [18]. Another explanation could be attempted based on the theoretical arguments made by Porfiri *et al.* [39] in the context of the stability of dynamical systems. Within a mathematical model of collective behavior of zebrafish pairs that accounts for visual and hydrodynamic interactions, the authors found that side-by-side swimming is dynamically unstable in an infinitely extended domain without a flow. Specifically, any perturbation to a side-by-side configuration would lead to the fish pair transitioning to another formation. It is possible that in the presence of a background flow and hydrodynamic interactions with the channel walls, a side-by-side configuration would emerge as a stable formation, in the sense that any perturbation to it would be automatically recovered by the pair.

Our study is not free of limitations, which call for future research along several directions. First, our entire analysis focused on collective behavior in two dimensions, thereby discarding zebrafish motion along the water column. It is known that the behavioral repertoire of zebrafish unfolds along three dimensions, with diving motions playing a particularly salient role on the quantification of stress-related behaviors [48, 49]. Future research shall attempt at the integration of video feed from multiple cameras to offer a more complete quantification of zebrafish behavior. Second, without access to metabolic rate information it is difficult to offer conclusive support in favor of any argument about hydrodynamic advantage afforded by shoaling. The use of custom-made or commercial respirometry systems could be a viable choice [50, 51], although these systems can only quantify energy expenditure of the pair, not of the individuals. Third, our analysis of swimming trajectories cannot unequivocally isolate hydrodynamic from visual interactions. Additional information would be needed, which is presently beyond or at the very front of the state of the technology in behavioral scoring. For example, accessing the gaze of the animals could help understand better visual interactions [52]. Likewise, probing the pressure field in the flow [53] (in real-time) via particle image velocimetry could assist in the quantification of hydrodynamic cues.

Despite its limitations, this study offers new insight into the combined role of illumination and current on the collective behavior of a model species with ubiquitous use in the study of behavior. Overall, our findings point at a rich interplay between illumination and current, affecting the multi-sensory processes that support living in a group.

Ethics

All animal procedures were approved by the University Animal Welfare Committee of New York University under protocol number 13–1424.

Authors' contributions

Both the authors designed the research, performed statistical analysis, and wrote the paper. DB performed the experiments and extracted the swimming trajectories. MP supervised the research, secured the funding, and prepared the final version of the manuscript. Both the authors reviewed and approved the manuscript.

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Data availability

Datasets used in the analyses are at `https://github.com/Dynamical-Systems-Laboratory/CollectiveBehaviorFlowLight.git`

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