THE LATERAL LINE IS NECESSARY FOR BLIND CAVEFISH RHEOTAXIS IN NON-UNIFORM
FLOW

Matthew R. Kulpa

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Committee:

Sheryl Coombs, Advisor

Paul Moore

Moirra van Staaden

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ABSTRACT

Sheryl Coombs, Advisor

When encountering a unidirectional flow, many fish exhibit an unconditioned orienting response known as rheotaxis. This multisensory behavior can reportedly involve visual, vestibular, tactile and lateral line cues. However, the precise circumstances under which different senses contribute are still unclear and there is considerable debate, in particular, about the contributions of the lateral line. In this study, we investigate the rheotactic behavior of blind cavefish under conditions of spatially non-uniform flow (a jet stream), which in theory, should promote reliance on lateral line cues. The behavior of individual lateral line enabled and disabled fish was videorecorded under IR light in a square arena that prevented streamwise biases and that contained a narrow jet stream in the center of the tank. Whereas the stream's peak velocity (8 cm/s) declined very little in the streamwise direction, it declined steeply in the cross-stream direction ($\sim 3 - 4.5$ cm/s/cm). Lateral line enabled fish showed higher levels of orientation to the stream and its source (a 1 cm-wide nozzle) when in the central (jet stream) region of the tank compared to surrounding regions, whereas lateral line disabled fish showed a random orientation in all regions of the tank.

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TABLE OF CONTENTS

	Page
INTRODUCTION.....	1
MATERIALS AND METHODS.....	3
Experimental animals.....	3
Experimental setup.....	3
Flow field measurement.....	4
Methods for blocking the lateral line and verifying blocking effectiveness.....	4
Experimental procedure.....	6
Data analysis.....	7
Statistical analysis.....	8
RESULTS.....	10
DASPEI results.....	10
Behavioral reactions of blind cavefish to the test arena and the jet stream.....	10
The effects of the acclimation period on wall following behavior and swim speed in no flow conditions.....	12
The effects of time and treatment on behavior in the central (jet stream) region of the test arena.....	13
DISCUSSION.....	15
Velocity gradients in the lives of fish.....	15
The impact of flow generation methods on the spatial characteristics of flow and the sensory basis of rheotaxis.....	16

Temporal evolution of behavioral responses to flow.....	18
Aminoglycoside treatments to block the lateral line: implications for rheotaxis and other behaviors.....	20
Evidence against global toxic effects.....	22
Long acclimation periods for reducing exploratory behaviors.....	23
REFERENCES.....	25
APPENDIX A: FIGURE 1.....	28
APPENDIX B: FIGURE 2.....	29
APPENDIX C: FIGURE 3.....	30
APPENDIX D: FIGURE 4.....	31
APPENDIX E: FIGURE 5.....	32
APPENDIX F: FIGURE 6.....	33
APPENDIX G: FIGURE 7.....	34
APPENDIX H: FIGURE 8.....	35
APPENDIX I: FIGURE 9.....	36
APPENDIX J: IACUC LETTER.....	37

INTRODUCTION

When encountering a unidirectional flow, many fish orient upstream. This unconditioned rheotactic response has several potential benefits for fish, including interception of downstream drifting prey or odors, energetic cost savings, and directional guidance for long-range migrations (reviewed in Montgomery et al. 1997). Although the flow-sensing lateral line system of fish would seem to be ideal for rheotaxis, vision and other senses are clearly involved (reviewed in Arnold 1974). However, the precise role played by any one of these senses in relation to the others is still poorly understood. Moreover, there is continuing uncertainty and debate about the importance of the lateral line for rheotaxis. Some studies have indicated that lateral line deprivation results in severe deficits in rheotactic performance, indicating that the lateral line is quite important (Montgomery et al. 1997; Baker and Montgomery 1999a,b; Bak-Coleman et al., 2014; Suli et al. 2012). Others have shown little to no effect of lateral line deprivation on rheotaxis, indicating that the lateral line is of very little, if any importance (Dijkgraaf 1963; Van Trump and McHenry 2013; Bak-Coleman et al., 2013, 2014).

There are a number of important differences between the various studies that might explain these rather discrepant results. These include the number of fish tested (groups of 4 – 5 fish vs. solitary fish), the shape of the experimental arena (rectangular vs. square), species-specific differences in behavior or sensory capabilities, and the spatial characteristics of the flow (non-uniform vs. uniform)(Bak-Coleman et al., 2013; 2014).

One behavioral factor that has recently been shown to affect lateral line contributions to rheotaxis is the degree to which fish are sedentary and coupled to the substrate (Bak-Coleman et al., 2014). That is, lateral line deprivation was shown to have a negative impact on rheotactic responses to uniform flow in a benthic, sedentary species (the three-lined corydoras, *Corydoras*

trilineatus), but not in a highly mobile, epibenthic species (the blind cavefish, *Astyanax mexicanus*). These findings reveal that species-specific differences can certainly affect the degree to which the lateral line contributes to rheotaxis, but they cannot explain the conflicting results obtained from the same species (blind cavefish) in three separate studies (Montgomery et al, 1999b; Van Trump and McHenry 2013; Bak-Coleman et al. 2014).

One important difference between blind cavefish studies not yet explored is the method of current generation, which is likely to impact the spatial characteristics of the flow field. Both Bak-Coleman et al. (2013; 2014) and Van Trump and McHenry (2013) utilized recirculating flow tanks that were designed to minimize turbulence and spatial non-uniformities in flow speed (Vogel and LaBarbera, 1978). In contrast, Montgomery and collaborators (Montgomery et al. 1997; Baker and Montgomery 1999a,b) utilized pumps with small outlets to generate flow. Although it is impossible to know precisely how or if flow fields differed in the various studies, it is certainly conceivable that some of the confusion about the relative importance of the lateral line may be due to differences in the spatial uniformity of flows.

In this study, we test the hypothesis that the lateral line plays an important role in rheotaxis when fish are in spatially non-uniform flow by measuring the rheotactic performance of blind cavefish to a non-uniform flow with steep velocity gradients (a jet stream) when the lateral line is intact and pharmacologically blocked. We compare our results in non-uniform flow with previous results obtained in uniform flow in the same species using identical measures of performance (Bak-Coleman et al, 2014). Taken together, these results suggest that the lateral line plays a much more important role in rheotaxis in spatially non-uniform flow than uniform flow.

MATERIALS AND METHODS

Experimental animals

Mexican blind cave fish, *Astyanax mexicanus* (44–53 cm in standard length) were obtained from commercial aquarium suppliers. Blind cave fish inhabit lightless, limestone caves in East Mexico (Espinasa et al. 2001) and thus, rely heavily on their lateral line system to survive in their subterranean environment (Teyke 1990). Fish were housed communally (2–8 fish) in 20 gallon aquaria at the Bowling Green State University's animal facility maintained at 17–24° C on a regular light/dark cycle (12 hours light/12 hours dark). Fish were fed commercial flake food (Tetramin) *ad libitum* 6 days a week. Plastic-lined nets were used for the transfer of fish to and from home tanks to provide a cushion of water around the fish in order to minimize physical damage to the lateral line. Protocols for the care and experimental use of fish were approved by the BGSU Institutional Animal Care and Use Committee.

Experimental setup

Fish were tested in a square working arena (25 x 25 cm) within a larger rectangular tank (53 x 27 x 10 cm) (Fig. 1). The upstream and downstream ends of the working arena were bounded by flow-through tank dividers (Penn Plax, Hauppauge, N.Y., USA). A narrow jet stream down the center of the working arena was generated by a Hydor Seltz L20 water pump, which pumped water through a 1 cm diameter nozzle at one end of the tank while draining it from the opposite end via a recirculating line of plastic tygon tubing of similar diameter (ID = 12.7 mm). Flow speed was controlled with an inline PVC ball valve ($\frac{5}{8}$ inch diameter). A collimator made of 3 cm long straws at the upstream end of the jet stream helped to streamline the jet and reduce overall circulation in the tank. A matrix of infrared (IR)-emitting diodes below the working area provided upwelling light (diffused by a white plastic sheet) so that fish behavior

could be video recorded with a Sony Handycam (DCR-HC42 Digital Video Camera Recorder) positioned above the flow tank. The flow tank was housed in a light-tight, single-walled sound attenuating chamber (Industrial Acoustics).

Flow field measurement

Particle image velocimetry (PIV) was used to track the movements of tiny, neutrally buoyant polystyrene particle (PLIOLITE VTAC-H, Goodyear Chemical) particles (0.023mm < diameter < 0.35) in order to characterize the flow field in the experimental arena. Particles were illuminated with a horizontal light sheet, generated by a slide projector (Kodak Ektagraphic III A) through a customized slide with a narrow (1 mm) horizontal slit. The light sheet transected the center of the jet stream at the level of the nozzle. The illuminated flow field was video recorded at 30 fps using a high-definition camera (Canon Vixia HF M500) positioned above the flow tank. Videos were captured to disk and converted to grayscale bitmap image sequences using VirtualDub 1.9.11 and a custom MATLAB (Version r2009b, Mathworks, U.S.A) program. Image sequences were analyzed with PIV software (PIVlab [W. Thielicke & E.J. Stamhuis]) adapted for Matlab. This software characterized the two-dimensional flow field in the jet stream (Fig. 1) at a spatial resolution of one vector per a 4.5 x 4.5 mm area. Velocity vectors were time-averaged over 240 frame sequences (8 s video @ 30 fps).

Methods for blocking the lateral line and verifying blocking effectiveness

Two different aminoglycoside antibiotics, streptomycin and neomycin, were used to disable lateral line hair cells in two, independent treatment groups. Fish in the first group were immersed in a 5.5 L treatment of 343 μ M streptomycin (dissolved in home tank water) for 3 h (Montgomery and Baker, 1999a,b; Bak-Coleman et al., 2013) prior to the 24-h acclimation and testing periods (Fig. 2). The second (neomycin) treatment group was added because DASPEI

verification methods (see below) revealed incomplete blocking of the lateral line in the first (streptomycin) group, possibly because hair cells had begun to regenerate during the lengthy acclimation period (see discussion). To increase the likelihood of complete lateral line blockage in the second treatment group, we took three steps. One, we switched to neomycin due to extensive documentation of dose-response relationships (Harris et al., 2003, Murakami et al., 2003, Buck et al., 2012; Coffin et al., 2014) and the fact that this particular aminoglycoside had the lowest IC_{50} (concentration causing a 50% reduction in DASPEI measures of hair cell functionality (Buck et al., 2012). Two, we minimized the potential for hair cell regeneration by delivering the drug one hour prior to testing. This was done by remote injection of a concentrated solution of neomycin (total volume = 100 μ l or < 0.2 % of the total volume in the test system) into a region downstream of the working arena twenty-three hours into the acclimation period and one hour before testing began. This allowed the drug to slowly diffuse to its full working concentration (250 μ M) before the flow was turned on and the drug-infused water was circulated. Fish in this treatment group were thus immersed in the neomycin-treated water for 1 hour before testing and 23 minutes during testing. Three, we treated and tested fish in calcium-free, deionized water supplemented with salts [25 μ M KCl, 50 μ M KNO_3 , 50 μ M Na_2HPO_4 , 200 μ M NaCl, 100 μ M $MgSO_4$ (Karlsen and Sand 1987)] to maximize the sensitivity of hair cells to neomycin's blocking effects (Coffin et al, 2009; 2014). Low calcium reportedly increases the probability that hair cell transduction channels will be open and thus, that the drug will be taken up by the transduction channels (Coffin et al., 2014). To prevent osmotic stress, the same salt solution, but with added $CaCl_2$ (1.3 mmol), was used to house this group of fish prior to treatment and testing. Finally, both control and neomycin-treated groups received a 3 hr sham treatment similar to that given to streptomycin-treated fish. That is, they were placed in a 5.5L

tank of their home tank water (without any drug) prior to being placed in the experimental arena (Fig. 2).

DASPEI (2-(4-(dimethylamino)styryl)-*N*-ethylpyridinium iodide) was used to verify the effectiveness of drug treatment in all experimental animals. DASPEI is a pyridinium dye that is actively taken up by the mechanoelectrical transduction channels of the healthy lateral line hair cells. Uptake of the dye thus confirms functionality of the hair cells (Van Trump et al. 2010). Shortly after each experimental run with any given fish, the fish was immersed in a solution of 0.016% DASPEI in home tank water and kept in the dark to prevent photobleaching of the fluorescent dye. After 10 minutes, the fish was removed from the DASPEI solution and anesthetized in a solution of Ethyl 3-aminobenzoate methanesulfonic acid (MS-222, 0.1%) in home tank water. The fish was then examined under a stereomicroscope (Olympus SZX12, New Hyde Park, NY) fitted with a GFP filter (excitation 450–490 nm, barrier 515 nm). Each fish was then scored according to whether canal (CN) and superficial (SN) neuromasts in specific locations on the head (preopercular region) and body (trunk scales) exhibited strong, partial or little to no labeling of hair cells (Fig. 3)(see DASPEI results section for scoring criteria).

Experimental procedure

The sequence of experimental steps are summarized in Fig. 2. Individual fish were transferred from their home tanks to a 5.5 l tank, where they remained for 3 h for either a streptomycin or sham (control and neomycin groups) treatment. Fish were then transferred from the treatment tank to the working arena of the experimental flow tank and the door to the light-tight, sound attenuating chamber was closed. The fish's behavior was video recorded for 3 min (10 fps) in no flow under IR light. Fish were then allowed to acclimate to the no-flow test environment for 24 hours before testing in flow began. The long acclimation period was

designed to reduce the wall-following tendencies of blind cavefish, which could theoretically dampen rheotactic responses to flow (Bak-Coleman et al., 2013; 2014). Blind cavefish typically respond to novel environments with an increase in swim speed and wall-following behavior, which presumably serve an exploratory function (Teyke 1989, Sharma et al., 2009). By providing fish with sufficiently long periods to explore their new environment, we hoped to minimize their wall-following tendencies, while also maximizing the potential for responses to a novel flow, introduced after the 24 hour acclimation period. After the 24 hour acclimation period, fish were again videotaped for another no-flow trial before the flow was turned on (Fig. 2). Following a 2 min ramp-up period for the jet stream to reach its maximum flow speed (peak speed in the center ~ 8 cm/s)(Fig. 1), the fish's behavior was video recorded again for a 3 min period. This step was repeated for 3 more flow trials, separated by 2 min intertrial periods.

Data analysis

Custom-written Matlab software (Butali, S. & Bak-Coleman, J.B.) was used to automatically track the spatial position and orientation of the fish with respect to the flow direction in the jet stream. The tracking software utilizes MATLAB's image processing toolbox, which first fits an ellipse around the body of each fish and subsequently determines the centroid, major and minor axis of each ellipse in a Cartesian coordinate system relative to the video frame. The fish's position was determined by the x,y coordinates of the centroid in each video frame, whereas a vector drawn from the centroid to the leading (front) edge of the ellipse was used to characterize fish orientation.

To reveal the spatial preferences of the fish in the working arena relative to the jet stream, two-dimensional scatter plots of the fish's streamwise and crosswise position were generated for all individuals in all treatment groups and for all test trials. In addition, the time spent, mean

streamwise position and orientation of the fish in the central (jet stream) region of the test arena (Fig. 1a) were measured for both flow and no-flow trials. Orientation in the jet stream was characterized by the rheotactic index (RI). RI is a measure of the overall degree to which fish head upstream and represents the area encompassed by the cumulative frequency distribution (CFD) of observed fish headings and the CFD based on a theoretical distribution drawn from random headings (Bak-Coleman et al. 2013). The RI is a continuous variable that varies between +1 (perfect upstream orientation) to -1 (perfect downstream orientation).

In addition, distance to the nearest wall and swim speed were measured in no flow (across all tank regions) to determine if the acclimation period had the intended effect of reducing swim speed and wall-following behaviors. The fish's instantaneous swim speed was measured as its displacement in the horizontal plane divided by the time between consecutive video frames in cm s^{-1} .

Statistical analysis

Because the neomycin (but not streptomycin) treatment achieved the intended effect (complete blocking of the lateral line) and differences between streptomycin and neomycin treatment regimens make differential effects between these two treatments difficult to interpret, only the control and neomycin treatment group results were analyzed statistically. However, results from all three treatment groups are presented. Statistical analyses were performed using IBM SPSS Statistics (Statistical Package for the Social Sciences). To ensure that there were no treatment-dependent orientational preferences before the onset of flow, an ANOVA was performed on RI's computed for the post-acclimation, no-flow trial for behaviors restricted to the central region of the tank. In this case, an RI of 1 indicates a 'perfect' upstream heading with respect to an absent jet stream. A repeated measures MANOVA was used to compare wall

distances and swim speeds for the pre- and post- no flow trials to determine if the acclimation period had any effects on these variables. Lastly, a repeated measures MANOVA was used to determine if RI, time spent, and SW position differed among treatment groups across the 4 flow trials.

RESULTS

DASPEI results

Sham-treated (LL+) fish showed strong labeling of hundreds, if not thousands of superficial and canal neuromasts all over the head and body surface (top row, Fig. 3). In streptomycin treated fish, DASPEI labeling of both superficial and canal neuromasts was reduced in comparison to sham treated fish (middle row, Fig. 3). Although the majority of streptomycin-treated fish exhibited little to no labeling of hair cells in superficial or canal neuromasts, a significant number of fish nevertheless exhibited partial labeling of both SNs and CNs (42 and 25% of this treatment group, respectively) (Fig 4). Nearly all neomycin-treated fish showed little to no labeling of hair cells in either SNs or CNs (third row, Fig 3). In streptomycin-treated fish, it was often the case that a few strongly-labeled spots were visible within the paler surround of the neuromast (e.g. trunk scale SNs in streptomycin-treated fish as illustrated in the middle row of Fig. 3). We interpret the faint (pale) labeling as endocytotic uptake of the dye by the entire neuromast (including support cells) and strongly labeled ‘spots’ as active uptake via transduction channels in a limited number of viable hair cells. Fish having neuromasts like these were scored as having partially labeled SNs, whereas fish with predominantly ‘pale’ neuromasts or no visible neuromasts at all were scored as having little to no SN labeling. Similarly, canal neuromasts in this treatment group often showed weak labeling on the edges of the canal neuromast (e.g. trunk scale CN in middle row, Fig. 3). Fish with this pattern were scored as having partially labeled CNs.

Behavioral reactions of blind cavefish to the test arena and the jet stream

This first section provides a qualitative description of how fish behaved in the test arena before and after the presentation of the jet stream. Subsequent sections describe the effects of the

acclimation period, lateral line blocking treatments and time on various behavioral metrics. As expected, the tendency of blind cavefish to follow the walls of the novel arena was somewhat reduced after the 24 hour acclimation period, and fish began venturing more into the central regions of the tank (Fig. 5). All treatment groups showed similar trends, as illustrated by the spatial distribution plots for a single (representative) individual (Fig. 5a), as well as by the spatial density plots pooled across all individuals (Fig. 9b). In flow, both sham-treated controls and streptomycin-treated fish showed similar spatial patterns, tending to spend more time near the source (nozzle) of the jet and also near the left, downstream corner of the arena, where some of the water from the jet was recirculated along the downstream end and side walls of the test tank (see Fig. 1A). In contrast, neomycin-treated fish tended to avoid both of these regions, spending more time in low-velocity regions outside the jet stream and away from the region of strong recirculation (Fig. 5). Although no statistically-significant time-dependent effects were revealed, the attraction of sham-treated controls and streptomycin-treated fish to the jet stream source did not typically develop until after the first flow trial and appeared strongest during the last flow trial, as evidenced by spatial density plots (Fig. 5b).

Blind cavefish exhibited varying reactions when crossing from the surrounding (low velocity) regions into the jet stream (Fig. 6). During the first flow trial, both lateral line enabled (Fig. 6A) and disabled (neomycin-treated) (Fig. 6b) fish were displaced downstream by the flow (trajectories 1, 3 and 4 in Fig. 6A and trajectories 1, 2, 4 and 5 in Fig. 6b). In addition, lateral line enabled, but not lateral line disabled, fish occasionally oriented upstream (trajectories 2 and 5 in Fig. 6a). In some cases, fish from both groups also exhibited what might be described as an avoidance reaction – i.e. turning away from the stream after entering it (trajectory 3 in Fig. 6b). By the time of the fourth flow trial, however, lateral line enabled fish were frequently turning

upstream after crossing into the jet stream region of the tank (trajectories 1 – 5 in Fig. 6c), whereas lateral line disabled fish largely continued to be displaced downstream (trajectories 1 and 3 in Fig. 6d), sometimes as far as the downstream barrier (trajectory 4 in Fig. 6d) and/or to turn away from the jet stream altogether (trajectories 2 and 5 in Fig. 6d).

At the population level, fish in both LL+ and LL+/- (streptomycin-treated) groups showed a higher probability of being within $\pm 30^\circ$ of upstream when located within ± 3 cm of the jet stream center compared to LL- (neomycin-treated) fish (Fig. 7). Moreover, due to a slight left/right asymmetry in the flow field (Fig. 1A,B), there was also some tendency for lateral line enabled and partially disabled fish to orient downstream in the left, downstream corner of the tank where a strong region of recirculation was located. In essence, they oriented upstream to the current at this location.

The effects of the acclimation period on wall following behavior and swim speed in no flow conditions

A significant multivariate interaction between time (pre- and post- acclimation) and treatment (sham-treated controls and neomycin treated) was observed (Wilks' $\lambda = 0.486$, $F_{2,13}=6.874$, $P = 0.009$). Subsequent univariate tests revealed that this interaction pertained to swim speed ($F_{1,14}=14.691$, $P = 0.002$) but not distance to the wall ($F_{1,14}=0.671$, $P = 0.426$). Specifically, the swim speeds of sham-treated control fish were significantly lower during the post-acclimation no-flow trial compared to the pre-acclimation no-flow trial (Fig. 8a). In contrast, there were no significant pre and post acclimation differences in neomycin treated fish (Fig. 8a). Though not significant at the $p < 0.05$ level, there was also a slightly significant increase in the average distance to the wall in post-acclimation no-flow trials compared to pre

acclimation trials in both sham-treated controls and neomycin-treated fish ($F_{1,14}=3.480$, $P = .083$)(Fig. 8b).

The effects of time and treatment on behavior in the central (jet stream) region of the test arena

The RI for the post-acclimation, no-flow trial did not differ significantly among sham-treated controls and neomycin-treated groups (one-way ANOVA, $F_{1,15}=2.035$, $P = 0.176$). In flow, however, there was a significant effect of treatment (Wilks' $\lambda = 0.267$, $F_{3,12}$, $P = 0.001$), but no effect of time (Wilks' $\lambda = 0.260$, $F_{9,6}$, $P = 0.224$) on RI, time spent and streamwise position in the jet stream (repeated-measures MANOVA). Subsequent univariate tests showed that sham-treated controls exhibited higher RIs when compared to neomycin treated fish ($F_{1,14}=18.834$, $P = .001$). Furthermore, the streamwise position of untreated (LL+) fish was slightly more biased towards the upstream end of the jet stream than that of neomycin-treated fish ($F_{1,14}=5.654$, $P = 0.032$) .

Despite the absence of a statistically significant effect of time on any of the dependent variables, there were nevertheless some interesting trends in the temporal evolution of both RI and the proportion of time spent in the jet stream (Fig. 9). Time spent in the central region of the working arena was similar to that expected by chance alone (dashed line in Fig. 9c) for the no-flow trial (NF-A in Fig. 9c). However, as soon as the flow was turned on (F1 in Fig. 9c), time spent in the jet stream dropped to below chance levels in all three treatment groups. In sham treated (LL+) fish, but not neomycin-treated (LL-) fish, the time that fish spent in the jet stream gradually rose to chance or slightly above chance levels over the course of the next three flow trials. The temporal evolution of this metric in streptomycin-treated (LL+/-) fish was somewhat intermediate between sham-treated control (LL+) fish and neomycin-treated (LL-) fish.

Similarly, the mean RI for each treatment group was near zero in no-flow, indicating that fish were randomly oriented in all directions when in the central region of the tank (NF-A in Fig. 9a). However, after the flow was turned on, the mean RI in both sham- (LL+) and streptomycin-treated fish gradually rose to its maximum level (~ 0.6 for control fish and ~ 0.4 for streptomycin-treated fish) by the time of the second flow trial (F2, Fig. 9a). In contrast, the mean RI remained near zero in neomycin-treated fish for all flow trials.

DISCUSSION

The main finding of this study is that blind cavefish have an unconditioned, upstream orienting response to non-uniform flow that is abolished when the lateral line system is blocked. Dijkgraaf (1933, 1963) reported similar findings for blinded minnows responding to a narrow (5 mm diam) current, but these were based on casual observation rather than any quantitative measures. These findings, in combination with findings from two other studies in which lateral line deprivation failed to have an effect on the rheotactic response of blind cavefish to uniform flow (Bak-Coleman et al, 2013; Van Trump and McHenry, 2013) indicate that the spatial characteristics of flow, in particular spatial differences in flow velocity (velocity gradients), play a critical role in determining the extent to which fish use lateral line cues for current orientation.

Velocity gradients in the lives of fish

Velocity gradients are likely very prevalent in many aquatic environments. A narrow constriction in a stream bed, for example, would be expected (by Bernoulli's principle) to produce a jet-stream-like phenomenon, with flow velocity increasing in the narrow region relative to the wider region. In addition, velocity gradients are always present in the boundary layer interface between a stationary solid and moving fluid. Although the spatial extent of the boundary layer can be quite small (a few mm)(depending on flow speed and characteristic length of the solid) and thus of questionable relevance for rheotaxis, the interactions between a stationary solid and moving fluid can also generate velocity gradients beyond the boundary layer region. For example, a bluff body in a stream results in increased pressure at the leading edge and reduced pressure (and velocity) at the trailing edge. As a result, there is a spatial velocity gradient between the reduced velocity region in the rear and the surrounding free stream region. In this respect, it is interesting to note that the ability of trout to hold station (entrain) to the left

or right of a bluff body, thereby positioning themselves in a steep velocity gradient, is dramatically reduced after denervation of the lateral line (Przybilla et al., 2010; Sutterlin and Waddy, 1975). Spatial velocity gradients also create ripe conditions for vortex formation, as expected for a bluff body in a stream (Liao 2007; Vogel 1994) and as observed in the jet stream used in this study (Fig. 1).

Velocity gradients can also be created by the ram and suction behaviors of predatory fish. Much like a stationary bluff body in a stream, a fish swimming in still water causes an increase in pressure at the leading edge and reduced pressure at the trailing edge, which causes water to be pushed out in front of the fish and around the sides to the rear. As a result, the spatial flow field around the fish is characterized by differences in both flow speed and direction, with flow speed in front of the fish being approximately twice that on its side.

The impact of flow generation methods on the spatial characteristics of flow and the sensory basis of rheotaxis

It is reasonable to suggest that lateral line deprivation effects, reported to be absent in some studies (Van Trump and McHenry 2013, Bak-Coleman et al. 2013, Bak-Coleman et al. 2014), but present in others (Montgomery et al. 1997; Baker and Montgomery 1999a,b) depend on the effectiveness of the flow as a lateral line stimulus. In order for the lateral line to be stimulated, there must be net motion between the fish and the surrounding water (Dijkgraaf, 1963; Kalmijn, 1988). For a benthic, sedentary fish that is able to withstand downstream displacement, a spatially uniform current is likely to be an effective stimulus to the lateral line simply because the fish stays stationary and the water moves relative to the fish. Under these circumstances, it is clear that the lateral line plays an important role in rheotaxis (Bak-Coleman et al., 2014). However, uniform flow is not likely to be a very effective stimulus to the lateral

line if it displaces the fish downstream, especially if the fish and the surrounding water move together in the same direction and speed, effectively reducing or even eliminating any net motion between fish and water. In this case, fish without visual cues must rely on vestibular (body acceleration) or tactile (slippage past a stationary frame of reference) cues. However, when fish are in spatially non-uniform flows, the many, spatially-distributed sense organs of the lateral line system are ideally suited to register spatial differences in flow speed over the body surface (Denton and Gray 1989, Kalmijn, 1988).

There is good reason to suspect, based on flow generation methods alone, that the flow field used in various rheotaxis studies has varied from spatially uniform to non-uniform. Van Trump and McHenry (2013) and Bak-Coleman et al. (2013, 2014) used flow tanks modeled after Vogel and LaBarbera (1978) to minimize spatial heterogeneities. Design features included an impeller-driven flow in a recirculating tank with nearly constant diameter throughout and upstream and downstream collimators (i.e. series of parallel pipes or soda straws) to reduce turbulence and straighten the flow across the width and depth of the flow channel. Particle imaging velocimetry was used in both studies to confirm the uniform nature of the flow throughout the tank, except near the side walls, where thin boundary layers are inescapable. In contrast, Baker and Montgomery (1999a,b) used a different flow tank design in which the outlet of an aquarium filter pump was used to generate a recirculating flow around a length-wise partition inserted into the fish's home tank (Montgomery et al. 1999b). While particle- and dye-tracking techniques were used to measure average flow speed, the spatial characteristics of the flow field were not reported in any detail. However, this design is similar to that used in the current study in that the current was produced from a pump with a narrow outlet. Thus, it is not unreasonable to suspect that the flow field in the Baker and Montgomery (1999) study, which

concluded that the lateral line played a role in rheotaxis, was less uniform than the flow field of either the van Trump and McHenry (2013) or the Bak-Coleman et al. (2014) study, which drew the opposite conclusions.

Temporal evolution of behavioral responses to flow

When blind cavefish were first presented with a novel flow stimulus, fish in all treatment groups spent less time than expected by chance in the jet stream (central region) of the tank (Fig. 9c). As time progressed, however, control and streptomycin-treated fish spent more time in the area of flow, while also exhibiting positive rheotaxis (Fig. 9c). Thus, while all treatment groups exhibited an initial avoidance response to the flow, only groups with partially or completely enabled lateral lines ventured back into the central (jet stream) region of the tank. One possible interpretation of these results is that the neomycin-treated group spent less time in the flow region because they were unable to orient upstream and thus, unable to negotiate the flow in an energy-efficient manner. In other words, it became too energetically costly for them to stay in the flow region. A second and not necessarily mutually exclusive explanation is that the neomycin group lost its ability to detect the jet stream entirely. The fact that both control and streptomycin-treated fish initially avoided, but then entered the jet stream, spending more time near the source than elsewhere, is strong evidence that fish in these two groups not only detected the jet stream, but were also able to successfully negotiate it. Because both vestibular and tactile cues can theoretically inform blind cavefish about the current's presence, however, it is possible that the neomycin-treated group of fish could still detect, but not appropriately react to the jet stream. The apparent avoidance of some neomycin-treated fish to the jet stream is further evidence of their ability to detect it.

The reason for the initial avoidance of the jet stream during the first flow trial is unclear. One explanation is that fish have an unconditioned avoidance response to novel flows. A novel flow could signify a predator or some other potential danger that needs to be initially avoided until further explored. Alternatively, the surprise introduction of the relatively strong jet stream could have simply required some time for fish to adapt to its presence.

A second, interesting finding of this study is that blind cavefish in control and streptomycin-treated groups spent higher than expected amounts of time in the upstream, central region of the test tank very near the current source. Dijkgraaf (1933, 1963) reported similar behaviors for blinded minnows, which he described as swimming *vigorously to the current outlet, as if drawn to a magnet* (as translated by Arnold, 1974). This behavior in blind fish can be interpreted in one of at least two ways – that fish are (a) naturally attracted to the current source or (b) using the upstream barrier as a tactile frame of reference for maintaining streamwise position in the absence of a visual frame of reference. Arguing in favor of the second explanation is the fact blind cavefish also exhibit upstream spatial preferences in uniform (Bak-Coleman et al., 2013; Van Trump and McHenry, 2013) and turbulent (Van Trump and McHenry, 2013) flows. Furthermore, sighted species (including the sister morph of blind cavefish, the Mexican tetra) also exhibit upstream spatial preferences when tested in the dark, but not in the light (Bak-Coleman et al., 2013; Elder and Coombs 2013). In these cases, however, it would have been difficult for fish to seek low-velocity refuges from the current because it was spatially uniform. Thus, the only recourse for fish in these particular studies was to reduce energy expenditure by orienting upstream and trying to maintain position. In the current study, however, fish were free to spend time in either the central, jet stream region of the tank or in surrounding, lower velocity regions. Under these circumstances, it is more difficult to explain the strong spatial preference

for the jet stream source as tactile compensation for the absence of vision for the purpose of maintaining an upstream orientation and position. Furthermore, fish also exhibited a second spatial preference for being near the downstream, left-hand corner of the tank, where water was recirculated to form what might arguably be described as a secondary (weaker) jet stream from a phantom source. Fish in this region of the tank exhibited positive rheotaxis to this secondary stream, the direction of which was opposite to that of the main (central) jet stream. As a final consideration, it is important to point out that fish in this study have undergone a lengthy acclimation period in no flow and thus, it is conceivable that they are responding to the novelty of the flow by trying to find (explore) its source. If that is the case, it can be said that the exploratory behavior to localize the source of the primary jet does not habituate over the short time period during which behavioral responses were measured (flow trials 1 – 4, ~16 min total); in fact, it appears to grow in magnitude (Fig. 5).

Aminoglycoside treatments to block the lateral line: implications for rheotaxis and other behaviors

Many aminoglycoside antibiotics, including streptomycin (Wersall and Flock, 1964; Blaxter and Fuiman, 1989; Baker and Montgomery, 1999), neomycin (Harris et al., 2003, Murakami et al., 2003, Buck et al., 2012; Suli et al. 2012; Coffin et al., 2014), and gentamycin (Song et al., 1995; Coombs et al., 2001; Santos et al., 2006; Van Trump et al., 2010) have been used to block the function of the lateral line system. In this study, streptomycin was found to cause partial blocking of the lateral line, whereas neomycin caused complete or nearly complete blocking. The unintended partial blocking by streptomycin underscores the precautionary notes raised by Van Trump et al. (2010) and Brown et al. (2011) about the need for anatomical (e.g. DASPEI) or other means of verifying the effectiveness of the drug. Differences between the

efficacy of streptomycin and neomycin treatments in this study could have been due to any one or combination of factors, including drug concentration (343 vs 250 μM), exposure time (3 vs 1.5 hr), the presence (streptomycin) or absence (neomycin) of calcium in the treatment water and elapsed time between the end of drug treatment and behavioral testing in flow (24 vs 0 hrs). The dose/duration combinations used in this study falls well within the range of those reported to cause nearly complete blockage of the lateral line in larval zebrafish, as judged by dose-response curves generated with a DASPEI scoring system (Harris et al., 2003; Murakami et al., 2003; Coffin 2009; Buck et al., 2012). However, the extent to which the dose response-curves of larval zebrafish are generalizable to adult fish or other species is presently unknown. Furthermore, the dose-response curve of larval zebrafish can be shifted upward by the presence of calcium in the treatment water such that higher doses are needed in the presence of calcium to produce the same effects (Coffin, 2009). Thus, streptomycin's ability to produce complete blocking in this study could have been compromised by the presence of calcium in the treatment water.

An alternative, but not necessarily mutually exclusive explanation for streptomycin's partial blocking affects is that streptomycin-treated, but not neomycin-treated fish had a 24 hour recovery period during which hair cells could have regenerated. Harris et al. (2003) demonstrated that a 24 hr recovery period is sufficient for a nearly complete restoration (regeneration) of lateral line hair cells in larval zebrafish after initial destruction with neomycin treatments. Thus, it is possible that the partial blocking effects observed in streptomycin-treated fish was actually a result of an initial complete blockage, followed by partial regeneration of hair cells over the 24 hour acclimation period.

An additional consequence of the 24 hour acclimation period is that hair cells in SNs may have regenerated at higher rates than those in CNs. Our DASPEI results are certainly indicative

of such a trend in that a higher proportion of streptomycin-treated fish showed little to no DASPEI labeling of CNs compared to SNs (Fig. 4). These results are somewhat consistent with results showing a preferential blocking of CNs relative to SNs by the aminoglycoside gentamycin (Song et al., 1995). From a functional point of view, a rapid regenerative capacity for SNs, which are superficially located and thus, more vulnerable to physical damage, makes sense. In any event, the fact that more SNs than CNs were present in streptomycin-treated fish is consistent with Montgomery et al.(1997)'s conclusion that SNs, but not CNs underlie rheotactic abilities.

Evidence against global toxic effects

One could argue that the failure of neomycin-treated fish to exhibit rheotaxis was due to global toxic effects (Janssen 2000), rather than specific blocking effects on the lateral line system. However, the treatment duration was short (1.5 hour) and the dose (250 μ M) was half the threshold level (500 μ M/1 hr) reported to elicit > 20 % mortality in larval zebrafish (Supplementary Table S1 in Buck et al., 2012). The only deaths that occurred during this study were those in which fish failed to recover from anesthesia during the post-experiment DASPEI procedure. These were typically associated with long observation periods (i.e. fish remained under anesthesia for > ~ 10 minutes), which were most frequent for streptomycin-treated fish, the group with the greatest mortality rate (50%). In contrast, the neomycin-treated group had the lowest mortality rate (12.5%). Fish that survived the DASPEI procedure were all feeding within 1 or 2 days. Furthermore, there was very little, if any, evidence for comorbid effects, such as increased swim speeds and copious mucous production, as reported by Janssen (2000) for blind cavefish treated with high doses of cobalt chloride. In fact, swim speeds were well within the normal range reported for blind cavefish (Teyke 1988) and post-treatment swim speeds were not

different from those measured 24 hours earlier (Fig. 8a). Nevertheless, we cannot exclude the likely possibility that swim speed declined during the 23 hour period before neomycin drug treatment and thus, that swim speeds right after drug treatment actually increased relative to those just before treatment. Such a result is to be expected based on results from the control group, which received the same treatment as the neomycin group up until the 23rd hour and which showed a decline in swim speed after 24 hours. A gradual decline in swim speed following an initial increase in response to a novel environment is a well-documented phenomenon related to active flow sensing and exploratory behaviors of blind cavefish (Teyke 1985; 1989) (see *Long acclimation periods for reducing exploratory behaviors* section).

Long acclimation periods for reducing exploratory behaviors

The tendencies of blind cavefish to increase their swim speed and follow walls after first being introduced into a novel environment is a well-documented phenomenon (Gertychowa 1970, Teyke 1988; 1989, Burt De Perera 2004; Sharma et al, 2009). To ensure that these tendencies did not interfere with the response of fish to the jet stream, a 24 hour acclimation period was instituted to diminish these behaviors. As predicted, fish showed a reduction in both behaviors after the 24 hour acclimation period. For example, spatial density plots reveal that both control and streptomycin-treated (but not neomycin-treated) were spending less time near the walls and more time in central areas of the experimental arena in comparison to when first introduced to the arena (Fig. 5b). Similarly, both control and streptomycin-treated (but not neomycin-treated) fish showed reduced swim speed after the 24 hour acclimation period. (Fig. 8a).

On the other hand, neomycin-treated fish continued to spend time the near walls of the arena (Fig. 5) and showed similar swim speeds to when the fish were first introduced to the novel

environment (Fig. 8a). One explanation for these behaviors is that fish were trying to compensate for decreased lateral line sensitivity with tactile senses by continuing to follow the wall surfaces. Another explanation is that the fish's learned spatial map of its environment was subsequently challenged when deprived of lateral line input. Thus, the fish responded as if the environment had changed, by renewing its exploratory behavior.

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APPENDIX A: FIGURE 1

Figure 1: Diagram of working arena with PIV image of jet stream velocity (A) and vorticity (B). Jet-stream velocity profiles in the streamwise (C) and cross-stream (D) directions. Mesh dividers (black vertical dashed lines) enclose the upstream and downstream ends of the square working arena; an upstream collimator helped to straighten out the flow. For the purpose of data analysis, the working arena was divided into three regions: the jet stream region in the center (C) and the surrounding regions to the left (L) and right (R) of the jet stream. Streamwise velocity profiles are shown for three different transects: one down the center of the jet stream, one on the very edge of the jet stream, and one just outside the jet in the surrounding (L) region. Similarly, cross-stream profiles are shown for three streamwise locations: 11, 17, and 25 cm downstream from the nozzle. Positive numbers indicate a flow direction from nozzle to drain, whereas negative numbers indicate the opposite direction. Note especially the recirculation zones in the left and right downstream corners and the slight (leftward) asymmetry in flow characteristics.

APPENDIX B: FIGURE 2

Figure 2: A flow chart representing the temporal course of drug treatment (solid line boxes) and video-taped test trials (dashed line boxes) during the experiment. The duration of each no-flow and flow trial was 3 min with a 2 min hiatus between trials. All treatment groups received either the 3-hour sham or streptomycin treatment, whereas only the neomycin group was treated in the test arena during the final hour of the 24 hour acclimation period.

APPENDIX C: FIGURE 3

Figure 3: Photomicrographs depicting extent of DASPEI labeling of canal (CN) and superficial (SN) neuromasts at different head (preopercular, PreOP) and body (trunk scale) regions in the three treatment groups. Labeling of both CNs and SNs was (1) strong in untreated fish, indicating a fully functioning lateral line system (LL+), (2) partial in streptomycin-treated fish, indicating only partial blocking of the lateral line (LL+/-) and (3) barely visible in neomycin-treated fish, indicating nearly complete blocking of the lateral line (LL-).

APPENDIX D: FIGURE 4

Figure 4: Proportion of fish for which superficial and canal neuromasts exhibited strong, partial or little to no labeling.

APPENDIX E: FIGURE 5

Figure 5: (A) Two-dimensional scatter plots to show the positions held (and when discernable, the pathways followed) by a single individual (one from each treatment group) during each of the six trials, beginning with the no-flow before (NF-B) and after (NF-A) the acclimation period and ending with the sequence of 4 flow trials (F1 - F4). Treatment groups: sham-treated controls with lateral line intact (LL+)(top row); streptomycin-treated fish with lateral line partially blocked (LL+/-)(middle row) and neomycin-treated fish with lateral line completely blocked (LL-)(bottom row). Crosswise location of the jet source and streamwise direction of the flow indicated by the red nozzle and arrow to the right of each treatment row. (B) Spatial density plots that show the composite density of positions held by all individuals for each experimental group and trial.

APPENDIX F: FIGURE 6

Figure 6: Individual examples of how swimming trajectories are altered when lateral line enabled (A,C) and disabled (B,D) fish cross into the jet stream region during the first (A,C) and fourth (B,D) flow trials. During the first flow trial, lateral line enabled fish show instances of being swept downstream (1,3 and 4) as well as orienting upstream (2 and 5), whereas disabled fish are more typically displaced downstream (1,2,4,and 5) after which they often quickly exit the stream region (e.g. 3 and 5). During the final flow trial, lateral line enabled fish tend to orient and even travel upstream (1-5), whereas disabled fish continue to be displaced downstream (1,3 and 4) and turn away from the jet stream (2 and 5).

APPENDIX G: FIGURE 7

Figure 7: Scatter plots of fish orientation as a function of cross-stream position for sham-treated control fish (LL+), streptomycin-treated fish (LL+/-) and neomycin treated fish (LL-) during the no-flow trial after the acclimation period (NF-A) and during the first (F1) and fourth (F4) flow trials (see Fig. 2). In NF-A, LL+ and LL+/- (but not LL-) fish show distinct orientation patterns that correspond to wall-following behaviors. By the time of the fourth flow trial, LL+ and LL+/- (but not LL-) fish show a high density of upstream (0 deg) orientations when in the central (jet stream) region of the tank. Left/right asymmetries in the density of orientations near 180° in surrounding regions is due to the tendency of LL+ and LL+/- fish to orient to recirculating currents that were prominent in the left, downstream corner of the tank (see Fig. 1). The conspicuous absence of orientations near 90° at the extreme left (-12 cm) and right (12 cm) sides of the tank for all groups and conditions is an artifact of how position is measured (by the centroid of an ellipse fit to the fish).

APPENDIX H: FIGURE 8

Figure 8: Mean swim speed (A) and distance to the wall (B) before and after the 24 acclimation period for untreated (LL+) fish (blue), and streptomycin (LL-Strep)(red), and neomycin (LL-Neo)(green) treated fish.

APPENDIX I: FIGURE 9

Figure 9: Mean RI (A), streamwise (SW) position (B) and proportion of time (C) spent in the jet stream by each of the treatment groups in the no-flow, post acclimation trial (NF-A) and each of the sequential flow trials (F1 – F4). The dashed line in A represents the RI value corresponding to random orientations, whereas that in B represents the dividing line between upstream and downstream positions. The dashed line in C represents the proportion of time spent in the central (jet stream) region expected by chance alone.

APPENDIX J: IACUC LETTER

Office of Research Compliance
309A University Hall
Bowling Green, OH 43403-0183
Phone: (419) 372-7716
Fax: (419) 372-6916
E-mail: hsrb@bgnet.bgsu.edu

August 28, 2014

Dr. Sheryl Coombs
Biological Sciences
Bowling Green State University

Re: Annual Renewal of IACUC Protocol 13-009

Title:
Information Processing by the Lateral Line System of Fish

Dear Dr. Coombs:

On August 20, 2014 the annual renewal for the above referenced protocol was **approved** after review by the IACUC. This renewal is in effect for one calendar year and expires on August 19, 2015. Please consult with the staff of the Animal Facility about any special needs you might have to continue with this project.

Comment(s):

The IACUC notes that you have used half of the number of approved Zebrafish in the first year.

Sincerely,

A handwritten signature in black ink that reads "Hillary Snyder". The signature is written in a cursive, flowing style.

Hillary Snyder, Ph.D.
IACUC Administrator