Going with, then against the flow: evidence against the optomotor hypothesis of fish rheotaxis

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Movement through an environment provides sighted organisms with dynamic visual cues known as optic flow. In flying insects, optic flow is important for collision avoidance, flight speed control and landing manoeuvres. The function of optic flow is much less understood in other taxa, particularly in fish. Despite a lack of quantitative studies, optomotor responses (OMRs) to optic flow are presumed to be nearly ubiquitously important for rheotaxis (orientation to currents), a widespread behaviour that confers a number of benefits, including energy conservation. Here we show that while very young larval zebrafish, *Danio rerio*, exhibit positive OMRs (swim in the direction of optic flow), thereby minimizing optic flow on the retina, older larvae and adults exhibit negative OMRs that increase optic flow. While the reason for this reversal remains unclear, negative OMRs are inconsistent with optically driven positive rheotaxis (orienting upstream). Furthermore, when optic flow cues are placed in conflict with nonvisual (mechanical) cues generated by water currents, adult fish largely ignore the optic flow cues relying instead on nonvisual cues. These results challenge a century-old belief that optic flow and the resulting OMRs are a dominant sensorimotor mechanism in rheotaxis, highlighting the importance of future work on life history changes in OMRs and the possible modulatory influence of learned, multisensory expectations.

Keywords:

Danio rerio

optic flow

optomotor response

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zebrafish

As sighted animals move through their environment, the apparent motion of the stationary surround across the retina generates optic flow cues. Optic flow has been implicated in a wide variety of behaviours across animals as diverse as insects (Collett et al., 1993; Egelhaaf et al., 2012; Srinivasin, 2011) and humans (Bruggeman et al., 2007; Warren et al., 2001). In flying insects, optomotor responses (OMRs) to optic flow have been well studied and play a pivotal role in flight control (Duistermars & Frye, 2012; Srinivasan et al., 1999), serving, among other things, a compensatory function to minimize destabilizing influences; for example, a bumblebee making a corrective steer to the right in order to compensate for a gust of wind that causes it to veer to the left.

Although the role of optic flow and OMRs in fish behaviour is much less well studied and understood, there is nevertheless an entrenched belief, originating from the classic optic flow manipulations of Lyon (1904), that OMRs are of paramount importance to rheotaxis and station-holding abilities of fish in currents (Arnold, 1974). Rheotaxis is a widespread, if not universal, behaviour among fish that has many potential benefits (Arnold, 1974), including directional guidance for migratory behaviours (Thorpe & Morgan, 1978; Tytler et al., 1978), improved interception of downstream-drifting prey and odours (Baker et al., 2002; Gardiner & Atema, 2007) and energetic cost savings (Montgomery et al., 1995). Although rheotaxis is typically positive (orienting upstream) (Arnold, 1974; BakColeman et al., 2013; BakColeman et al., 2014; Lyon, 1904; Montgomery, 1997), especially among stream- or river-dwelling species (Clausen, 1931), it can also be negative (orienting downstream), as is true for diadramous species like salmonids, which are negatively rheotactic during their downstream migration to the sea as juveniles and positively rheotactic during their upstream migration to their natal

freshwater spawning sites as sexually mature adults (Thorpe & Morgan, 1978; Veselov, 1998).

The evidence in support of OMRs underlying the more typical positive rheotaxis rests largely on behavioural responses of fish to two types of optic flow stimuli in the absence of any water currents: (1) translational optic flow consisting of horizontally oriented black and white stripes moving underneath the floor of a rectangular-shaped test tank (Lyon, 1904; Orger & Baier, 2005; Orger et al., 2000; Neuhauss et al., 2009) and (2) rotational optic flow consisting of vertically oriented black and white stripes revolving around a cylindrical tank (Harden-Jones, 1963; Kraus & Neumeyer, 2003; Maaswinkel & Li, 2003; Rock & Smith, 1986). In both cases, fish reportedly respond by following (swimming with) the direction of the optic flow, a response that has also been observed in tethered insects (Srinivasan, 1999). The prevailing explanation of this response is that animals reflexively move to stabilize the displacement of the visual image on the retina. Since downstream displacement of a fish by a water current creates optic flow that is opposite in direction to that of the current, swimming with the optic flow has thus been interpreted as heading upstream (positive rheotaxis) in order to maintain spatial position in the virtual (hydrodynamic) current.

Unfortunately, little attention has been given to the fundamental differences between translational and rotational optic flow stimuli and whether or not they adequately simulate the visual experience of a fish being swept downstream. For example, many rotational (optomotor drum) stimuli are bilaterally asymmetric and probably create a visual experience more akin to rotation (yaw) than to downstream displacement. In flying insects, bilateral asymmetries are crucial for eliciting collision avoidance responses when

approaching an obstacle from one side (Egelhaaf et al., 2012). Thus, it is presently unclear whether the apparent following response to this type of stimulus is relevant to rheotaxis or some other behaviour (e.g. collision avoidance).

Furthermore, recent studies using translational optic flow were performed on young (5–8 days postfertilization, dpf) larval zebrafish tested in large groups of 50–200 individuals (Neuhauss et al., 2009; Orger & Baier, 2005; Orger et al., 2000), leaving open the question of whether or not additional sensory cues from the movements of neighbouring fish are involved and whether results are generalizable to adult fish of the same or different species. Although older studies (Clausen, 1936; Lyon, 1904) used translational optic flow to test both juvenile and adult fish in different species, conclusions were based on qualitative observations without benefit of any quantitative measures or tests of statistical significance. In summary, the entrenched belief that OMRs are pivotal to rheotaxis relies largely on two untested assumptions: (1) that the biological significance of positive OMRs to optic flow is independent of whether fish are tested individually or in groups and (2) whether translational or rotational optic flow stimuli are used.

Finally, the optomotor hypothesis of positive rheotaxis makes several predictions that have essentially gone untested for over a century. First and foremost is that fish should follow in the direction of the optic flow (i.e. exhibit positive OMRs that are consistent with positive rheotaxis). Second, is that if positive OMRs are the principal sensorimotor mechanism for positive rheotaxis, then optic flow cues should have a greater influence on rheotaxis than nonvisual cues, which include lateral line, tactile and vestibular cues (Arnold, 1974; Bak-Coleman & Coombs, 2014; Bak-Coleman et al.,

2013; Montgomery et al., 1997). Third, species that exhibit positive rheotaxis throughout their life history should likewise exhibit positive OMRs throughout their life history.

In this study, we perform a series of optic flow manipulations in both the absence and presence of hydrodynamic flow (Table 1) to test the assumptions and predictions of the optomotor hypothesis of rheotaxis. In essence, we measure the behavioural outputs (OMRs and rheotaxis) from the brain in response to manipulations of the stimulus inputs to the brain (optic and hydrodynamic flow). Zebrafish, *Danio rerio* (both larval and adults) were chosen for this study for several reasons, including (1) their native stream-dwelling and diurnal life styles and (2) their use in investigations to determine how OMRs are used in the adaptive control of locomotion (Ahrens et al., 2012; Portugues & Engert, 2011). Finally, this increasingly popular vertebrate animal model for investigating the sensorineural basis of behaviour offers several practical advantages (e.g. ease of breeding and transparent larvae for optical imaging of in vivo neural activity; Friedrich et al., 2013; Sumbre & de Polavieja, 2014). Thus, this species can readily be used in follow-up studies to further explore the neural or genetic underpinnings of rheotactic behaviours.

METHODS

Experimental Animals

Adult (wild-type) zebrafish were housed communally in 75.7-litre aquaria. They were maintained on a 12:12 h light:dark cycle at 22 ± 1 °C and fed daily with

commercially available (Tetramin) food flakes. Adult fish used in experiments, as well as those used in breeding larval fish were both obtained from commercial suppliers.

However, breeding adults were housed separately from experimental fish under different temperature and light conditions (see below). Standard breeding protocols were followed (<https://wiki.zfin.org/display/prot/Breeding>). To induce breeding, six males and six females were fed a rich diet of bloodworms and were housed together in a 43-litre tank maintained at 26 ± 1 °C under an LD 14:10 h cycle. Eggs were collected daily over a 4-day period and examined under a microscope in order to remove unfertilized eggs. Each batch of 15–25 embryos was transferred to a 250 ml beaker filled with 100 ml of water and labelled with the collection date. Days postfertilization (dpf) were estimated from the collection date as a proxy for the fertilization date, assuming a margin of error of 2–3 days. Larvae were fed Sera Micron Fry food (a combination of *Spirulina* algae, krill and shrimp meal). Embryos and early-stage larvae (<9 dpf) were maintained in temperature baths at 25–27 °C. Once larvae were transferred from nursery to rearing containers, they were acclimated to lower temperatures (21 ± 1 °C) over a 24 h period and thereafter maintained on an LD 12:12 h photoperiod. Both larvae and adults were thus tested at the same range of temperatures and during the same time period, between 1200 and 1700 hours. This research was approved by the Institutional Animal Care and Use Committee of Bowling Green State University (protocol no. 13-009) and adhered to the guidelines of ASAB/ABS and of the National Institutes of Health (Public Health Service) for the use and care of live animals in research.

Test Arenas

We used three different test arenas. In the absence of hydrodynamic flow (optic flow cues only; experiments 1 and 6), a small rectangular tank (9.8×2.8 cm) filled with water to a depth of 0.7 cm was used to test both young (9–11 dpf) and old (16–18 dpf) larvae, whereas a larger arena (22×9 cm) filled to a depth of 2.5 cm was used to test adult fish. In experiments involving both hydrodynamic and optic flow (experiments 2–5), adult fish were tested in a square (25×25 cm), flow-through working area in the main channel of a custom-built flow tank filled to a depth of 25 cm. The flow tank was modelled after the design of Vogel and LaBarbera (1978) and consisted of a rectangular Plexiglas channel ($154 \times 28 \times 38$ cm) fitted with a 20 cm diameter PVC pipe at both ends to recirculate the water below the main channel. Flow was generated just below the downstream end of the channel by a 13 cm impeller inserted into the PVC pipe and rotated by a chemistry stirrer (IKA Labortechnik RW 20DZM). The motor assembly was clamped to a drill press stand, which was placed on rubber mats to isolate the vibrations of the motor from the tank. Motor speed (revolutions/min) was displayed on an LCD and controlled manually by a dial on the stirrer.

Collimators at the upstream and downstream end of the channel were used to reduce impeller-driven turbulence in the flow and to promote uniform flow across the entire width and depth of the tank. Collimators were made out of plastic egg-crate lighting panels (1 cm^2 grid) and bundles of 3 cm length straws. Flow speed was measured using Janus green dye streaks and particle image velocimetry (PIV), as described previously (Bak-Coleman & Coombs, 2014; Bak-Coleman et al., 2013).

Optic Flow Stimuli

Optic flow stimuli were presented from one or more 60 cm diagonal computers (ASUS VS247H-P, aspect ratio 16:9 and spatial resolution of 1920×1080 , set to default settings) in one of three different configurations: (1) on the floor of the tank; (2) on the sides of the tank; or (3) on both the floor and sides of the tank (Table 1, Fig. 1). Stimuli were displayed at 60 Hz, above the flicker–fusion threshold of adult and larval zebrafish (Branchek, 1984). Stimuli consisted of a series of dark (dark grey, hex code: no. 3F3F3F) and light (white, hex code: no. FFFFFFFF) bars that were either horizontally (floor stimuli) or vertically (side stimuli) oriented. We used dark grey bars rather than black bars to facilitate visualization of larval fish. Bar orientation was always perpendicular to the direction of optic flow, and bar width was either 5 cm (experiments 1–5) or 1.9 cm (experiment 6). Because the distance between the free-roaming fish and the optic flow source varied, the visual angle subtended by one stimulus cycle (dark + light bar) and the corresponding spatial frequency (cycles/degree) varied within a restricted range of values for each experiment (Table 1).

Experimental Design and Test Procedures

We performed six experiments (Table 1, Fig. 1) to test specific predictions and assumptions underlying the optomotor explanation of rheotaxis, as described for each experiment below. Experiments 1–5 were performed on adult zebrafish to see whether translational optic flow could elicit positive OMRs under any of several different optic

flow conditions, both in the absence (experiment 1) and presence (experiments 2–5) of water currents. Experiment 5 additionally examined whether the testing of solitary fish versus groups of fish influenced the OMR in any way. Failing to elicit positive OMRs under any optic flow or solitary versus group testing condition, we performed a final experiment (experiment 6) using stimulus conditions identical to those shown to be effective in eliciting positive OMRs from larval zebrafish tested in groups (Orger et al., 2000). This final experiment was performed on both larval and adult fish under identical stimulus conditions to determine whether life history stage affects OMRs in solitary fish.

To eliminate the confounds of social cues associated with group testing, all tests were done on solitary fish except those in experiment 5, for which group size (1 or 8 fish) was a key independent variable. Other key independent variables and optic/hydrodynamic flow characteristics are summarized in Table 1 for each experiment, whereas dependent variables and statistical tests are described with the results. In all cases, fish were given a 10 min period without optic or hydrodynamic flow to acclimate to the novel test arena before testing began. In addition, testing was done with all room lights off and with black curtains surrounding the test arena to minimize the use of surrounding visual cues. Thus, the only light available for videotaping was provided by the upwelling and surrounding light of the computer monitors displaying the optic flow stimuli. Finally, the fish's behaviour was recorded with a video camera (Nikon D5100 for larval experiments and Sony DCR-HC42 camcorder for all else) from above the test arena at the rate of 30 frames/s. The stimulus duration and thus the time period over which behaviour was recorded and analysed ranged from 1 to 3 min, depending on the experiment (see below for further details of each experiment). Position and orientation

were tracked manually by selecting a location along the midline corresponding to the snout and the widest part of the fish. The vector between the two points determined orientation, whereas the x - y coordinates at the widest part of the fish defined position. Video was manually tracked at one frame/s in experiments 1, 2, 4 and 5, at 0.2 frames/s in experiment 6 and automatically at 30 frames/s using custom-written MATLAB scripts (as in Bak-Coleman et al., 2013) for experiment 3. Fish used in each set of experiments (experiments 1–6) were not recycled from other experiments.

Experiment 1: Effects of Optic Flow Speed on the OMRs of Solitary Adult Fish

To determine whether optic flow speed affects the polarity or magnitude of the OMR, adult fish were exposed to different speeds of a two-dimensional translational optic flow on the floor of the tank (Fig. 1a). This experiment is one of several that tests the main prediction of the optomotor hypothesis of rheotaxis (i.e. that fish will follow in the direction of optic flow to exhibit a positive OMR). Each stimulus presentation lasted 3 min and flow was either in a left or right direction (along the long axis of the tank) at one of four flow speeds (0, 5, 10, 15 cm/s) (see Table 1 for further information on optic flow characteristics). A 3 min period without optic flow (i.e. stationary bars) preceded and followed each stimulus presentation. A different group of eight fish was tested at each of the four flow speeds (32 fish total; Table 2), with the order of flow direction being counterbalanced across individuals within each of the groups and each individual within the group being tested at the same flow speed.

Experiment 2: Effects of Placing Optic and Hydrodynamic Flow Cues in Conflicting (Opposite) Directions

The primary goal of this experiment was to test the prediction that optic flow cues will dominate the rheotactic response if OMRs are the prevailing sensory mechanism. A secondary goal was to test the possibility that three-dimensional translational stimuli might be more effective in eliciting OMRs than the two-dimensional stimuli used in experiment 1. One monitor below the floor and one on each side of the tank were used to produce a whole-field translational optic flow in which the floor and the side bars were in spatial and temporal register (Fig. 1b). Three stimulus conditions were used: (1) optic flow only condition, in which the dark and light bars moved but the water did not; (2) hydrodynamic flow alone, in which the water moved but the bars did not; (3) both optic and hydrodynamic flow condition, in which the computer-generated optic flow was at the same speed and in the same downstream direction as the water current. The last condition presents a cue conflict situation in which the optic flow direction is opposite to that normally expected for the self-generated optic flow of a fish displaced downstream. Each fish was presented with a sequence of the three possible stimulus conditions, each stimulus presentation and video-recording session lasting 1 min. The order of stimulus conditions was counterbalanced across individuals in each of four groups ($N = 6$ per group), one group for each of the four flow speeds tested (0, 2.5, 5 and 10 cm/s; Table 2).

Experiments 3 and 4: Effects of Optical Yaw and Slip Illusions on Rheotaxis

The primary goal of these experiments was to test for the possibility that optic flow, while perhaps not necessary for rheotaxis, might nevertheless play a role in increasing the stability and/or precision of upstream orienting behaviour, as observed in visually enabled compared to visually deprived giant danio, *Devario aequipinnatus* (Bak-Coleman et al., 2013). This increased performance could be due to optic flow, whereby fish attempt to minimize rotational optic flow (indicative of yaw instabilities) or transverse translational flow (indicative of sideways displacement or slip instabilities). In principle, this experiment is similar to experiment 2 in that the direction of the optic flow is different from that of the hydrodynamic flow and thus, is also a test of the assumption that optic flow cues will dominate hydrodynamic flow cues if the optomotor hypothesis is valid. In experiment 3, we used a shear stimulus to generate the optical illusion of horizontal body rotation (yaw). Two monitors were used, one on each side of the tank to produce optic flow in opposing (shearing) directions, one with and one against the hydrodynamic flow (Fig. 1c). The optic flow on one side of the tank was thus in the same direction and speed (5 cm/s) as the hydrodynamic flow, whereas that on the other side was at the same speed, but in the opposite direction. The floor of the tank was uniformly white. After the acclimation period, which consisted of stationary bars on the sides of the tank with no water current, the hydrodynamic flow was turned on and allowed to ramp up to full speed over a 1 min period. The ramp-up period was followed by 1 min of hydrodynamic flow only, in which white and black bars were present, but stationary, on the sides of the tank. In the final 1 min, the optic flow shear stimulus was either added to the hydrodynamic flow (treatment group, $N = 8$) or not (control group, $N = 8$) (Table 2). The direction of optic flow on the sides of the tank was counterbalanced across

individuals such that optic flow was downstream on the left side and upstream on the right side for half of the treatment group and vice versa for the other half.

In experiment 4, we used a cross-stream (transverse) optic flow to generate the optical illusion of sideways slip. The optic flow configuration and procedures for conducting this experiment were identical to those for experiment 3, except that the optic flow stimulus added during the final 1 min was in a cross-stream direction along the floor of the tank (Fig. 1d). For this experiment, the sides of the tank were black (to minimize external visual cues) and the direction of the cross-stream optic flow (leftward or rightward) was counterbalanced across individuals in the treatment group ($N = 8$) (Table 2). Light and dark bars remained static for the control group ($N = 8$).

Experiment 5: Effects of Group Testing on the Use of Optic Flow Cues in Rheotaxis

This experiment is a test of the assumption that the optomotor mechanism of rheotaxis is independent of whether fish are tested individually or in groups. Given that many previous OMR studies have been done on groups of fish rather than solitary fish and the possible significance of optic flow cues to group behaviours like schooling, it is important to determine whether optic flow cues are more likely to affect the rheotactic performance of grouped fish compared to solitary fish. The design and procedures for this experiment were similar to those used in experiment 2 except that only one flow speed (5 cm/s) was used for each of two groups of animals, one in which solitary individuals were tested ($N = 6$ individuals) and one in which groups of eight fish were tested simultaneously ($N = 6$ groups or 48 individuals total) (Table 2).

Experiment 6: Effects of Life History Stage on the OMR of Solitary Fish

This experiment had two goals. One was to test the assumption that the optomotor mechanism of rheotaxis is independent of whether larval fish are tested individually or in groups, as determined for adults in experiment 5. This was done by providing data on the OMRs of solitary larvae to the same optic flow stimulus used to elicit positive OMRs from grouped larvae (Orger et al., 2000). The second objective was to make direct comparisons between solitary larvae and adults to test a second prediction: species that exhibit positive rheotaxis throughout their life history should likewise exhibit positive OMRs throughout their life history. Although we did not measure the rheotactic behaviour of larvae to water currents in this study, there are several published reports that larval zebrafish exhibit both positive rheotaxis (Olzewski et al., 2012; Suli et al., 2012) and positive OMRs (Neuhauss et al., 2009; Orger & Baier, 2005; Orger et al., 2000).

For this experiment, we adopted the stimulus and response measurement approach used to elicit strong positive OMRs from groups of 5–8 dpf larvae by Orger et al. (2000). Solitary larval and adult fish were exposed to a two-dimensional translational optic flow on the floor of the tank with a spatial frequency of 0.01 cycles/degree moving at 3.8 cm/s (1 cycle/s) in the absence of any hydrodynamic flow (Fig. 1a). The square wave stimulus reversed directions every 30 s for a total of five reversal cycles. Thus, this stimulus served as an additional control against the possibility that the unidirectional stimuli used in experiments 1–5 may have dampened the magnitude of the OMR. That is, the measured response of a fish traversing the length of the tank to follow a unidirectional

optic flow might have declined over time once the fish reached the end of the tank and could no longer follow the flow. Three groups of fish, adults ($N = 8$), as well as young (9–11 dpf, $N = 8$) and old (16–18 dpf, $N = 8$) larvae, were tested. The starting direction of the optic flow stimulus (leftward or rightward along the long axis of the tank) was counterbalanced across individuals in each of the three groups.

RESULTS

Experiment 1: Adult Zebrafish Exhibit Negative OMRs Over a Wide Range of Optic Flow Speeds

Fish moved in a direction that was more against the flow than with it. As a result, their position in the tank shifted to the upstream end of the tank, although the magnitude of the shift varied with flow speed (Fig. 2a). The average positional bias of the fish towards one end of the tank or the other was measured as the mean streamwise position of the fish (averaged over the second minute of the 3 min optic flow stimulus) minus the mean streamwise position of the fish in the absence of optic flow, which was assumed (and observed, intercept of Table 3) to be unbiased and on average, at the centre of the tank. Although positional shift provides a reliable measure of the OMR, it does not capture the fundamental nature of rheotaxis, which is orientation to the current, not position in space. Therefore, we added a second metric, the rheotactic index (RI) (Bak-Coleman et al., 2013), which is the average angular deviation from upstream converted to a signed index. Given that there is no real (hydrodynamic) current in this experiment, RI

is measured with respect to the fictive (illusory) current that is presumably induced by optic flow. The fictive current is assumed to be of the same magnitude but of opposite direction to that of the optic flow. Like the positional shift index, RI varies from -1 to 1, with negative values indicating a downstream orientation (i.e. going with the fictive current, but against the optic flow) and positive values indicating an upstream orientation (i.e. going against the fictive current, but with the optic flow). Both RI and positional shift were negative and varied in a nonlinear way with flow speed (Fig. 2a, b, Table 3), such that the 10 cm/s condition caused the biggest (negative) RI and positional shift (multivariate polynomial regression: $r^2 = 0.40$, Pillai's trace = 0.45, $F_{4,58}=4.243$, $P = 0.004$). RI and positional shifts pooled across different flow speeds, and individuals were also found to be highly correlated (Pearson correlation: $r = 0.69$, $P < 0.0001$; Fig. 2c), as might be expected if fish are always oriented in the direction of their movements (i.e. do not move sideways or backwards). Thus, if movements are always in the opposite direction of the optic flow, thus yielding a negative positional shift, RI should approach its maximum negative value (-1), indicating negative rheotaxis to the fictive current induced by optic flow.

Experiment 2: Water Currents Produce Strong Positive Rheotaxis That Is Unaffected by Out-of-phase Optic Flow Cues

Hydrodynamic flow alone reliably produced a robust, positive rheotactic response, which increased in magnitude as a function of current speed (linear mixed effects: $b = -0.080$, $t_{45} = 7.69$, $P < 0.0001$; Fig. 3). As with experiment 1, optic flow alone

produced a significant negative response, with RI to the fictive hydrodynamic flow decreasing as a function of increasing flow speed ($b = -0.025$, $t_{45} = -2.39$, $P = 0.021$). When visual and nonvisual cues were 180° out of phase (i.e. optically induced fictive and real hydrodynamic currents were in opposite directions but of equal speed), fish showed a positive rheotactic response to the actual water flow with the magnitude of the response being indistinguishable from that caused by hydrodynamic flow alone (Fig. 3).

Experiments 3 and 4: Rheotaxis Is Not Affected by Optic Flow Designed to Create Yaw or Slip Illusions

The rheotactic performance of adult fish, as measured by RI, was unaffected by the optic flow shear stimulus designed to create the illusion of yaw (Fig. 1c). RI in the presence of the optic flow shear stimulus was not significantly different from that measured in the static control, either in the short term (measured at 30 frames/s during the first 3 s after stimulus onset; Student's two-tailed t test: $t_{14} = 1.07$, $P = 0.301$; Fig. 4a) or in the long term (measured at one frame/s for 1 min after stimulus onset; Student's two-tailed t test: $t_{14} = 0.234$, $P = 0.818$). Similarly, exposure to translational optic flow across the bottom of the tank in a direction transverse to the current did not degrade rheotactic performance in either a transient (Student's t test: $t_{14} = 0.42$, $P = 0.680$; Fig. 4b) or long-term fashion ($t_{14} = 0.488$, $P = 0.632$).

Experiment 5: Group Testing Suppresses Negative OMR to Optic Flow Alone

In the presence of hydrodynamic flow, groups of fish and solitary individuals performed similarly and without evidence of effects due to conflicting optic flow cues (Fig. 5, Table 4). However, in response to optic flow alone, the magnitude of the negative OMR was significantly smaller in groups of fish than in solitary individuals (linear mixed effects model: $b = 0.038$, $t_{20} = 2.23$, $P = 0.038$), although the direction remained significantly negative (one-sample, two-tailed t test: $t_5 = 3.0619$, $P = 0.028$).

Experiment 6: Larval and Adult Zebrafish Differ in the Magnitude and Polarity of Their OMRs to Translational Optic Flow

Young larval fish (9–11 dpf, mean $\pm$ SE = 3.82 ± 0.17 mm in total length) exhibited a robust positive OMR that consisted of a rapid turn in the direction of optic flow at stimulus onset, followed by burst-and-coast swimming from one end of the tank to the other in the direction of the optic flow; a 180° reversal of the optic flow direction caused a corresponding reversal in orientation and direction of the positional shift (Fig. 6a). In contrast, older larvae (16–18 dpf, mean $\pm$ SE = 4.45 ± 0.32 mm in total length) and adults (mean $\pm$ SE = 33.40 ± 1.4 mm in total length) tended to orient and swim in the opposite direction, exhibiting a more or less negative OMR (Fig. 6b, c, Supplementary Videos S1, S2, S3).

The magnitude and direction of the positional shift was quantified as the difference between the fish's position at the beginning and end of each 30 s segment (as a proportion of maximum possible excursion). This measure was similar to that used in experiment 1 in that a proportional shift of 1 means that the fish swam from one end of

the tank to the other, either with (+1) or against (-1) the optic flow. A shift of 0 indicated that the fish's position remained unchanged. A Kruskal–Wallis test revealed that age significantly affected proportional shift ($H_2 = 10.76$, $P = 0.004$; Fig. 7). Consistent with previous results on 5–8 dpf larvae (Orger & Baier, 2005; Orger et al., 2000), young larvae (9–11 dpf) exhibited strong positive shifts (Fig. 6a, d, Fig. 7a). However, older larvae (16–18 dpf) exhibited negative shifts that differed significantly from those of young larvae (ranked Tukey HSD: $P_{\text{adj}} = 0.0006$; Fig. 6e, Fig. 7). Mature adults likewise exhibited a shift that was significantly more negative than that of young larvae ($P_{\text{adj}} = 0.013$), but not significantly different from that of older larva ($P_{\text{adj}} = 0.225$; Fig. 6b–f). Young larval shifts were significantly positive (Wilcoxon signed-ranks test: $V = 21$, $N = 8$, $P = 0.031$), whereas shifts of pooled older larvae and adult fish were significantly negative ($V = 19$, $N_1 = 16$, $P = 0.039$).

DISCUSSION

While multiple behavioural functions have been proposed for the OMRs of flying insects (e.g. monitoring ground speed, avoiding collisions; Collett et al., 1993; Srinivasan, 1999), none have been more widely accepted for fish than rheotaxis (Arnold, 1974; Lyon, 1904). Multiple experiments in this study nevertheless demonstrate that optic flow cues have little impact on the rheotactic behaviour of adult zebrafish. First, in response to optic flow alone, adult zebrafish exhibited negative OMRs that were inconsistent with positive rheotaxis. Second, in the presence of real currents, several potentially destabilizing forms of optic flow (out-of-phase streamwise flow, transverse

flow and bilateral shear flow) all failed to disrupt the rheotactic response of adult fish to the ongoing current, demonstrating that optomotor responses do not exert undue influence on rheotaxis and that nonvisual cues are sufficient to override the conflicting optic flow cues. Third, although young larval fish had positive OMRs consistent with positive rheotaxis, older larval and adult fish had negative OMRs inconsistent with positive rheotaxis. Thus, there is clear evidence for an ontogenetic change in the polarity of the OMR that is inconsistent with the prediction that the sensorimotor mechanism for positive rheotaxis will remain the same throughout the life history of the fish. Finally, translational optic flow stimuli within the same range of temporal and spatial frequencies reported to produce positive OMRs from adult zebrafish in optomotor drum studies (Krauss, 2003; Maaswinkel & Li, 2003) elicited negative OMRs from adult zebrafish in the present study. The dependence of OMR polarity on ontogenetic stage and optic flow type (translational versus rotational) raises interesting questions about the biological function of OMRs throughout the life cycle of fish and the factors that contribute to OMR polarity.

The Behavioural Relevance of Optic Flow and OMRs

To consider the behavioural relevance of optic flow, it is first important to recognize that optic flow can be generated in one of two ways: by self-generated motion or via motion produced by exogenous forces, such as air or water currents. Whereas optic flow generated by self-propelled forward motion will always be front to back, that imposed by a current can be quite different (e.g. from back to front for a fish that is

displaced downstream while maintaining an upstream orientation). Thus, appropriate behavioural responses require that animals have knowledge about the visual consequences of their movements in space and whether the optic flow is self-generated or exogenously imposed. Given that the larvae of this study were not exposed to any currents, it is unlikely that they could have learned the association between optic flow and downstream displacement. Thus, zebrafish are probably born with an innate positive OMR.

In theory, such an early and instinctual positive OMR, consistent with positive rheotaxis, is likely to serve larval zebrafish very well in several respects. In the presence of ambient currents, for example, a positive OMR results in an upstream orientation that reduces drag, thereby providing energetic cost savings. In response to currents generated by suction predators, positive OMRs result in the larvae moving away from the suction source in order to avoid predation (Olszewski et al., 2012; Stewart et al., 2013). Finally, positive OMRs should theoretically facilitate the ability of fish to keep pace with their schooling neighbours (Imada et al., 2010; Shaw & Tucker, 1965).

Developmental Factors Affecting the OMR

By the time larvae reach the age of 16–18 dpf, the polarity of the OMR to translational optic flow has switched from positive to negative, meaning that the older larvae tend to move more against the optic flow than with it, as is the tendency of adult zebrafish. One possible explanation for the polarity reversal is that the sensory abilities of fish have changed. As zebrafish age, for example, the ratio of eye diameter to standard

length decreases (Figure 4 in Parichy et al., 2011) and the eyes become more lateralized (J. Bak-Coleman, personal observation). Thus, younger larval fish may have wider fields of frontal vision compared to older larvae and adults. A wide field of frontal vision appears to be important for the processing of transverse optic flows by flying insects, which are believed to use positive OMRs to help stabilize yaw during upwind flight (Srinivasan, 1999). Fruit flies, *Drosophila melanogaster*, heading upwind, for example, respond to a transverse optic flow by turning in the direction of the flow, thus eliminating transverse flow, the proposed ‘rule’ for heading upwind (Collett et al., 1993; David, 1986). In contrast, the adult zebrafish of this study did not appear to follow the same rule. That is, when adult zebrafish were heading upstream, their upstream heading was not disrupted by transverse optic flow (Fig. 4b). While it remains possible that visual feedback still plays a role in stabilizing rheotactic behaviour by minimizing slip or yaw instabilities, the effect is probably small (Bak-Coleman et al., 2013) and may be a secondary consequence of stabilizing the streamwise or cross-stream position for station holding rather than stabilizing the orientation of the fish. In any event, it remains to be seen whether wide-field frontal vision and the ability to integrate bilateral inputs from the two eyes to form a panoramic view (Duistermars & Frye, 2010) are required for eliciting a positive OMR.

There are several other, well-documented developmental changes that could impact the relative contribution of optic and hydrodynamic flow cues to rheotaxis. One is swim bladder inflation, which results in decreased specific gravity and a substantial reduction in the flow stimulus to the lateral line in response to suction currents generated by predators (Stewart & McHenry, 2010). If anything, inflation of the swim bladder

should increase the probability of being carried downstream by a current, theoretically making optic flow cues more relevant, not less relevant. Moreover, it is difficult to see how the sequential inflation of the two-chambered swim bladder at approximately 4 (posterior chamber) and 17 (anterior chamber) dpf (Parichy et al., 2011; Robertson et al., 2007) can explain the positive-to-negative shift in the OMR between 11 and 16 dpf.

The OMR polarity reversal is similarly difficult to explain based on milestone events in the development of lateral line organs (neuromasts). With the exception of secondary neuromasts on the tail, which appear between 10–17 dpf (standard length, SL ~5 mm), the majority of primary neuromasts on the head and trunk develop by 4 dpf (SL = 3–4 mm) (Ledent, 2002; Parichy et al., 2011). Most importantly, swimming behaviour can be elicited by stimulating a subset of terminal neuromasts near the tail in 4–6 dpf larvae (Liao & Haehnel, 2012), indicating the establishment of functional connections to the brain at this early age.

Another, perhaps more likely explanation for the developmental change in OMR polarity is that older larvae and adults have acquired, through learning and/or other developmental changes, an ability to distinguish between self-generated and exogenously imposed (current-induced) optic flow. Such an ability is thought to involve the correlation of motor-corollary discharge with sensory reafference to form sensory expectations (memories) of self-generated movements (Bell et al., 1997; Cullen, 2004).

Seen from this perspective, exogenously generated optic flow stimuli presented in the absence of matched expectations in other senses could lead to a sensory confusion of sorts. The behavioural outcome of such confusion might thus be expressed as a reversal in the polarity and/or a reduction in the magnitude of the OMR in adult fish, which have

theoretically had sufficient time to develop expectations, but not young larval fish, which have not. For example, when fish are presented with optic flow stimuli in the absence of a water current, swimming with the optic flow (positive OMR) will cause the apparent flow on the retina to cease (stabilize), signifying no motion, whereas sensory reafference from nonvisual systems will continue to signify forward motion (i.e. front-to-back water flow along the lateral line, forward acceleration as sensed by the vestibular system, etc.). In fact, swimming against the flow (negative OMR), as older larvae and adults do, may actually reduce the degree of mismatch by producing the correct expected direction of optic flow (front-to-back) to match the expected directions for nonvisual senses. Thus, the observed negative OMR in larval fish might arguably be interpreted as a compensatory movement to minimize violations of multisensory expectations. By the time fish reach adulthood, they are largely able to ignore optic flow stimuli that produce out-of-register inputs to the visual system (Figs 3, 4, 5), perhaps because the weight of matched expectations in multiple nonvisual senses overrides the single irregularity from the visual system to resolve these kinds of ambiguities (Green & Angelaki, 2010). Future studies will be necessary to assess the role of development, learning and multisensory expectations for sensory reafference in the modulation of OMR magnitude and polarity.

When considering developmental factors that may impact the OMR, it is worth noting that fish standard length is reportedly a much better measure of postembryonic development than age (Ledent, 2002; Parichy et al., 2011). Standard lengths of larvae in this study were measured post hoc from video frames and thus, subject to small depth of field errors. Nevertheless, the range of SLs for early and young larvae was within the range of those reported for 9–17 dpf larvae (Figure 2 in Parichy et al., 2011). However,

the SLs of young (3.4–4.5 mm) and older (3.5–5.7 mm) larvae overlapped, making it possible that the two groups were closer in postembryonic development than indicated by their chronological age (dpf). Arguing against this possibility is the fact that only one out of eight individuals in the group of older larvae exhibited a positive OMR, whereas no individuals in the early larval group exhibited a negative OMR. Thus, it is likely that the two groups were largely, but perhaps not perfectly, segregated according to developmental stage as well as age.

The Potential Influence of Nondevelopmental Factors on the OMR

A number of additional factors may affect the direction and/or magnitude of OMRs to optic flow. These include temperature (Veselov et al., 1998), which may serve as an environmental trigger for changes in the optomotor and rheotactic behaviour of diadromous species, and various optic flow characteristics, such as wavelength (Orger & Baier, 2005) and spatial or temporal frequency (Maaswinkel & Li, 2003). However, none of these factors offer plausible explanations of the results reported here. Temperature is an unlikely factor because both larval and adult zebrafish were maintained and tested over the same narrow range of temperatures. Stimulus characteristics are likewise unlikely for several reasons. First, visual angles were well above the reported threshold angle ($\sim 9^\circ$) for eliciting positive OMRs to translational flow in larval zebrafish (Orger et al., 2000). Second, spatial (on average, 0.01 cycles/s) and temporal (0.5–1.5 Hz) frequencies were well within the range of spatiotemporal frequency combinations known to produce positive OMRs in adult zebrafish, as measured from optomotor drum studies

(Maaswinkle & Li, 2003). Interestingly, the temporal frequency of optical stimulation (i.e. the number of dark or white stripes crossing the photoreceptors per second) is the single most important parameter determining OMR magnitude in flying insects (Got, 1975; Srinivasin et al., 1999). The strength of the OMR response is tuned to the temporal frequency such that low and high temporal frequencies produce weak OMRs, whereas intermediate temporal frequencies produce strong OMRs (Srinivasin et al., 1999). Thus, it would be very interesting to see how or if positive and negative OMRs are tuned to temporal frequencies in fish.

A third argument against stimulus characteristics as being an explanatory factor for the observed OMR phase reversal is that the stimulus characteristics were the same for both young and old larvae, between which the reversal was observed (experiment 6). Furthermore, the same stimulus was used to elicit positive OMRs from groups of young (5–8 dpf) larvae in previous studies (Orger & Baier, 2005; Orger et al., 2000). The whole-field (floor + sides) stimulus used in experiment 2 (Fig. 1b) also served to reduce potential concerns that the two-dimensional floor stimulus by itself was simply insufficient for eliciting a positive OMR. Finally, experiments 1 and 2 differed in many respects, both in terms of optic flow characteristics and other factors (e.g. tank size, shape and water depth, etc.), yet the effects of optic flow speed on adult zebrafish OMRs were nevertheless similar, indicating that a negative response to optic flow is robust across a range of experimental conditions. Similarly, differences in tank size between larval and adult studies are unlikely to account for the polarity of the OMR, as both young and old larvae (differing by only a few millimetres in length) were tested in the same small tank, yet exhibited robust differences in the polarity of their OMRs.

Conclusions

Given that adult zebrafish exhibit weak, negative OMRs to optic flow alone and that optic flow cues do not disrupt rheotaxis when they are in conflict with hydrodynamic cues, we conclude that OMRs play little if any role in rheotaxis in this species. Whether or not these results are peculiar to this species or extend to other species remains to be seen, but the absence of OMR-based rheotaxis in a highly visual and diurnally active species raises serious doubt about the validity of the optomotor hypothesis. The stark difference in the polarity of adult zebrafish OMRs to translational (this study) and rotational (previous optomotor drum studies) optic flow stimuli of similar spatial and temporal characteristics raises serious questions about how these data are interpreted with respect to rheotactic behaviours. The positive OMRs reported for optomotor drum studies may be associated with other behaviours besides rheotaxis, such as collision avoidance or schooling behaviour. Finally, because there appears to be an early and perhaps irreversible shift in the polarity of the OMR that cannot currently be explained by any developmental milestones or other environmental or stimulus factors, we raise the possibility that OMRs can be modified by learning and experience. Clearly, additional studies and the use of more biologically realistic optic flow stimuli will be necessary to shed further light on these important questions.

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Supplementary Material

Supplementary Material associated with this article can be found, in the online version, at doi:

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Table 1

Summary of optic/hydrodynamic flow manipulations and characteristics for each experiment

Experiment	Key factors	Optic flow location	Cycle width (cm)	Visual angle (°/cycle)	Spatial frequency (cycles/°)	Temporal frequency (cycles/s)	Optic/hydro flow speeds (cm/s)
1	Optic flow speed	Floor	10	78–98	0.010–0.013	1	0, 5, 10, 15/NA
2	Streamwise optic flow to create out-of- phase instability	Floor and sides of tank	10	20–136	0.007–0.05	0.1, 0.25, 0.5	0, 1, 2.5, 5 for both
3	Optic shear flow to create rotational instability	Sides of tank	10	20–136	0.007–0.05	0.5	5 for both

4	Cross-stream optic flow to create transverse instability	Floor	10	20–136	0.007–0.05	0.5	5 for both
5	Group size (1 vs 8)	Floor and sides of tank	10	20–136	0.007–0.05	0.5	5 for both
6	Life history stage (larvae vs adults)	Floor	3.8	87–124	0.008–0.016	1	3.8/NA

Table 2

Within- and between-group variables, number of experimental groups and sample size, N (number of replicates) for each experiment

Experiment	Within-group variables	Between-group variables	Number of experimental groups	Sample size (N) per group	Total number of fish tested
1	None	Flow speed (4 levels)	4	8	32
2	Optic/hydrodynamic flow conditions (3 levels)	Flow speed (4 levels)	4	6	24
3	None	Presence or absence of shear optic flow	2	8	16
4	None	Presence or absence of transverse optic flow	2	8	16
5	Optic/hydrodynamic flow conditions (3 levels)	Number of fish tested simultaneously (1 or 8)	2	6 (6 individuals or 6 groups of 8)	54
6	None	Fish age (9–11 and 17–19 dpf larvae and adults)	3	8	24

dpf: days postfertilization.

Table 3

Results of multivariate and univariate polynomial regression for the effect of optic flow speed on the rheotactic index (RI) and positional shift of fish

Regression	Order	$b \pm SE$	t_{29}	P
Multivariate	Intercept	0.0320 $\pm$ 0.2495	1.28	0.209
	1st	-0.0320 $\pm$ 0.0080	-3.99	<0.001
	2nd	0.0017 $\pm$ 0.0051	3.32	0.002
RI	Intercept	0.0320 $\pm$ 0.0249	1.28	0.209
	1st	-0.0320 $\pm$ 0.0080	1.284	<0.001
	2nd	0.0017 $\pm$ 0.0051	3.321	0.002
Position	Intercept	1.3120 $\pm$ 0.8207	1.60	0.121
	1st	-0.9966 $\pm$ 0.2635	-3.78	<0.001
	2nd	0.0532 $\pm$ 0.0168	3.16	0.004

A second-order model provided the best possible fit. Bold values indicate significance of $P < 0.05$.

Table 4

Results of a linear mixed effect model comparing the response of solitary and grouped individuals to optic flow, hydrodynamic flow, and when both were in conflict

Effect	$b \pm SE$	df	t	P
Hydrodynamic	-0.05 $\pm$ 0.06	20	-0.90	0.381
Optic	-0.45 $\pm$ 0.06	20	-7.67	<0.001
Group size	-0.06 $\pm$ 0.06	10	-1.01	0.337
Hydrodynamic*group size	-0.02 $\pm$ 0.8	20	-0.21	0.834
Optic*group size	-0.18 $\pm$ 0.08	20	-2.23	0.378

Values (e.g. b , t) are relative to conflict/individual conditions. Bold values indicate significance of $P < 0.05$.

Figure 1. Optic flow manipulations in various experiments. (a) Experiments 1 and 6: black and white stripes were translated along the bottom of the tank in either one continuous direction (experiment 1) or alternating directions (square wave optic flow stimulus, 30 s in each direction for five cycles; experiment 6). Optic flow direction is indicated by black solid arrows, whereas the virtual hydrodynamic flow is depicted by dashed blue arrows. (b) Experiments 2 and 5: a combination of optic (black solid arrows) and hydrodynamic (blue solid arrows) flow conditions were used (from left to right): (1) optic flow alone, in which horizontally oriented stripes were translated along the bottom of the tank in register with the movement of vertically oriented stripes along the sides; (2) hydrodynamic flow in the presence of stationary stripes along the bottom and sides of the tank; (3) both hydrodynamic and optic flow moving in the same direction, meaning that the optic flow direction was opposite to that expected for a downstream-displaced fish (blue dashed arrows). (c) Experiment 3: fish were presented with a hydrodynamic flow in the presence of stationary vertical stripes on the side walls of the tank (pretest control), which during the test phase of the experiment, moved in opposite directions (the optic flow shear stimulus) to simulate the visual experience of a fish that is rotated by the flow. (d) Experiment 4: the sides of the tank were solid black and optic flow under the floor of the tank moved in cross-stream (transverse) direction relative to the hydrodynamic flow.

Figure 2. Effect of flow speed on optomotor responses (OMRs) of adult zebrafish (experiment 1). Changes in the (a) positional shift and (b) rheotactic index as a function of flow speed. (c) Relationship between positional shift and rheotactic index across all flow speeds.

Figure 3. Importance of optic flow cues to rheotaxis (experiment 2). Rheotactic performance (mean $\pm$ SE) as a function of flow speed for each of three experimental conditions (see Fig. 1b): (1) optic flow alone (dashed line, squares); (2) hydrodynamic flow alone (solid line, circles); (3) optic and hydrodynamic flow cues about downstream displacement in conflict (dotted line, triangles).

Figure 4. Effects of destabilizing optic flows on rheotaxis (experiments 4 and 5). Rheotactic performance to hydrodynamic flow (5 cm/s) in the presence of (a) a destabilizing shear or (b) orthogonal optic flow stimulus (as illustrated in Fig. 1c, d). Box hinges correspond to the first and third quartiles, as well as the median. The upper and lower whiskers extend to the highest and lowest values (respectively) within 1.5 times the interquartile range (IQR). Notches correspond roughly to 95% confidence intervals ($1.5 \times \text{IQR}/\text{square root}(N)$).

Figure 5. Effects of group testing on the role of optic flow in rheotaxis (experiment 6). Rheotactic performance (mean $\pm$ SE) of solitary fish (light grey) and groups of eight fish (dark grey) under each of three experimental conditions of experiment 3 (see Fig. 1b): (1) optic and hydrodynamic flow cues in conflict (conflict); (2) hydrodynamic flow alone (hydro); (3) optic flow alone (optic). Optic and hydrodynamic flow speeds were fixed at 5 cm/s.

Figure 6. Optomotor responses (OMRs) to the square wave optic flow stimulus in fish of different ages (experiment 6). OMRs from two individual larva, (a) one young and (b) one old, and (c) one adult fish, illustrating changes in the fish's streamwise position (red functions: left-hand axis) and orientation (blue functions: right-hand axis) relative to the changing directions of the optic flow stimulus (black function). In (d), (e) and (f), the mean cycle positional shift (as a proportion of the streamwise length of the tank) and orientation relative to virtual upstream are plotted as a function of time for the same individuals as in (a–c) illustrated on the left. The young larval fish (a, d) oriented in the direction of the optic flow while shifting its position to the opposite end of the tank in a manner consistent with positive rheotaxis (i.e. swimming with the optic flow). The older larval fish (b, e) did nearly the opposite, in a manner consistent with negative rheotaxis. The OMR of the adult fish (c, f) was more like that of the older larva than the young larva, but the positional shift was slightly less negative and with the exception of the first cycle, the mean orientation was neither upstream nor downstream.

Figure 7. Developmental plasticity in optomotor responses (OMRs) of zebrafish (experiment 6). Mean proportional shift in position along the tank during the OMRs of adult fish compared to both young (9–11 days postfertilization, dpf) and old (16–18 dpf) larvae in response to the square wave optic flow stimulus (as illustrated in Fig. 2). Box hinges correspond to the first and third quartiles, as well as the median. The upper and lower whiskers extend to the highest and lowest values (respectively) within 1.5 times the interquartile range (IQR). Notches correspond roughly to 95% confidence intervals ($1.5 \times \text{IQR}/\text{square root}(N)$).

a **b**

Rheotactic Index

a **b** **c** **d** **e** **f**

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