

RESEARCH ARTICLE

Movements of *Rana catesbeiana* tadpoles in weak current flows resemble a directed random walk

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SUMMARY

Current flow is an important biological stimulus for larval anuran amphibians, but little is known about how it is perceived. We quantified behavioral responses to controlled water flow in the bullfrog tadpole (*Rana catesbeiana*) at developmental stages prior to metamorphic climax, and examined the contribution of a functioning lateral line system to these behaviors. Tadpoles at these developmental stages show a significant preference for the sides and bottom of a flow tank. In response to water flow at three different rates, they exhibit a significant, time-dependent tendency to move downstream, away from the source of the flow, and to remain in areas where flow is minimized. The consistency of these behaviors at all tested flow rates suggests that the animals are not simply passively pushed by the current; instead, they actively swim away from the current source. Tadpoles do not exhibit positive rheotaxis towards the source of the flow at any flow rate but as a group are randomly oriented. Treatment with cobalt chloride, a known blocker of superficial neuromast function, significantly reduces the tendency to move downstream, but does not alter the preference for the sides and bottom of the tank. Tadpoles' movements under flow are consistent with a model of locomotion based on a directed random walk.

Key words: flow sensing, lateral line, *Rana catesbeiana*, rheotaxis, tadpole.

INTRODUCTION

The life cycles of many species of anuran amphibians (frogs and toads) include a free-swimming, larval (tadpole) period with its own characteristic suite of behaviors (Hoff et al., 1999). Little is known about how these animals process biologically relevant sensory signals (Muntz, 1963; Shelton, 1971; Jaeger and Hailman, 1976; Stehouwer, 1988; Hoff et al., 1999; Simmons et al., 2004; Roberts et al., 2009). Because tadpoles are wholly aquatic but inhabit diverse ecological niches, examining their behavioral responses to local changes in water flow presents an excellent model for comparative studies of adaptive sensory processing over development.

The lateral line system of fish codes velocity, direction and acute accelerations of water flow relative to the body of the animal, and thus facilitates prey detection, swimming, schooling, spawning and maintenance of position in a current (Dijkgraaf, 1963; Coombs and Montgomery, 1999). In fish, orientation into or against currents, known as positive rheotaxis, is largely mediated by the superficial neuromasts of the lateral line; the canal neuromasts, in contrast, discriminate pulsatile or high velocity stimuli (Montgomery et al., 1997; Baker and Montgomery, 1999; Kanter and Coombs, 2003; Coombs and Grossman, 2006). Fish exhibit considerable species and developmental diversity in the number, location, size and shape of superficial and canal neuromasts (Van Netten and Kroese, 1987; Van Trump and McHenry, 2008). This diversity reflects adaptation to different ecological niches and contributes to the wide range of behaviors related to lateral line function (Dijkgraaf, 1963; Webb, 1989; Engelmann et al., 2000; Carton and Montgomery, 2004; Coombs and Grossman, 2006).

Larval anurans also possess a lateral line system, with considerable species differences in the number and distribution of neuromasts (Lannoo, 1987). Although tadpole neuromasts seem to resemble the superficial neuromasts of fish, there are only limited data on their detailed morphology (Jande, 1966; Lannoo, 1987; Roberts et al., 2009). Behavioral data on lateral line function in these animals are also sparse. Larval *Xenopus laevis*, at developmental stages prior to hindlimb formation, exhibit positive rheotaxis to current flow (Shelton, 1971; Simmons et al., 2004; Roberts et al., 2009). Whether similar behavioral responses to flow are also characteristic of other species of tadpoles, with their different developmental trajectories, external morphologies and ecologies (Hoff et al., 1999; McDiarmid and Altig, 1999), is unknown.

Here, we present results of three experiments quantifying responses of bullfrog tadpoles, at developmental stages prior to full differentiation of the hindlimbs, to controlled water flow. We show that larval bullfrogs at these developmental stages do not respond to water currents in the same manner as do either *Xenopus* tadpoles or many species of adult fish. Instead of consistently orienting positively towards the source of current, these tadpoles adopt positions downstream from the source, with orientations that are random with respect to the direction of flow. This downstream positioning is sensitive to pharmacological inactivation of the lateral line neuromasts. To understand these results, we asked whether these responses are the result of active locomotion or simply of passive influences of the current. The tadpoles' spatial distribution in the presence and absence of the current as well as the effect of lateral line disruption suggest that moving downstream is an active behavioral response to the flow.

MATERIALS AND METHODS

Animals

Rana catesbeiana, Shaw 1802, tadpoles were obtained from a commercial supplier (Dozier Lester, Duxon, LA, USA) or collected from a natural breeding site in Rhode Island (State of RI collection permit no. 2009-67). Animals were between developmental stages 26 and 35 (Gosner, 1960) and ranged in length from 40 to 75 mm. At these stages [encompassing the early larval period and the initial part of the late larval period as defined by McDiarmid and Altig (McDiarmid and Altig, 1999)], tadpoles are active swimmers, with long tails, absent to small hindlimb buds, no hindlimbs or toes, and no forelimb buds. They were housed in groups in polycarbonate aquaria filled with buffered, dechlorinated aerated water (pH 7–8) and were fed cooked spinach and dried fish flakes *ad libitum*. The colony room was maintained on a 12h:12h light/dark cycle at temperatures of 22–24°C. Experimental procedures were approved by the Brown University Institutional Animal Care and Use Committee and are consistent with federal guidelines.

Procedures

Animals were tested in a custom-built recirculating freshwater flow tank (overall dimensions 105×15×20 cm, testing area

68×15×16 cm; Fig. 1A), which was used previously in a study of rheotaxis in *Xenopus* tadpoles (Simmons et al., 2004). The basic design of the tank is similar to that described elsewhere (Vogel and LaBarbera, 1978; Kanter and Coombs, 2003). For testing, the tank was filled to a depth of 16 cm with dechlorinated, buffered water (pH 7–8; temperature 21–25°C). The bottom of the tank was covered by a white grid (2.54 cm squares) to facilitate visual estimation of the tadpole's position during the experiment. Plain white paper with position calibration markings covered the far side wall of the tank. Illumination was provided by red lights (655 nm) mounted separately above and to the sides of the tank. Rana (*R. temporaria*, *R. pipiens*, *R. clamitans*) tadpoles at similar developmental stages to the *R. catesbeiana* tadpoles tested here exhibit maximal sensitivity to green light (~550 nm) and minimal phototactic response to frequencies above 600 nm (Muntz, 1963; Jaeger and Hailman, 1976). Assuming that bullfrog tadpoles have a visual sensitivity similar to that of these other rana species, they are unlikely under these illumination conditions to resolve features of the tank. The entire tank, lighting and camera system was enclosed by opaque vinyl in order to reduce any shadows or extraneous visual cues. Thus, visual cues should not be available to affect positioning behaviors.

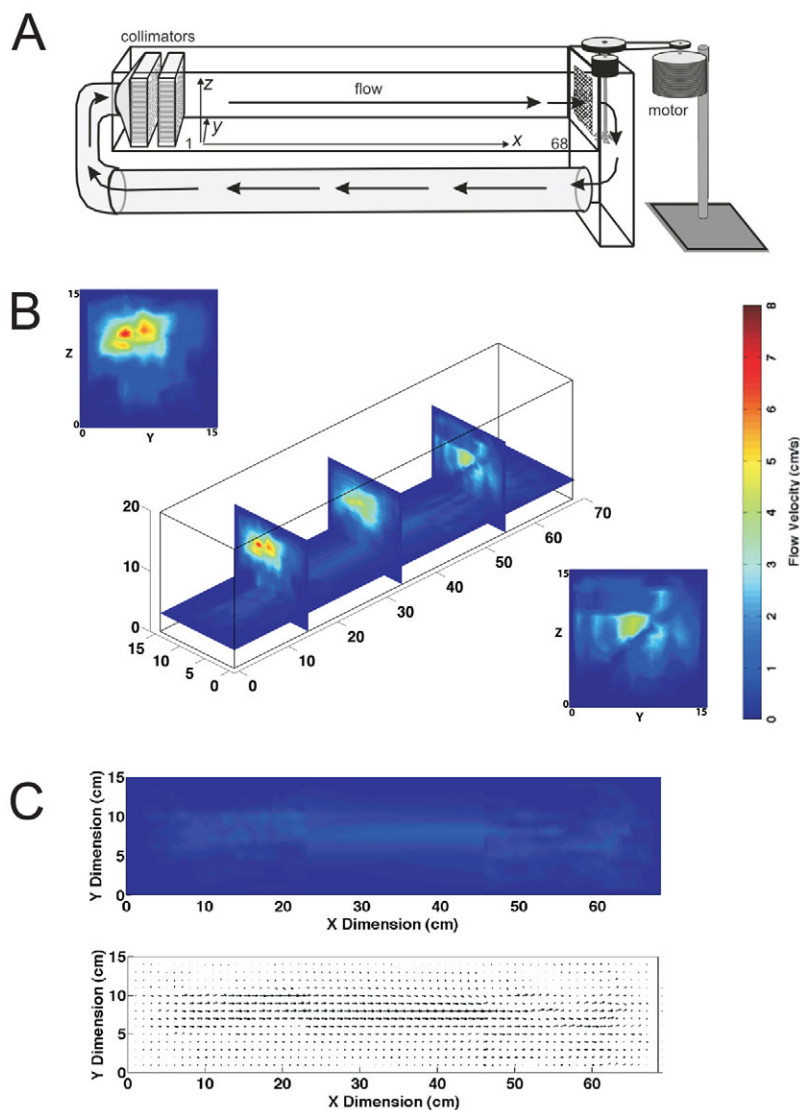


Fig. 1. Flow tank design and digital particle image velocimetry (dPIV) flow measurements. (A) Schematic diagram of the flow tank. A motorized impeller mounted on a separate stand near the downstream end of the tank produced the water flow, which traveled (arrows) through a polyvinylchloride tube, a funnel, two collimators and a mesh screen at the upstream end. The water flowed out of the tank at the downstream end through another mesh screen and then back into the polyvinylchloride tube for recirculating. The areas available for tadpole movements are 68 cm in the X-dimension, 15 cm in the Y-dimension and 16 cm in the Z-dimension. The entire tank system was supported by a metal stand covered with neoprene cushioning material and placed over a granite counter and sink. (B) The X-component of flow velocity (measured at a flow rate 8 cm s^{-1}) at three cross-sections (from left to right: $X=15 \text{ cm}$, $X=35 \text{ cm}$ and $X=55 \text{ cm}$) illustrated in a rotated three-dimensional (3D) view of the tank. The vertical bar on the far right shows the color code. In each cross-section, the flow is maximal in a central area between approximately $Z=4\text{--}12 \text{ cm}$ and is considerably reduced near the bottom and along the sides. The left ($X=15 \text{ cm}$) and right ($X=55 \text{ cm}$) cross-sections are expanded to show the flow pattern more clearly. (C) Top: flow velocity in the X- and Y-dimensions at $Z=3 \text{ cm}$, near the bottom of the tank. Bottom: a flow field showing the direction and magnitude of the flow in this horizontal section. The flow rate was dramatically reduced near the bottom of the tank, especially along the sides and in the corners.

Experiments were recorded by four synchronized digital cameras (BW DSP CCD and Philips LTC 0500/20; Supercircuits, Austin, TX, USA); two mounted on a separate housing 42 cm directly above the tank and two mounted on a single beam 35 cm apart and 112 cm perpendicular to the near side wall of the tank. The four video feeds were multiplexed and recorded onto tape with a Sony Digital Video Walkman (Sony Corp., New York, NY, USA) at 30 frames s^{-1} . The four cameras were calibrated for three-dimensional (3D) reconstruction by digitizing the positions of 24 calibration points for a direct linear transform (DLT) algorithm (Hatze, 1988). DLT was implemented in a custom-designed MATLAB (MathWorks, Natick, MA, USA) routine, which included code from the DLTdv3 toolkit (Hedrick, 2008).

The noise level generated by the impeller motor producing the flow (Fig. 1A) was measured by a hydrophone (Fishphone; Bioacoustics, Woods Hole, MA, USA; sensitivity $-148 \pm 0.7 \text{ dBV Pa}^{-1}$ in the frequency range 50–2000 Hz) placed at the downstream end. Acoustic pressure measurements were taken both with the motor off and with the motor on and producing current at rates from 4 to 10 cm s^{-1} . The hydrophone output was recorded (Zoom H4, Japan; www.zoom.co.jp), digitized, and analyzed using Adobe Audition 3.0 (Adobe, San Jose, CA, USA). The mean noise level at all flow rates was $-61.40 \text{ dB re } 0.1 \text{ Pa}$, approximately 20 dB below the tadpoles' neural thresholds in this same frequency range (Boatright-Horowitz and Simmons, 1997). We can thus rule out any influence of extraneous auditory cues on the tadpoles' behavior.

Flow analysis

Water flow was measured using digital particle image velocimetry (dPIV) (Drucker and Lauder, 1999). The movements of silver-coated glass spheres (mean diameter $12 \mu\text{m}$, density 1.3 g cm^{-3}) suspended in the water were illuminated by a pair of 80 mW line lasers mounted collinearly so that they illuminated a single plane. Flow data were captured using a high-speed digital video camera (Casio EX-FH100, Tokyo, Japan) recording at $120 \text{ frames s}^{-1}$ at a mean spatial resolution of $26 \text{ pixels cm}^{-1}$. The line lasers were placed on a sliding mount above the tank so that the illuminated plane was parallel with the X -axis and could be moved along the Y -axis. To capture flow measurements throughout the water column, video was recorded with the illuminated plane located at a stack of Y -positions separated by 1 cm. This procedure was repeated at two camera positions to capture velocimetry data throughout the tank.

Videos were analyzed using a custom-written MATLAB routine that cross-correlated regions in the videos between successive frames using code from the openPIV software package (Taylor et al., 2010). For each position of the illuminated plane, a flow field was generated by analyzing data from 150 frames of video. 3D data were obtained by combining velocimetry measurements from the stack of videos. The flow was measured in sequential cross-sections through the tank and the flow data were interpolated in order to produce evenly spaced measurements that faithfully reproduce the measured data. These measurements (Fig. 1B, flow speed of 8 cm s^{-1}) demonstrate that the flow stimulus consisted of a narrow fast-moving stream surrounded by a decreasing gradient of flow. The flow rate was maximal in a central area in the water column and reduced along the bottom (Fig. 1C), corners and sides of the tank. The pattern of flow velocity was similar at all three flow rates tested.

Experimental procedures

Three experiments were conducted to quantify behavioral responses to water flow. Testing was conducted at the same time each day to avoid any circadian effects. The water in the tank was completely

drained and changed after each individual animal was tested in order to eliminate any lingering chemical cues. After each trial, animals were measured (length from snout to tip of tail) and staged. Animals in separate experiments were of similar developmental stages, and those animals that were tested twice did not change developmental stage between trials.

Experiment 1 – responses to steady flow

We quantified responses to existing water flow in one group of tadpoles ($N=18$; stages 26–35) under two different test conditions – first untreated and then treated with cobalt chloride to inactivate the lateral line neuromasts (Karlsen and Sand, 1987). In the first condition, untreated tadpoles were individually released from a small, water-filled glass beaker placed at the water surface in the center of the tank ($X=14 \text{ cm}$, $Y=3.5 \text{ cm}$, $Z=6 \text{ cm}$). The beaker was tilted so that animals swam out into the tank of their own accord. At the time of release, the water in the tank was already moving at a measured rate of 10 cm s^{-1} . The animal's behavior in this flow was videotaped for 5 min. Forty-eight hours after this initial testing, each tadpole was immersed in a 2 mmol l^{-1} solution of buffered, calcium-free cobalt chloride (pH 7.2; Sigma, St Louis, MO, USA) for 1 h and then immediately released back into the flow tank, again with the current already moving at the time of release. Ten days later, six cobalt-treated animals were re-tested to verify that the cobalt treatment did not permanently affect positioning or swimming behaviors (Janssen, 2000). In a control experiment, a separate group of tadpoles ($N=20$) was tested once under no flow (flow rate of 0 cm s^{-1}); these animals were not treated with cobalt chloride.

Experiment 2 – responses to changes in flow at three flow rates

In experiment 2, tadpoles (stages 26–29) were allowed to swim into the tank from the release point when the flow rate was set to 0 cm s^{-1} (5 min trial; no flow condition); then, at the end of this 5 min period, flow was adjusted to 6, 8 or 10 cm s^{-1} and the animals' behaviors monitored for an additional 5 min (flow condition). Fourteen tadpoles were tested at 6 cm s^{-1} , 40 tadpoles were tested at 8 cm s^{-1} and 39 tadpoles were tested at 10 cm s^{-1} .

Experiment 3 – responses to changes in flow after cobalt treatment

Of the 39 animals that were tested at 10 cm s^{-1} in experiment 2, 35 were re-tested 48 h later, after immersion in either amphibian Ringer's solution (1 h; $N=15$) or 2 mmol l^{-1} cobalt chloride (1 h; $N=20$). The remaining four tadpoles were tested for 10 min without any water flow but with impeller noise present.

Data analysis

Videotapes were digitized and edited using Pinnacle Studio 9 video editing software (Avid Technology, Mountain View, CA, USA). Two analysis methods were employed. For manual analysis, still frames were generated from the digitized videotapes at 30 s time intervals from the beginning to the end of each 5 or 10 min trial. The position of the tadpole in X -, Y - and Z -coordinates was measured from these images with reference to the grid and calibration markings in the tank. The orientation of the animal's head in circular coordinates with respect to the current source was recorded from overhead bitmap images to 10 deg accuracy using a protractor. Images were analyzed by two of the authors and a third rater unaware of the experimental hypothesis or animal treatment. Reliability between raters was statistically assessed using SPSS v14 (SPSS Inc., Chicago, IL, USA), and found to be excellent ($r > 0.88$

in all comparisons). Data from one rater were used for further statistical analyses.

Computer-assisted manual analysis was performed on a randomly selected subset of data using a custom-written MATLAB interface that required raters to click on the position of the tadpole in each camera view at 30 s intervals. The program automatically calculated the 3D position of the tadpole at each time point using DLT. The animal's orientation at each time point was calculated by asking the user to identify its tail and head. This analysis was performed by one of the authors and by a second rater unaware of the hypothesis or animal treatment. Agreement between the two raters was excellent ($r > 0.98$). In addition, agreement with data obtained from the fully manual analysis was also excellent ($r > 0.90$).

A randomly selected set of recordings from experiment 2 was analyzed frame-by-frame using a custom-written automatic tracking program in MATLAB. Using DLT to reconstruct 3D positions from the four camera views, the tadpole's position was recorded at a rate of 30 frames s^{-1} over the duration of each 10 min trial. Using a custom-written routine, position data were low-pass filtered and numerically differentiated to calculate the tadpoles' velocity over the course of a trial.

For all analysis and discussion, $X=1$ cm designates the most upstream position (at the front of the tank near the collimators) and $X=68$ cm designates the most downstream position in the tank (at the back near the pump); by 'upstream', we refer to X -positions of 1–34 cm and by 'downstream', we refer to X -positions of 35–68 cm (Fig. 1A). $Y=1$ cm designates the near side wall of the tank closest to the cameras and $Y=15$ cm designates the far side wall of the tank. $Z=1$ cm denotes the bottom of the tank and $Z=16$ cm indicates the surface of the water. An animal directly facing the flow source is considered to be oriented at a 0 deg (or 360 deg) angle; an animal facing directly opposite the flow source is considered to be oriented at 180 deg; an animal facing the back side wall is oriented at 270 deg; and an animal facing the front side wall is oriented at 90 deg.

Statistical analyses

We observed that bullfrog tadpoles, unlike *Xenopus* tadpoles (Simmons et al., 2004), typically did not station hold in response to flow, but instead exhibited variable and frequent movements over the course of the trial, even when flow was on. We thus examined tadpoles' positioning behavior as a time series so as to quantify these changes in response. Positions in the X -, Y - and Z -dimensions at each 30 s time point were separately analyzed by repeated measures analysis of variance (ANOVA), with time as the repeated measure. Proportions of animals upstream or downstream (X -dimension), against the tank walls (Y -dimension) or on the tank floor (Z -dimension) were also analyzed with binomial tests. All statistical tests were performed using SPSS, with a criterion significance level of $P < 0.05$ or greater.

The tadpole's orientation with respect to the source of the flow was analyzed by circular statistics, using a commercial program (Oriana 3.0, Kovach Computing, Anglesey, UK). The mean circular orientation for each animal is the angle formed by the resultant vector of a set of unit vectors with orientations corresponding to the measured (observed) orientation, and not the linear mean of the measured angles (Batschelet, 1981). The statistical significance of the mean orientation vectors was analyzed using a modified Rayleigh test (Greenwood and Durand, 1955) that examined the hypothesis that tadpoles exhibited positive rheotaxis (orientation towards 0 or 360 deg) or negative rheotaxis (orientation towards 180 deg). A significance level of $P < 0.01$ or greater was adopted for the Rayleigh tests.

Visualization of neuromasts

After completion of behavioral testing, some animals ($N=18$) were killed by immersion in buffered 0.15% tricaine methanesulfonate (MS-222, pH 7.0; Sigma). One sacculle from each animal and a sample of skin from the supraorbital line of neuromasts were microdissected, flushed with 0.9% saline and cold shocked at -20°C for 20 min. Care was taken to dissect equivalent amounts of skin from the same region of the supraorbital line in all animals. Samples were incubated at room temperature for 20 min in 175 μl 0.9% saline and 20 μl defined equine serum (HyClone, Logan, UT, USA) to prevent non-specific binding, and then incubated for 30 min at room temperature in 5 μl of Alexa Fluor 594 phalloidin (A-12381; Invitrogen, Eugene, OR, USA) to visualize actin in the hair cells. Samples were whole-mounted using AquaMount (Polysciences, Warrington, PA, USA). Actin fluorescence was visualized using an Olympus BX-60 microscope (Olympus, Melville, NY, USA) equipped with an Olympus DP72 digital camera and DP2-BSW software, or a Leica TCS SP2 MP confocal microscope (Leica Microsystems, Bannockburn, IL, USA) and associated Leica software. Images were stored as uncompressed 24 bit RGB Tiff files and cross-adjusted to match gain. Image analyses were carried out on coded slides using ImageJ v1.43n (NIH, Bethesda, MD, USA) by an independent observer blind to the experimental hypothesis. Each image was split into red/green/blue channels, and red channels were thresholded using a dark background. Parameters were held constant for each image. Particle filters were constructed to regard entire neuromasts as a single particle and were calibrated against control skin sections. The number of red pixels was normalized within each image to give a relative measure of phalloidin label intensity in skin areas containing identifiable neuromasts. Because of errors in tissue preparation, only 16 skin samples were available for statistical analyses. Statistical differences in the intensity of phalloidin label between untreated and treated skin samples were assessed by unpaired two-tailed t -tests (SPSS).

RESULTS

Experiment 1 – steady flow

Animals that were released into the center of the tank with the flow already moving at 10 cm s^{-1} adopted positions in the downstream end of the tank. Control animals, tested under no flow, were more randomly positioned. After exposure to cobalt chloride, downstream movement in flow was significantly altered. Positive rheotaxis was not observed under any condition. There were no differences in behavior related to developmental stage or body size.

XYZ-position in the tank

Differences in X -position between untreated animals tested in the presence of flow and control (untreated) animals tested under no flow conditions were statistically significant ($F_{1,36}=20$, $P < 0.001$). Animals tested in the presence of flow moved downstream (towards $X=68$ cm) over the course of the trial, while animals tested in the absence of flow remained distributed around the release point (Fig. 2A).

We then compared X -position during flow between untreated animals and animals treated with cobalt chloride. Two-way repeated measures ANOVA using time and treatment as factors showed a significant main effect of time ($F_{9,153}=2.1$, $P < 0.05$) and a marginally significant treatment by time interaction ($P=0.07$), but no significant main effect of treatment. When exposed to flow, both untreated and treated animals moved downstream, away from the flow source (Fig. 2A). Because the variable of time showed a significant quadratic trend ($F_{1,17}=4.97$, $P < 0.05$), data were collapsed across the

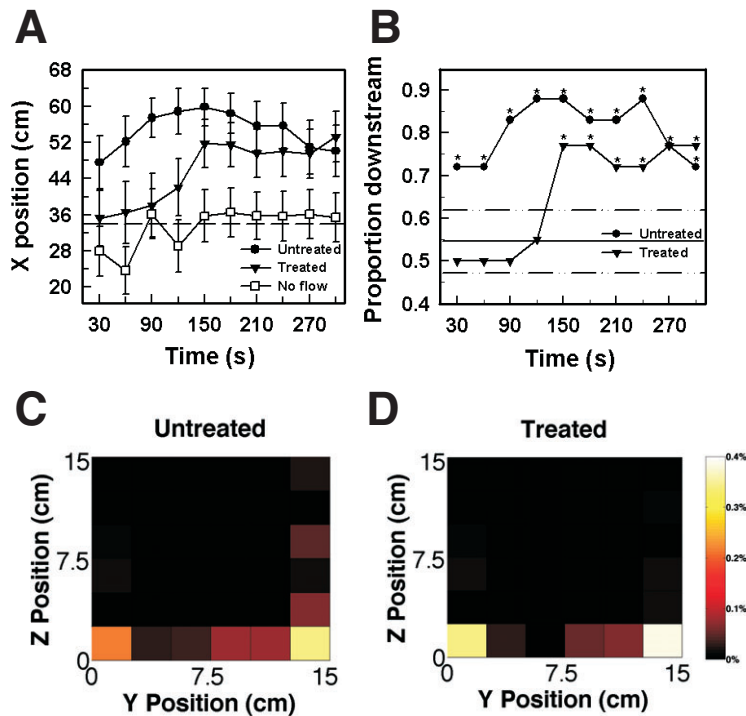


Fig. 2. Tadpoles respond to 10 cm s^{-1} flow by moving downstream. (A) X-position, plotted as mean and standard error, at each 30 s time point (from 30 to 300 s) in the 5 min trial for the three groups of tadpoles. The horizontal dashed line shows the release point (at $X=34 \text{ cm}$). Control (untreated) animals were tested under conditions of no flow. (B) Proportion of tadpoles downstream at each 30 s time point. The solid horizontal line shows the mean downstream proportion of the control animals averaged across all time points; the dashed horizontal lines show the 95% confidence intervals of this mean. Untreated animals are significantly more likely ($*P<0.05$) than control animals to be downstream in response to flow at all time points. Animals treated with cobalt chloride do not differ significantly in their downstream position from control animals until the 150 s time point. (C,D) Two-dimensional (2D) occupancy histograms displaying the tadpoles' position in the Y- and Z-dimensions under flow. YZ-position data from all animals at each 30 s time bin were sorted into $3 \times 3 \text{ cm}$ bins and the proportion of time spent in each bin was calculated. (C) YZ-occupancy of untreated tadpoles under flow. (D) YZ-occupancy of cobalt-treated tadpoles under flow. Both untreated and treated animals were predominantly located along the sides and the bottom of the tank. Colors are referenced to the vertical bar on the far right.

10 different time points into two summary times (the first half of the flow period from 30 to 150 s and the second half of the flow period from 180 to 300 s). The results of ANOVA on these categorized data showed a significant treatment by time interaction ($F_{1,17}=4.36$, $P=0.05$), suggesting that cobalt treatment affected X-position only during the first half of the flow period. Mean X-position of the untreated animals was 55.17 cm in the first half of the flow period and 54.15 cm in the second half. In contrast, mean X-position of the treated animals was 40.74 cm in the first half of the flow period and 50.77 cm in the second half. Thus, cobalt-treated animals tended on average to remain near the release point during the first part of the trial before eventually moving downstream, while untreated animals on average adopted more downstream positions even at the first time point tested (30 s).

Because individual animals showed variability in their downstream movements, data were converted into a binomial distribution: an animal located in the upstream half of the tank ($X=1\text{--}34 \text{ cm}$) was given a score of 0 while an animal in the downstream half of the tank ($X=35\text{--}68 \text{ cm}$) was given a score of 1. One-way binomial tests were calculated (separately at each time point) to compare the observed proportion of animals located downstream with the null hypothesis of equal proportions of animals located upstream and downstream. The results showed that untreated animals in the control (no flow) group were randomly distributed throughout the tank ($P>0.2$ at all 10 time points). The mean X-position (with 95% confidence intervals) was calculated for these control animals and compared with the movements of animals tested in flow (Fig. 2B). Untreated tadpoles tested in flow were significantly more likely to be located downstream ($P<0.05$ at all time points). Animals that were not located downstream in flow tended to be upstream in one of the two front corners of the tank closest to the collimators (at $X=1 \text{ cm}$, $Y=1 \text{ cm}$ or 15 , $Z=1 \text{ cm}$). This is an area of reduced current flow (Fig. 1C). Tadpoles treated with cobalt chloride were randomly distributed during the first 120 s of the 5 min flow period (Fig. 2B), but were significantly likely to adopt downstream positions at time points from 150 to 300 s ($P<0.05$ at these times).

Tadpoles' positioning in the Y- and Z-dimensions, unlike that in the X-dimension, did not exhibit significant effects of time in flow. To examine positioning in these coordinates, we created two-dimensional (2D) occupancy histograms showing the proportion of time the animals spent at each location in the YZ-plane. YZ-occupancy histograms demonstrate that both untreated (Fig. 2C) and cobalt-treated (Fig. 2D) animals spent the vast majority of the time positioned on the bottom and along the side walls of the tank. For statistical analysis, Y-position data were placed into two groups: along a side wall ($Y=1\text{--}2$ or $14\text{--}15 \text{ cm}$) or in the center of the tank ($Y=3\text{--}13 \text{ cm}$). One-sample binomial tests showed a significant preference for a side wall at 9 of the 10 time points ($P<0.01$) for untreated animals, and at 10 of the 10 time points for treated animals ($P<0.01$). Binomial tests analyzing differences in the proportion of tadpoles at position $Y=1\text{--}2 \text{ cm}$ compared with $Y=14\text{--}15 \text{ cm}$ showed that neither untreated nor treated tadpoles had a significant preference for either the far or near side wall. Z-position data were also transformed into two groups: the bottom of the tank ($Z=1\text{--}2 \text{ cm}$) or in the water column ($Z=3\text{--}16 \text{ cm}$). All 10 time points in both untreated and treated conditions returned a significant preference for the bottom of the tank ($P<0.001$ for all tests). The slight differences in Z-position between untreated and treated tadpoles in Fig. 2C,D are not statistically significant.

Orientation vectors

The mean orientation vectors for each animal across the entire 5 min test were analyzed with a modified Rayleigh test to examine the hypothesis that the animal was oriented towards 0 deg (360 deg). None of the control, untreated or cobalt-treated animals showed significant positive orientation in either no flow or flow. Tests for negative rheotaxis (orientation towards 180 deg) did not return any statistically significant results in control, untreated or treated conditions.

Experiment 2 – changing flow

Three groups of tadpoles were tested. All were initially observed under no flow conditions for 5 min; while they remained in the tank,

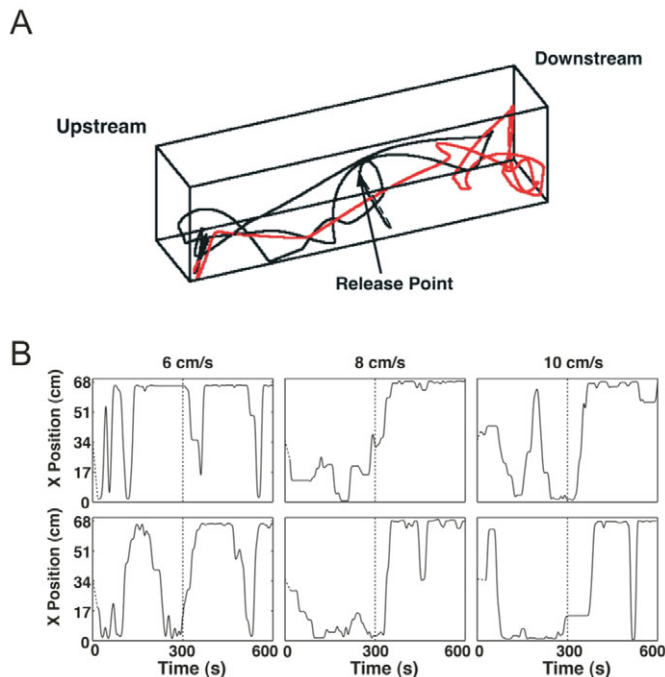


Fig. 3. Tadpoles respond to 6, 8 and 10 cm s^{-1} flow by actively moving downstream. (A) Movements of one animal from the 10 cm s^{-1} condition, reconstructed in 3D over the course of the 10 min trial using automatic frame-by-frame tracking and direct linear transform (DLT) analysis. The release point is indicated by the black arrow. This tadpole moved over the length of the tank during the no flow period (black line) but was predominately downstream in the flow period (red line), although it continued to move actively and even swam upstream. (B) Changes in X -position over time in no flow and flow of two representative tadpoles at each of the flow rates (6, 8 and 10 cm s^{-1}). The dashed vertical line in each graph divides periods of no flow (1–300 s) and flow (330–600 s). The behavior of the animals was variable even under flow, but they tended to move downstream at all three flow rates. Upstream movements and changes in position under flow suggest that the tadpoles were not simply passively pushed by the current.

flow was then set to one of three measured rates (6, 8 or 10 cm s^{-1}) and the animals were observed for an additional 5 min. Behavior was similar for all three groups – tadpoles tended to move downstream in response to flow, and they did not consistently show positive orientation towards the source of the flow. Under both no flow and flow conditions, tadpoles tended to position themselves near the walls and on the bottom on the tank, in areas of reduced current flow. Body size and developmental stage were not significantly correlated with any behavioral measure.

XYZ-coordinates

A 3D depiction of the behavior of a representative animal in the 10 cm s^{-1} group, as identified by the automatic tracking program, is shown in Fig. 3A. This animal moved considerably throughout the tank during the no flow period (black line), but exhibited a clear preference for the downstream end during flow (red line). Even under flow, this animal changed its position, even swimming upstream at some points. Fig. 3B shows the variability of X -position during no flow and flow periods for two representative animals at each of the three flow rates. These graphs demonstrate that tadpoles moved downstream in the presence of flow, but did not station hold. Some animals swam upstream, against the current flow, even at the strongest flow rate tested.

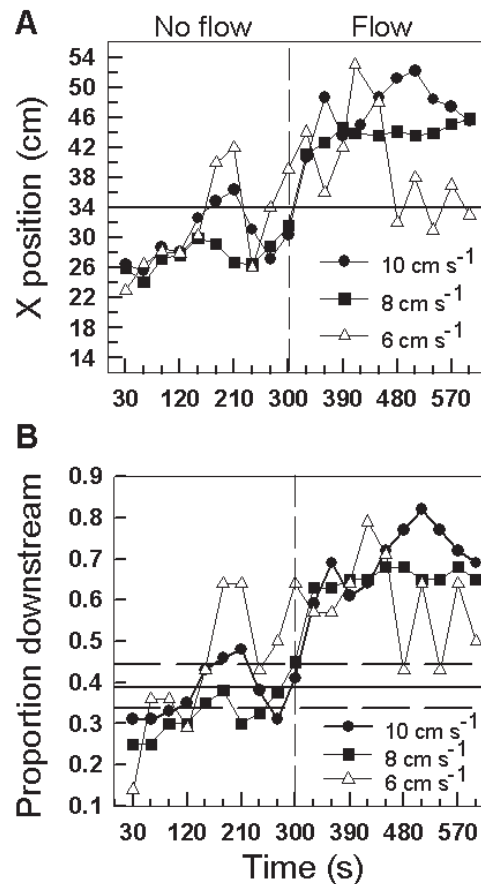


Fig. 4. Quantification of data from experiment 2. (A) Mean X -position during both no flow and flow. Standard error bars are omitted for clarity. The dashed vertical line shows the onset of flow (directly after the 300 s time point). The horizontal solid line shows the release point. In response to flow at all three rates tested, tadpoles are more likely to be downstream than upstream or randomly distributed. (B) Proportion of tadpoles in the downstream portion of the tank at all three flow rates. The solid horizontal line is the mean proportion of four control animals (tested in no flow) downstream at all time points. Dashed lines are the 95% confidence intervals of this mean value. A higher proportion of tadpoles is downstream rather than upstream during flow.

At all three flow rates tested, X -position was significantly influenced by flow (6 cm s^{-1} : $F_{1,13}=5.27$, $P<0.05$; 8 cm s^{-1} : $F_{1,39}=19.83$, $P<0.001$; 10 cm s^{-1} : $F_{1,38}=21.29$, $P<0.001$). During no flow (30–300 s), tadpoles were distributed around the release point or were slightly upstream; after flow was initiated (at 300 s), they moved downstream (Fig. 4A). The main effect of time was significant at 10 cm s^{-1} ($F_{9,342}=2.42$, $P=0.01$), only marginally significant at 8 cm s^{-1} and not significant at 6 cm s^{-1} . The absolute X -positions of tadpoles tested at 8 and 10 cm s^{-1} did not differ significantly from each other at any time, and X -position under flow at 6 cm s^{-1} did not differ from that at 8 and 10 cm s^{-1} at flow times of 30–150 s. In the 6 cm s^{-1} condition, tadpoles tended to move back towards the release point around 180 s after flow onset, consistent with the individual data shown in Fig. 3B. Mean X -position was not at the back wall ($X=68\text{ cm}$) of the tank at any flow rate, because some tadpoles swam upstream. Analyses of individual tracks indicated that these animals were positioned in one of the two front corners of the tank, in areas of reduced flow (Fig. 1B).

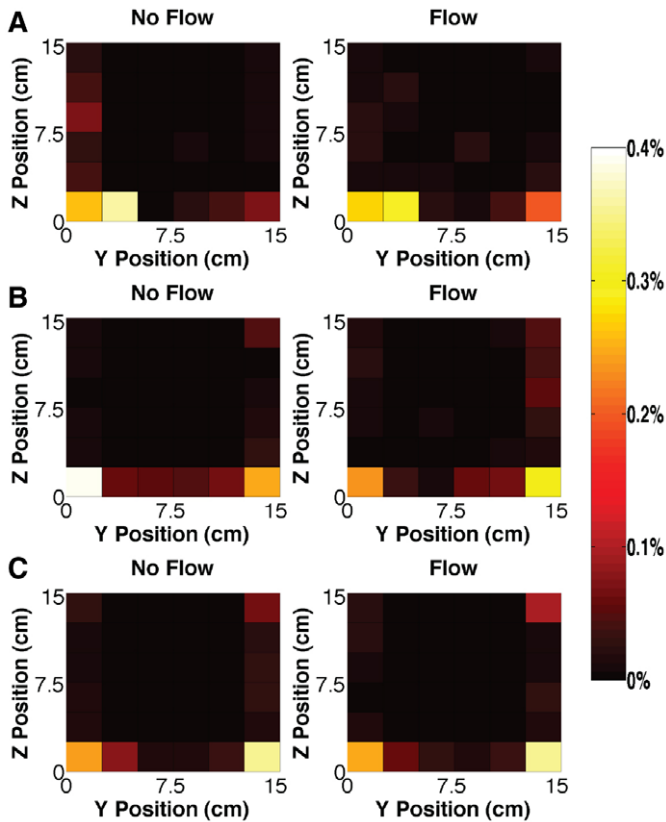


Fig. 5. Tadpoles prefer the bottom and sides of the tank in both no flow and flow conditions. YZ-position data from all animals at each 30 s time point were sorted into 3×3 cm bins and the proportion of time spent in each bin was calculated for both the no flow period and the flow period. (A) YZ-occupancy in the 6 cm s^{-1} condition. (B) YZ-occupancy in the 8 cm s^{-1} condition. (C) YZ-occupancy in the 10 cm s^{-1} condition. At all three flow rates, the tadpoles were predominantly positioned along the sides and the bottom of the tank, and were most likely to be in the corners. Comparing these data with Fig. 1 indicates that the animals were rarely in the fast moving flow stream.

The results of binomial tests showed significant differences in the proportion of tadpoles positioned downstream between no flow and flow conditions (Fig. 4B). In no flow conditions, the 6 cm s^{-1} group showed a significant preference ($P < 0.05$) for the upstream portion of the tank at two of the 10 time points. In 6 cm s^{-1} flow, these animals preferred the downstream portion of the tank at two of the 10 time points. The 8 cm s^{-1} group showed a significant preference for the upstream portion of the tank at seven of the 10 no flow time points. In contrast, during flow, these animals showed a significant preference for the downstream portion of the tank at eight of the 10 time points. The group of animals tested at 10 cm s^{-1} showed a significant preference for the upstream portion of the tank at three of the 10 time points during the no flow period. During flow, these tadpoles showed a significant preference for the downstream portion of the tank at nine of 10 time points. Statistical results suggest that tadpoles responded more strongly to flow at 8 and 10 cm s^{-1} than at 6 cm s^{-1} , although trends were similar at all flow rates. The smaller sample size at 6 cm s^{-1} made achieving statistically significant results more difficult.

YZ-occupancy histograms (Fig. 5) show that, at all flow rates, tadpoles were predominantly distributed along the side walls, corners and bottom of the tank in both the presence and absence of

flow. The results of binomial tests show that these preferences for the sides and bottom were statistically significant (no flow: $P < 0.05$ at all time points for each dimension for each group; flow: $P < 0.01$ for the Y-dimension at all flow rates; $P < 0.001$ for the Z-dimension for all flow rates, again at all time points). These preferences for the side walls of the tank likely overrode full downstream movements, such that an animal positioned along a wall would be in an area of reduced flow, even if upstream (Fig. 1B). A position bias in the Y-dimension emerged in response to the 10 cm s^{-1} flow: tadpoles significantly preferred the far side wall in 10 out of 10 comparisons.

Orientation vectors

Consistent orientation either towards or away from the source of the flow did not occur at any flow rate (Fig. 6). For tadpoles tested at a flow rate of 6 cm s^{-1} , the largest vector (representing approximately 11% of the data points) was oriented between 0 and 30 deg in both no flow and flow. The similarity in the vector distribution under no flow and flow suggests that flow had little effect on orientation. For tadpoles tested at a flow rate of 8 cm s^{-1} , the largest vector was less than 10% in both no flow and flow, with a slight bias towards 330 and 30 deg, respectively. The distribution of orientation vectors is even more random in the 10 cm s^{-1} group.

Experiment 3 – effects of cobalt treatment during changing flow

In experiment 3, 35 of the 39 tadpoles tested at 10 cm s^{-1} in experiment 2 were re-tested 48 h later after immersion in either Ringer's solution or cobalt chloride for 1 h. Downstream movements in response to flow were significantly reduced in cobalt-treated animals, but cobalt treatment did not have a significant effect on swimming speed. The four remaining tadpoles were tested for 10 min in the tank with the motor disconnected from the pump, so that impeller noise was present but there was no water flow. These animals were randomly distributed in the tank.

XYZ-coordinates

The results of repeated measures ANOVA on X-position showed a significant main effect of flow ($F_{1,33}=18.51$, $P < 0.001$), a significant flow by treatment interaction ($F_{1,33}=16.01$, $P < 0.001$), and a significant flow by time by treatment interaction ($F_{9,297}=2.1$, $P < 0.05$). All tadpoles were more likely to adopt positions downstream in the presence of flow and were more randomly distributed during no flow (Fig. 7). Compared with untreated tadpoles, tadpoles exposed to cobalt chloride showed a reduced preference for the downstream end of the tank during flow. The results of binomial tests showed that untreated tadpoles were more likely to adopt positions upstream rather than downstream during no flow (5 of 10 comparisons significant at $P < 0.05$). In flow, these animals exhibited a preference for the downstream portion of the tank (binomial tests, $P < 0.05$ for 8 of 10 comparisons). This trend was suppressed after treatment with cobalt chloride (none of the 10 time points was significant).

There was no significant time-dependent effect of positioning in either the Y- or Z-dimensions. In both no flow and flow conditions, and under untreated and treated conditions, tadpoles were significantly more likely to be at the sides rather than the wall of the tank (Y-coordinate, $P < 0.01$ at all time points) and at the bottom of the tank rather than in the water column (Z-coordinate, $P < 0.01$ at all time points). There was no bias for either the far or near side wall for either untreated or cobalt-treated animals. Tadpoles tested in the absence of flow but with impeller noise present were

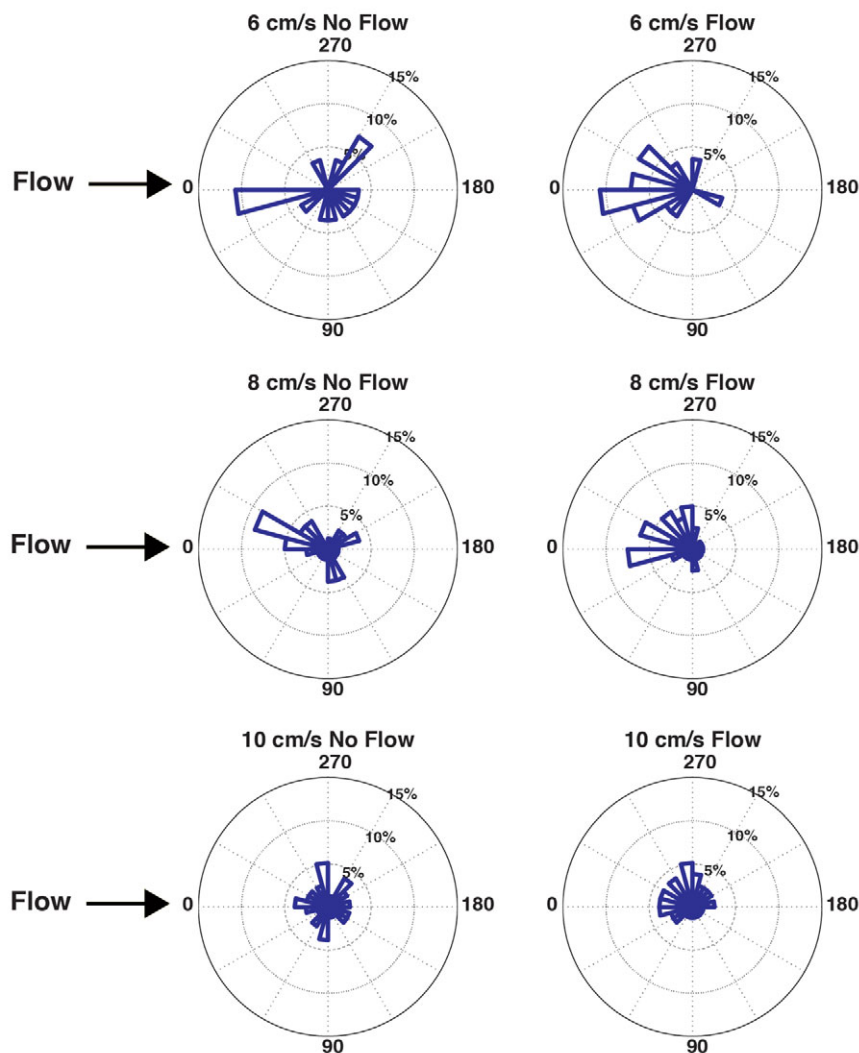


Fig. 6. Tadpoles do not exhibit positive rheotaxis at any flow rate. Orientation vectors from all data points (each tadpole at each 30 s time point) are displayed on circular coordinates and are normalized at each flow rate by the total number of data points. The vertical dotted lines in each circular display are at 30 deg intervals, and the circular dotted lines are at 5% intervals. Bin width is 10 deg. The direction of flow is indicated by the arrow. Orientations are mostly randomly distributed, under both no flow and flow.

randomly distributed throughout the tank, and showed no significant upstream or downstream preference.

Orientation vectors

Neither untreated nor treated animals displayed positive orientation towards the source of the flow. In addition, none of the tadpoles exhibited negative rheotaxis. Instead, as a group, both untreated and treated animals were randomly oriented both in the absence and in the presence of flow.

Swimming speed in no flow conditions

In some species of fish, interference with the lateral line through exposure to cobalt chloride increases swimming velocity (Hassan et al., 1992). To test whether cobalt chloride treatment had a similar influence on bullfrog tadpoles, we measured swimming velocity in the absence of flow, using the automatic tracking routine described above, for a random selection of five animals before and after immersion in Ringer's solution and for eight animals before and after immersion in cobalt chloride (animals were matched for developmental stage and size). As shown in Fig. 3, over the course of a trial, tadpoles typically exhibited periods of swimming behavior when they moved about the tank and periods of no swimming behavior when they remained in one position. We calculated the swimming speed when animals were moving faster than a threshold

of 1 cm s^{-1} and measured the proportion of time the animals spent in this swimming condition (Table 1). There were no significant differences in swimming velocity or the amount of time spent swimming between pre- and post-immersion conditions for either the untreated (Ringer's solution) group or the cobalt-treated group. The untreated group showed a higher mean swimming velocity pre-immersion than the pre-immersion cobalt animals, but also larger variability, so these comparisons were not significantly different. These data suggest that cobalt treatment had no measurable effect on the swimming velocity or time spent swimming in no flow conditions.

Immunohistochemical verification of hair cell damage

Examples of phalloidin-stained neuromasts from the supraorbital lines on the head of untreated and cobalt chloride-treated tadpoles are shown in Fig. 8. Tissue from untreated tadpoles showed clear, intense label of neuromasts, while tissue from animals exposed to cobalt chloride showed overall reduced fluorescence ($t_{14}=2.53$, $P<0.05$). Neuromasts in cobalt-treated samples were often disorganized in appearance, although some intact neuromasts could be found. The variability in the effects of cobalt chloride treatment likely reflects the relatively low dosage and duration of the cobalt treatment used in this experiment. Damage to hair cells in the saccule was never observed in either treated or untreated animals (data not shown).

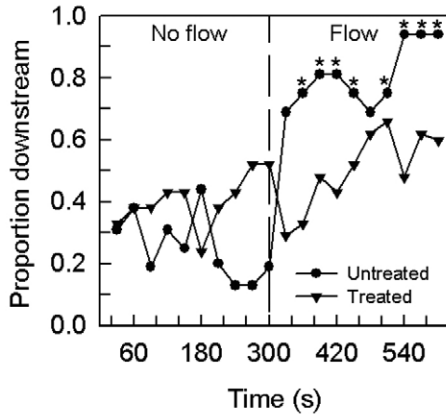


Fig. 7. Treatment with cobalt chloride affects downstream positioning. Proportion of animals positioned downstream during no flow (30–300 s) and flow (300–600 s). The vertical dashed line separates the no flow from the flow periods. Untreated animals were immersed in Ringer's solution; treated animals were immersed in cobalt chloride. Asterisks denote statistical significance of the downstream position ($P < 0.05$). Animals were tested at a 10 cm s^{-1} flow rate.

DISCUSSION

These experiments show that bullfrog tadpoles, at developmental stages prior to full hindlimb development, respond to weak current flows by moving downstream. Unlike *Xenopus laevis* tadpoles at comparable developmental stages or many species of adult fish, bullfrog tadpoles do not station hold, do not suspend in the water column, and do not exhibit positive rheotaxis against the current. Not only did bullfrog tadpoles not exhibit positive rheotaxis but also their orientation with respect to the current was statistically random. Downstream movements in current are consistent at the three different flow rates tested here, all of which are within the range of rates known to elicit positive rheotaxis in other species (Montgomery et al., 1997; Kanter and Coombs, 2003; Simmons et al., 2004; Coombs and Grossman, 2006). The results of the experiments involving disruption of the lateral line neuromasts by exposing tadpoles to cobalt chloride suggest that the lateral line system is the dominant mediator of these downstream movements.

Analyses of the animals' behavior in the *Y*- and *Z*-dimensions show that tadpoles consistently positioned themselves at the sides and bottom of the tank, regardless of current flow. These behaviors were consistent across all developmental stages and all tadpole body sizes. Areas along the sides and bottom are areas of reduced current flow, even in the upstream position. These positioning preferences are consistent with the spatial positioning of bullfrog tadpoles in their natural environments, where they are typically found in slow moving streams and situated along the bottom of ponds, near vegetation or rocks, rather than suspended in the water

column (McDiarmid and Altig, 1999; Nie et al., 1999). In our experiments, we observed that tadpoles sometimes exhibited a positioning bias towards the far over the near side wall. This might reflect the left turning bias previously observed after tadpoles surface for air (Wassersug et al., 1999), but its inconsistent appearance in our experiments (in one of three experiments testing responses to the 10 cm s^{-1} flow rate) makes it difficult to interpret. Adoption of positions near side walls has also been observed in some species of adult fish (Baker and Montgomery, 1999; Coombs and Grossman, 2006; Windsor et al., 2008; Sharma et al., 2009). Wall-following behaviors in these species emerge in novel environments, particularly when visual cues are unavailable, and may reflect exploratory or predator-avoidance functions (Sharma et al., 2009). Assuming that an approaching predator induces disturbances in the ambient current, bullfrog tadpoles' preferences for tank sides may also serve a predator-avoidance function. A preference for tank walls and bottom also highlights the importance of tactile cues in positioning behaviors. Coombs and Grossman suggested that orientation in a flow tank may be mediated not only by lateral line function but also by tactile cues contributed by the alignment of the animal's body along the sides of the tank (Coombs and Grossman, 2006). Our data show that such tactile cues are important in both no flow and flow conditions. But we also show that preferences for the side walls and bottom of the flow tank were not significantly affected by cobalt chloride treatment, suggesting that the lateral line did not markedly contribute to these behaviors.

Though bullfrog tadpoles do not exhibit the positive rheotaxis evident in other species tested under similar experimental conditions, rigorously defining their behavior presents a difficult problem. Whereas positive rheotaxis is easily defined as behavioral orientation in opposition to the flow of water, movement in the direction of flow does not necessarily imply an active behavior. These results raise the question of whether flow elicits an active behavioral response in bullfrog tadpoles, or whether their movements downstream are merely the result of a passive push by the current.

One simple way to think about passive movement in current is to consider the dynamics of an inanimate object such as a cork. Under the 'cork' model, animals under flow would be swept downstream at about the velocity of the flow, until they came to rest against the downstream wall of the tank. The tadpoles in this study clearly did not behave like inanimate objects. Rather than move directly to the back of tank upon the initiation of flow, many animals moved gradually downstream and continued to change their position throughout the course of the trial. Some animals even reversed position and swam upstream, where they positioned themselves in areas of reduced flow (Fig. 3).

The variability between individual animals and the apparent unpredictability of an individual's behavior during a trial suggest that their movements are comparable to a random walk through 3D space. In order to better understand the animal's response to flow, we must ask just what kind of random walk bullfrog tadpoles engage in, and how the nature of their random movements changes when they encounter flow. In a random walk, the walker moves in a random direction for some length of time before selecting a new direction and moving again. Many biological studies have employed a 'correlated random walk' to model animal movements (Codling et al., 2008). In a correlated random walk, a model animal maintains an orientation as well as a position and a velocity. On each iteration of a correlated random walk, the walker selects a new orientation through a probability distribution that is dependent on the previous orientation. As a result, the model animal will turn at random, but will tend to maintain a consistent heading for short periods of time.

Table 1. Swimming speed and time spent moving are not affected by cobalt chloride treatment

		Mean \pm s.e.m. swimming speed (cm s^{-1})	Proportion of time spent swimming
Ringer's solution immersion ($N=5$)	Pre	1.04 ± 0.61	0.26
	Post	0.88 ± 0.30	0.20
Cobalt chloride immersion ($N=8$)	Pre	0.68 ± 0.31	0.14
	Post	0.68 ± 0.47	0.12

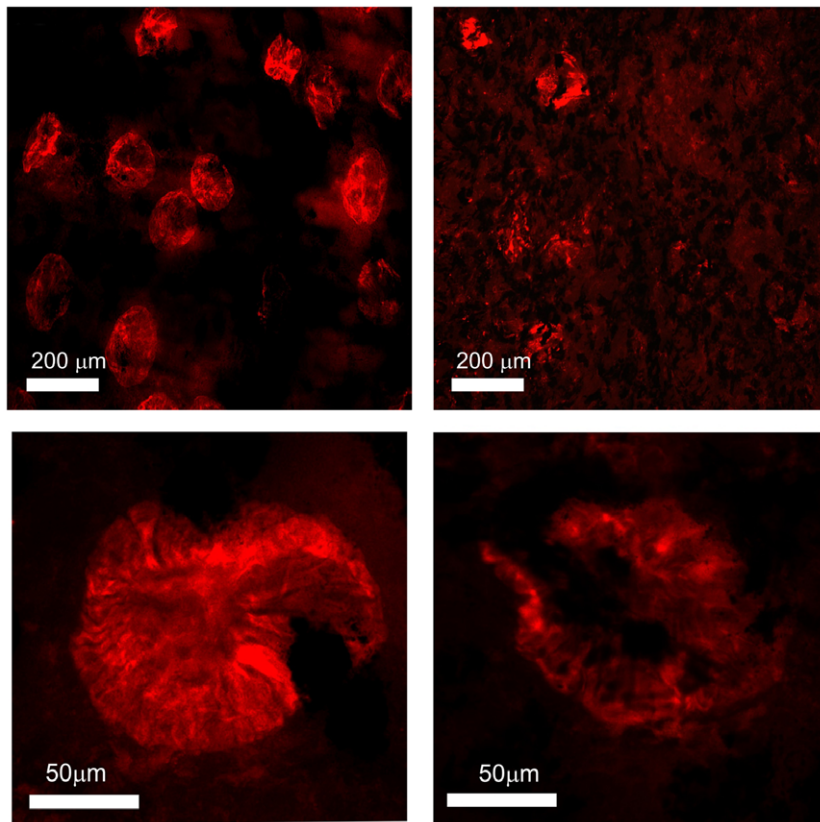


Fig. 8. Confocal images of phalloidin-stained (red) neuromasts from the supraorbital lines of an untreated (left, stage 30) and a cobalt-treated (right, stage 33) tadpole. Skin samples were matched for size. Fluorescence is reduced in the cobalt-treated sections. Black stains are pigment cells.

By considering the movement of bullfrog tadpoles in terms of a correlated random walk, we can separate the passive influence of water flow from an active behavioral change elicited by flow. In the absence of flow, the position of a random walker placed in the middle of a tank would be distributed randomly around the release point. Under flow, a random walker would initially adopt an identical random distribution, but its position would be shifted downstream by the push of the current. Without any active change in behavior, a random walker would continue to move randomly, but its position would be pushed towards the downstream end of the tank.

An alternative model is that a flow stimulus actively changes the process by which the animal selects its movements. In the absence of flow, an animal adhering to this model would behave identically to the passive random walker described above. Under the influence of flow, however, an active random walker would be biased to select a movement in the direction of the flow or to remain in place at the downstream end. In contrast to a random walk shifted by flow, a directed random walk requires a sensory mechanism to identify the direction of flow and a neural mechanism to regulate a locomotor reaction.

Our results strongly suggest that the downstream movements we observed involve an active change in behavior in response to flow. Under a passive random walk model, the degree of downstream movement would depend on the rate of flow: the higher the flow, the more the distribution of positions would be shifted downstream. Throughout the experiment, the tadpoles maintained positions along the sides and bottom of the tank, where the passive push from the current was lowest. Comparing measurements of flow in the YZ-plane (Fig. 1B,C) with YZ-occupancy histograms (Fig. 2C,D, Fig. 5) indicates that the animals were rarely in the regions of strong current flow. Rather than depend monotonically on the intensity of the flow stimulus, the tadpoles' tendency to move downstream was

comparable at all three rates tested, though the behavior was somewhat reduced in the 6 cm s^{-1} condition. Comparing across all three flow rates suggests that the behavior is an active response to the flow, which depends on a threshold at 6 cm s^{-1} or below.

The most convincing evidence that downstream movement results from an active behavior is that treatment with cobalt chloride damaged peripheral neuromasts on the supraorbital line and produced a significant reduction in the animals' movement downstream. Under a passive random walk model, one would expect that disrupting hydrodynamic sensation in this way would have no effect on the animal's behavior under flow. In contrast, treatment with cobalt chloride increased the latency of downstream movement after flow onset and increased the probability of tadpoles maintaining an upstream position throughout the trial. One possible explanation for these effects is an increase in the animals' locomotor behavior after treatment: if cobalt chloride increased the tadpoles' swimming speed, then treated animals would be more resistant to passive push by the flow. But we found no significant effect of cobalt treatment on the tadpoles' swimming speed in no flow conditions. These findings suggest that disrupting the sensation of flow by interfering with lateral line function interferes with an active preference for the downstream end of the tank.

Our experiments provide evidence of lateral-line-mediated behavior different from that of most other adult fish and, more importantly, another species of larval anuran, *X. laevis*. Differences in the behavioral responses of different species may reflect differences in the structure, function and biophysical properties of sensory receptors (Carton and Montgomery, 2004; van Trump and McHenry, 2008; Ward and Peichel, 2010) or of their central projections. The contribution of peripheral and central processing to tadpoles' responses to the biologically relevant stimulus of current flow remains to be determined.

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