THE SENSORY BASIS OF RHEOTAXIS IN TURBULENT FLOW

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ABSTRACT

Rheotaxis is a robust, multisensory behavior with many potential benefits for fish and other aquatic animals, yet the influence of different fluvial conditions on rheotactic performance and its sensory basis is still poorly understood. Here, we examine the role that vision and the lateral line play in the rheotactic behavior of a stream-dwelling species (Mexican tetra, *Astyanax mexicanus*) under both rectilinear and turbulent flow conditions. Turbulence enhanced overall rheotactic strength and lowered the flow speed at which rheotaxis was initiated; this effect did not depend on the availability of either visual or lateral line information. Compared to fish without access to visual information, fish with access to visual information exhibited increased levels of positional stability and as a result, increased levels of rheotactic accuracy. No disruption in rheotactic performance was found when the lateral line was disabled, suggesting that this sensory system is not necessary for either rheotaxis or turbulence detection under the conditions of this study.
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INTEGRATION

When placed into a water current, most fish and some other aquatic animals exhibit an unconditioned orientating response to currents, a widespread behavior known as rheotaxis (Arnold, 1974; Kozlowski et al., 2003). Rheotaxis is thought to confer a number of benefits, including reduced energetic costs and improved interception of downstream planktonic drift (reviewed in Arnold, 1974; Montgomery et al., 1997). Although the importance of vision (and in particular optic flow cues) in mediating rheotactic behavior has been championed by many (Arnold, 1974; Dijkgraaf, 1963; Lyon, 1904), fish are also able to use non-visual senses in complete darkness (Arnold, 1969; Bak-Coleman et al., 2013; Baker and Montgomery, 1999a, 1999b). One such non-visual sense is the lateral line, which has been shown to play a role in several species, especially at low flow speeds (Bak-Coleman and Coombs, in press; Montgomery et al., 1997; Olszewski et al., 2012; Suli et al., 2012). However, rheotaxis persists even in the absence of both lateral line and visual cues (Bak-Coleman et al., 2013) and additional lines of evidence suggest that fish make use of vestibular (Pavlov & Tyuryukov 1993), and likely tactile (Baker and Montgomery, 1999b; Lyon, 1904) cues. In addition, food odors have been shown to lower the current speed at which blind cavefish Astyanax fasciatus (Baker and Montgomery, 1999b) and some elasmobranchs (Gardiner and Atema, 2007; Mathewson and Hodgson, 1972) first exhibit rheotactic behavior, a phenomenon known as olfactory-released rheotaxis.

In summary, rheotaxis is a complex multisensory, but robust behavior that can withstand the loss of more than one sense (Bak-Coleman et al., 2013). Still largely unanswered, however, is the extent to which sensory contributions are context-dependent, i.e., do different senses contribute in different ways under various sensory (e.g. light vs dark) or flow (e.g. turbulent or non-turbulent) conditions? The majority of studies on the sensory basis of rheotaxis have been
performed in laboratory flow tanks designed to produce unidirectional flows of constant velocity. However, natural environments are hydrodynamically complex, and these studies are only able to provide limited insight into the sensory basis of rheotaxis.

A number of studies have examined the impact of turbulence on the behavior and spatial distribution of fish in flowing water (reviewed in Liao, 2007; Liao and Cotel, 2012; Webb et al., 2010). For example, the behavior of trout near a turbulence generating structure (a bluff body (cylinder) in otherwise uniform flow), has been well characterized under conditions that produce periodic vortex streets conditions ($10 < \text{Re} < \approx 100,000$) (Liao et al., 2003; Przybilla et al., 2010). The spatial disruptions in the flow caused by the cylinder are such that they produce three distinct flow regions: (1) the bow wake in front of the cylinder, (2) the suction region just behind the cylinder and (3) the vortex wake further downstream of the cylinder. Corresponding to these regions are three distinct types of station-holding behaviors (bow waking, entraining, and Karman gaiting, respectively) (Liao et al., 2003; Przybilla et al., 2010; see also reviews by Liao, 2007; Liao and Cotel, 2013). Although trout appear to orient upstream while performing these behaviors and hold station at all three locations, the effects of the turbulent wake on rheotactic performance was not directly measured in these studies.

Direct effects of turbulence on rheotactic performance have been measured in only two studies, one of which showed that rheotactic performance was enhanced in turbulence under both light and dark conditions (Pavlov et al., 2000) and one which showed no effect of turbulence (van Trump and McHenry, 2013). Given that the two studies used different species (the roach, *Rutilus rutilus*, and the blind cavefish, *Astyanax mexicanus*, respectively), different types of turbulence-generating structures, and different metrics of rheotactic performance (among other methodological differences), it is difficult to reconcile the results. Moreover, the group metric of
rheotactic performance used by Pavlov and colleagues (2000) (proportion of fish oriented upstream) suffers from several limitations, including arbitrary criteria for what range of headings are defined as ‘upstream’, a non-continuous variable with large (20%) step sizes for estimating rheotactic performance (e.g. 1 out of 5 fish, 2 out of 5 fish etc.), and the potential for confounding influences (e.g. sensory cues from other fish; streamwise biases caused by tank shape coupled with packing density of fish) (see Bak-Coleman et al., 2013; Bak-Coleman and Coombs, in press for further discussion).

In this study, we examine the effects of turbulence on the rheotactic performance of riverine Mexican tetra (*Astyanax mexicanus*) under both light and dark conditions using continuously-varying performance metrics. Given that the reported enhancement effects of turbulence appear to persist in the dark (Pavlov et al., 2000), we also compare the effects of turbulence on two treatment groups – one with and one without sensory input from the lateral line. To minimize complexities associated with different station holding behaviors in distinct flow regions around a single turbulence-generating structure, we use an array of equally-spaced, vertically-oriented cylinders to produce nearly isotropic turbulence in both vertical and horizontal planes. Under these conditions, the results of this study confirm that turbulence enhances rheotactic performance, but fail to find evidence that the lateral line enables this enhancement. This study also reveals that vision profoundly affects the ability of fish to maintain a fixed position in space, with fish in the light exhibiting site fidelity (strong station holding behaviors) and those in the dark exhibiting periodic and wide-ranging sweeping motions in the cross-stream direction. These behavioral differences are unaffected by turbulence.

**MATERIALS AND METHODS**

*Experimental animals*
Mexican tetras (*Astyanax mexicanus*, De Fillipi, 1853), (74.75±8.50 mm SL, 8.49±2.98 g) were used in the present study. Fish were wild-caught by Dr. Timothy Bonner of the University of Texas-San Marco. Fish were housed collectively in a 530 L “Living Stream” tank (FrigidUnits, Toledo, OH, USA) in no flow at 19-22° C on a 12:12 light:dark cycle and fed ad libitum on commercial flake food (Tetramin Tropical). Fish were handled using a plastic-lined net at all times in order to minimize physical damage to lateral line neuromasts. The BGSU Institutional Animal Care and Use Committee approved all protocols for experiments and fish care.

*A. mexicanus* is a benthopelagic, riverine species native to the Rio Grande and Guadeloupe river systems in Texas and Northern Mexico (Leavy and Bonner, 2009). These fish inhabit streams of medium to high velocity (>20 cm/s) (Leavy and Bonner, 2009), often associating with riffle environments (Kollaus and Bonner, 2012). Their usual habitat includes streams with cobble substrate and a moderate amount of woody debris (T.H. Bonner, personal communication). All of these habitat features suggest that *A. mexicanus* inhabit areas in which turbulence is likely to be an important feature of flow.

**Experimental design**

The present study was conducted as a mixed repeated measures and independent group design. Of the four independent variables, three within-subject variables were presented in a blocked and counterbalanced order: (1) visual condition (V+, under visible light, and V-, under infrared light), (2) turbulence level (turbulence-generating structure present, TGS+, or absent, TGS-) and (3) flow speed (0, 1, 2, 4, 7, & 12 cm/s presented in an ascending order to prevent carry-over effects). The final, between-subject independent variable, lateral line condition,
utilized independent groups: one group with a pharmacologically-disabled lateral line system (LL-) and the other, with an intact lateral line system (LL+).

**Experimental setup**

The flow tank used in these experiments, modeled after Vogel and LaBarbera (1978), consisted of a rectangular plexiglass channel (154 x 28 x 38 cm) fitted with a return loop underneath the channel constructed from a 20 cm diameter PVC water pipe. Flow is generated just below the downstream end of the channel by a 13 cm impeller inserted into the PVC pipe and rotated by a chemistry stirrer (IKA Laborteknik RW 20DZM, Staufen, Germany). The motor assembly was clamped to a drill press stand, which was placed on rubber mats to isolate the vibrations of the motor from the tank. Motor speed in RPM was displayed on an LCD and was controlled manually by a dial on the device.

Four collimators, three at the upstream end of the channel and one at the downstream end, were used to reduce impeller-driven turbulence (measured below) in the flow and to promote uniform flow across the entire width and depth of the tank. Two of the collimators (one each at the upstream and downstream ends) were made of plastic eggcrate lighting panels (1 cm² grid). The third collimator at the upstream end consisted of two, back-to-back pieces of eggcrate offset by 50% to give an effective mesh size of 0.5 cm². The fourth collimator was constructed of plastic drinking straws cut into approximately 3 cm lengths and bundled across the entire submerged area of the channel. Fish were placed in a cube-shaped working arena (25 x 25 x 25 cm) that was enclosed by two rigid aquarium divider screens (Penn-Plax, Hauppauge, NY, USA) on the upstream and downstream ends. The working arena was diffusely illuminated from below with fluorescent lamps (V+ condition) or an array of IR LEDs (V- condition).
The turbulence-generating structure (TGS) consisted of an array of equally-spaced, 1.27 cm diameter cylinders with inter-cylinder gaps equal to cylinder diameter. The TGS was inserted immediately upstream of the working area and spanned the entire cross-stream width and depth of the working area to produce nearly isotropic turbulence in both vertical and horizontal planes (measured below).

**Flow speed and turbulence measurement**

Flow speed and turbulence were measured using particle image velocimetry (PIV). To perform PIV, the flow tank was seeded with neutrally-buoyant vinyl toluene-acrylate copolymer particles (PLIOLITE VTAC-H, Goodyear Chemical, Akron, OH, USA) (0.07 mm<D<0.12 mm). The particles were illuminated by a horizontal light sheet (2 mm thick) generated by a slide projector through an opaque slide with a horizontal slit. The light sheet was placed at three elevations (6 cm above the tank floor, 6 cm below the water surface and an intermediate level between the two) to determine how or if turbulence in the horizontal plane varied with water depth in the working arena. Particle movements were videorecorded at 30 FPS with a high-definition (1080p) camera (Vixia HF-M500, Canon, Tokyo, Japan) suspended above the flow tank; these videos were captured directly to disk using a HDMI capture card (Game Broadcaster HD, AverMedia, New Taipei City, Taiwan). The videos were converted to grayscale bitmap image sequences using VirtualDub 1.9.11 (Avery Lee, USA) and a custom MATLAB (Version r2009b, Mathworks, Natick, MA, USA) program.

PIV software (PIVlab 1.32, Thielicke and Stamhuis, Bremen, Germany) was used to calculate a two-dimensional velocity vector field (53 x 36 vector values), from which flow speed and various turbulence metrics (see below) were calculated across the region of interest for the duration of each three-second recording. The software uses a multi-pass, multi-grid window
deformation technique to compute particle motion. Interrogation windows were 16.56, 8.28, and 4.14 mm$^2$ for the first, second, and third pass, respectively, with 50% step sizes for each. The areas within 2 cm of the walls were excluded from analysis to exclude boundary-layer effects. Flow speed and turbulence measures were computed using custom MATLAB programs (see below).

Two metrics (turbulence intensity and vorticity) were used to provide a more complete description of the turbulent nature of the flow as a function of flow speed and the presence or absence of the turbulence-generating structure. Turbulence intensity gives an overall measure of the variability in flow speed at a single location in space without taking flow direction into account and is a commonly used measure of turbulence in biological studies (e.g. Cotel et al., 2006; Pavlov et al., 2000; Smith, 2003). Vorticity provides information about the magnitude and directionality of local vortices, and can provide a larger-scale picture of turbulence if averaged across an area (Crowder and Diplas, 2002).

**Turbulence intensity** (Smith, 2003), a dimensionless quantity, is the ratio of root mean square of velocity fluctuations ($u'$) to average velocity ($U$), as follows:

$$I = \frac{u'}{U}$$  \hspace{1cm} (1)

Turbulence intensity for each interrogation window within the measured region was calculated from PIV data by computing the resultant flow vector at each location of the 56 x 36 vector field for each image pair over a 3 s time period at 30 fps. The mean and standard deviation of velocity was then computed for each location. The spatial average of these time-averaged values was then computed as the overall value of TI for a given condition.
**Vorticity** ($\tilde{\zeta}$) is the vector of rotation of an infinitely small particle of fluid about its axes in three dimensions (measured in radians per second) (Crowder and Diplas, 2002). Mathematically, it is defined as

$$\tilde{\zeta} = 2\vec{\omega} = \nabla \times \vec{v}$$  \hspace{1cm} (2)

where $\vec{\omega}$=rotation vector of fluid, $\vec{v}$=velocity vector of fluid, and $\nabla$ (del) = curl function. This makes $\nabla \times \vec{v}$ equal the curl of the velocity vector, which can be further defined by the equation

$$\nabla \times \vec{v} = \left( \frac{\partial w}{\partial y} - \frac{\partial v}{\partial z} \right) \hat{i} + \left( \frac{\partial u}{\partial z} + \frac{\partial w}{\partial x} \right) \hat{j} + \left( \frac{\partial v}{\partial x} + \frac{\partial u}{\partial y} \right) \hat{k}$$  \hspace{1cm} (3)

Where $u$=streamwise (x) velocity, $v$=cross-stream (y) velocity, $w$=vertical (z) velocity and i-hat, j-hat, and k-hat are unit vectors in x, y, and z directions, respectively. These experiments ignore the vertical dimension of vorticity, which simplifies equation 3 to

$$\tilde{\zeta} = 2\vec{\omega} = \left( \frac{\partial v}{\partial x} - \frac{\partial u}{\partial y} \right) \hat{k}$$  \hspace{1cm} (4)

By convention, counterclockwise rotation, as viewed from above, results in positive values for vorticity. To prevent regions with opposite rotational directions from canceling each other out, the absolute value of vorticity was computed for each 4.14 mm$^2$ window of PIV interrogation and each image pair. The spatial mean of these values across the entire 21.5 x 14.9 cm area of view was then computed for each image pair. As the final measure of vorticity magnitude at each experimental condition, the temporal mean of spatial means was computed over sequential image pairs.

*Disabling the lateral line system*

Fish in the lateral line deprived (LL-) group were placed in an aerated solution of streptomycin sulfate (0.5 g l$^{-1}$ in home tank water) for 3 h (Baker and Montgomery, 1999a). Streptomycin is an aminoglycoside antibiotic that is toxic to hair cells in the lateral line, hearing
and vestibular senses, but it is not absorbed through the gills or digestive tract when fish are
immersed in a solution of it (Blaxter and Fuiman, 1989). Thus, it affects only the canal and
superficial neuromasts of the lateral line system. Fish with intact lateral lines (LL+ group) were
sham treated (placed for 3 h into an identical aquarium that contained no streptomycin) to control
for handling effects.

Lateral line blocking was verified by staining fish with DASPEI ((2-(4-(dimethylamino)styryl)-N-ethylpyridinium iodide). DASPEI is a fluorescent vital dye that is
rapidly taken up by the mechanotransduction channels of healthy hair cells of the lateral line and
other hair cell systems (Meyers et al., 2003; Van Trump et al., 2010). Failure of dye uptake
indicates that the transduction channels have been blocked or otherwise compromised.
Procedures are modeled after those used by Van Trump and colleagues (2010). Immediately after
the second day’s behavioral trials, fish were immersed in a 0.008% DASPEI solution for 10
minutes and then anesthetized in a bath of ethyl 3-aminobenzoate methanesulfonic acid (MS-
222, 0.01%) until body movement ceased, but opercular movement remained. Fish were then
examined under epifluorescent illumination using a stereomicroscope (Olympus SZX12, New
Hyde Park, NY) fitted with a GFP filter (excitation 450–490 nm, barrier 515 nm) to confirm
labeling of neuromasts in LL+ animals and the lack of labeling in LL- animals. Data from fish
not meeting labeling expectations (hair cells labeled in streptomycin-treated fish or few hair cells
labeled in sham-treated fish, n=4) were discarded and replaced by an additional replicate.

Recording rheotactic behavior

The rheotactic behavior of fish was videorecorded from above at 10 frames per second
using a camcorder (DCR-HC42, Sony, Tokyo, Japan) connected to a PC video-capture card
(Winnov, Version 3.3, Santa Clara, CA, USA), which allowed the camera to be controlled from the capture computer and video sequences to be recorded directly to the hard drive.

Individual fish of each treatment group (LL+ and LL-) were presented with an ascending sequence of flow speeds (0, 1, 2, 4, 7, & 12 cm/s) in one of 4 possible sensory/turbulence combinations: V+/TGS+, V+/TGS-, V-/TGS-, V-/TGS. These conditions were presented in counter-balanced order across 8 individuals to control for order effects. A single fish was placed into the experimental tank and given 18 minutes to acclimate. The fish was then videorecorded for three minutes as a no-flow control. The flow was then switched on to 1 cm/s and three minutes were given for the current to reach a stable speed. The fish was then videorecorded for three minutes. The flow was increased to the next current speed, and this process repeated for all 5 flow speeds. All of the steps from tank acclimation through the 12 cm/s flow speed comprised a single sequence and each fish received two flow-speed sequences per day on two consecutive days to complete the total of 4 sensory/turbulence combinations.

Analyzing behavioral results

Image sequences were extracted from video recordings using Virtualdub. Custom MATLAB software was used to determine the orientation and position of the fish in the working arena at each frame of video. This software used MATLAB’s image-processing toolbox to fit an ellipse to the outline of the fish’s body. It then determined the centroid, major and minor axis of the fitted ellipse in a Cartesian coordinate system relative to the video frame (Butail and Paley, 2012). The known dimensions of the working arena were then used to calculate the position of the fish relative to the edges of the working arena, as well as the fish’s orientation relative to the upstream direction. The elevation of the fish was not tracked, so depth-of-field errors were possible. These were measured to be less than 4% of the streamwise and crosswise dimensions of
the working arena. Orientations and positions of fitted ellipses were verified by overlaying them on the original video and examining them frame-by-frame. Data in a small number of frames were lost due to failure of the tracking algorithm. Though infrequent (<1%), such losses typically occurred when the apparent shape of the fish changed dramatically, as might happen during vertical movements or bending of the body during sharp turning maneuvers. Therefore, the sample size (number of video frames in which the fish was successfully tracked) ranged from 1757 to 1800, with a mean of 1798.

Five metrics of rheotactic behavior were calculated for each three-minute trial: positive rheotactic index (PRI), rheotactic accuracy (% of samples within ±10° of upstream, hereafter RA), mean streamwise position, and cross- and streamwise variability (standard deviation from the mean). PRI is derived from the area encompassed by the cumulative frequency distribution (CFD) of fish headings within ±90° of upstream and the theoretical CFD for no upstream headings. This index is similar in principle to the rheotactic index (RI) described by Bak-Coleman and colleagues (2013) except that it is calculated using only orientations within 90° of upstream and therefore has positive values only, ranging from 0 (fish never oriented within ±90° of upstream) to 1 (fish always oriented directly upstream). A value of 0.253 on this scale corresponds to completely random orientations.

Statistical analysis

All statistical analyses were performed using SPSS 19 (IBM, Armonk, NY, USA). Univariate analyses of covariance (ANCOVA) were used to determine the effects of flow speed and presence/absence of the turbulence-generating structure on turbulence intensity and mean absolute vorticity. As the raw data for vorticity did not meet homoscedasticity assumptions for
ANCOVA (Levene’s test p=0.004), the data were Log_{10}-transformed to equalize variance among the groups.

Prior to analysis, bounded measures of rheotactic performance (PRI, rheotactic accuracy, and streamwise position) were arcsine-transformed in order to normalize their distributions. Principal component analysis (PCA) was used to reduce dimensionality, as several measures of rheotactic behavior were strongly correlated. A multivariate mixed-design analysis of variance (Mixed MANOVA) was performed on the extracted factors to analyze the effects of three within-group factors (flow speed, visual condition, turbulence condition) and one between-group factor (lateral line condition) on the extracted principal components. Out of a total of 640 data points (2 principal components x 8 replicates x 2 visual conditions x 2 turbulence conditions x 2 lateral line conditions x 5 flow speeds), three outliers were identified (values greater than 3 interquartile ranges from the next-nearest value). These values were not removed, but were replaced by the nearest value from another individual in the same condition. Across all conditions and variables, 13 distributions (out of a total of 80) were found to be significantly non-normal by Shapiro-Wilk’s test (p<0.05). Given the robustness of MANOVA to non-normality, no further transformation was employed.

Finally, the rheotactic threshold was calculated, based upon the relationship between the orientation metrics (PRI and RA) and flow speed. Because PRI and RA are related measures of rheotaxis, principal components analysis was used to reduce dimensionality. A single factor (Orientation Principal Component 1, hereafter OPC1) was extracted (Table 4). The rheotactic threshold was defined as the lowest flow speed that causes a two standard deviation rise in OPC1 above the mean OPC1 for the no-flow condition (Bak-Coleman and Coombs, in press). No-flow OPC1 values were compared across treatments using a generalized linear mixed model to
determine whether any differences between groups or individuals existed. As none were found, no-flow data were pooled across treatment groups to determine mean no-flow OPC1 and threshold value. If the threshold value fell between two flow speeds, linear interpolation was used to estimate the threshold flow speed (Bak-Coleman and Coombs, in press). A generalized linear mixed model (GLMM) was used to test for differences among treatments.

RESULTS

Flow and turbulence measurement results

Flow speed increased with motor speed in a linear fashion that was independent of turbulence condition and elevation (Figure 1A). Although variability in flow speed measures taken at different elevations increased slightly with flow speed, this was most likely due to decreased precision in motor speed control settings at higher RPMs. Mean turbulence intensity decreased logarithmically as flow speed increased in all conditions (Figure 1B). Both flow speed (F(1,118)=91.053, p<0.001) and TGS presence (F(1,118)=4.754, p=0.004) were found to have significant main effects on turbulence intensity. Mean absolute vorticity across the working area increased exponentially with flow speed in all TGS+ conditions (Figure 1C), and there was a significant effect of flow speed (p<0.001) and TGS condition (p=0.001) on mean absolute vorticity.

DASPEI verification of lateral line-disabling technique

All sham-treated individuals showed strong, uniform DASPEI labeling of superficial and canal neuromasts (Figure 2A). Very little, if any, labeling was present in streptomycin-treated fish (Figure 2B). Of the visible neuromasts in streptomycin-treated individuals, most showed faint labeling in the area surrounding the sensory epithelium, and was presumed to be a result of
endocytotic uptake in support cells, rather than rapid, transduction channel-mediated uptake of DASPEI (Meyers et al., 2003, Van Trump et al., 2010).

A summary of the main effects

A brief summary of the main effects from the factor analysis will be given first, followed by a more detailed description of the results and behavioral patterns that emerged. The Kaiser-Meyer-Olkin measure of sampling adequacy was used to determine the suitability of our data for factor analysis. This measure was 0.599, extremely close to the recommended value of 0.6, and furthermore, the data were found by Bartlett’s test to be significantly (χ² (10)=858.56, p<0.001) non-spherical. Additionally, all but two of the dependent variables (see Measures of rheotactic performance) were found to be significantly correlated with one another (p<0.025, Table 1), making factor analysis likely to reflect accurate trends in the data. Principal components analysis was chosen because it was believed that several of the metrics used (positive rheotactic index, rheotactic accuracy, and positional stability) measured different, but related and important features of rheotactic behavior. The two factors extracted collectively account for 78% of the variance (Table 2) and were the only factors that produced Eigenvalues greater than one, meaning that these factors accounted for more variance than expected if variance were shared equally across dependent variables. Varimax rotation was utilized for the final solution, as it produced a factor loading solution that matched behavioral expectations (Table 3). Low initial values of the first component (PC1), correspond to low variability in stream- and crosswise positions in the working arena and high rheotactic performance (PRI, RA). Higher values correspond to stronger rheotactic performance accompanied by stronger positional stability (Figure 3A, B). The second principal component (PC2) is heavily influenced by the streamwise
position of the fish, such that high values are associated with upstream positions and low values with downstream positions (Figure 3C, D).

Multivariate tests on selected principal components (PC1 and PC2) indicated a significant \( F(2,13) = 3.824, p = 0.049, \text{ partial } \eta^2 = 0.370 \) within-subjects main effect of TGS presence and a significant \( F(8,110) = 4.825, p<0.001, \text{ partial } \eta^2 = 0.260 \) within-subjects interaction effect between flow speed and visual condition (V+/V-). No other within or between-subjects factors produced significant multivariate effects. Subsequent univariate tests indicate that PCI was greater in the presence of the TGS than in its absence across all flow speeds and also greater in the vision enabled compared to the vision disabled condition, with V+/V- differences increasing with increasing flow speed (Figure 3A, B). In contrast, no test conditions resulted in a significant change in streamwise position (PC2) (p≥0.1, Figures 3C, D). Under no conditions was the performance of LL+ and LL- individuals significantly different (F(2,13)=1.449, p=0.27).

The presence of the TGS significantly (F(1,56)=7.007, p=0.011) decreased rheotactic threshold speed, from an overall TGS- mean of 3.59 cm/s to a TGS+ mean of 2.42 cm/s (Figure 5). No significant (p>>0.05) interaction effects or other main effects were present, including no effects of either visual or lateral line deprivation alone.

Spatiotemporal dynamics of rheotactic behavior

The best rheotactic performance (values of PC1 approaching 1.5, and values of PRI and rheotactic accuracy approaching 1) (Fig 3) occurred at high flow speeds (≥7 cm/s) in the light and with added turbulence, regardless of lateral line condition. Under these conditions, fish tended to swim close to a wall of the working arena, facing upstream with little change in orientation or position, as illustrated for a single individual in Figure 6. In some cases, these individuals would engage in bouts of “tail-propping”: resting their caudal fin against the
downstream netting and using pectoral fin movements in order to maintain an upstream orientation. In contrast, the lowest rheotactic performance (values of PC1 approaching -1.5, values of PRI approaching 0, and values of rheotactic accuracy approaching 0.2) occurred at low flow speeds (≤ 2 cm/s) in the dark and without added turbulence, regardless of lateral line condition. These individuals tended to swim around the working arena with a slight preference for remaining near a wall, but with little orientation preference (illustrated for a single individual in Figure 7).

At flow speeds greater than about 2 cm/s, a distinct crosswise “sweeping” behavior emerged for fish in the dark. During this behavior, the fish moved in a crosswise direction from one side of the tank to the other, while maintaining a slight angle (± 30°) with respect to upstream in the direction of movement (Figures 8, 10). The majority of fish exhibited very periodic sweep movements, repeating the cycle 5-15 times during a 3-minute experimental trial. To quantify this behavior, we computed the sweep frequency (cycles/min) by defining one cycle as a left/right cross-stream excursion greater than 5 cm. As figure 9 illustrates, there is a clear emergence of sweep behavior at flow speeds above 2 cm/s but only for fish in the dark (black lines) and irrespective of the TGS condition or availability of lateral line information.

Overall, the presence or absence of visual information dominated the spatial patterns of rheotactic behavior, as illustrated by the composite spatial density plots across conditions and treatment groups (Figure 11). In the light, regardless of lateral line or turbulence condition, fish in no flow tended to hover in place, with a slight preference for staying near walls. As flow speed increased, fish in the light became more and more stationary, exhibiting a strong pattern of site fidelity (station holding behavior), which involved steady swimming near a wall or tail-propping at the downstream netting of the working arena. By contrast, fish in the dark failed to exhibit any
site fidelity. At low flow speeds, they tended to swim around the arena without orientation preferences, but often spent more time near the walls than in the center of the arena. At higher flow speeds, they tended to exhibit crosswise sweeping behaviors. As noted previously, there was also a slight, but non-significant trend for V-/LL- fish to move further upstream.

**DISCUSSION**

*Effect of turbulence on rheotaxis*

The present study demonstrates that turbulence significantly lowers the threshold flow speed at which rheotaxis is initiated in both visually enabled and deprived fish (Figure 5). Pavlov and colleagues (2000) reported a similar turbulence-induced lowering of rheotactic thresholds in both visually enabled and disabled roach (Figure 12). However, the size of the effect for the roach (2 – 3 cm/s threshold shift) was considerably larger than that for the Mexican tetra (~1 cm/s shift) (Figure 5). Moreover, the overall level of rheotactic performance across flow speeds was not enhanced by turbulence as observed in the current study (e.g. Figure 3a, b; Figure 4). In contrast, van Trump and McHenry (2013) reported no effect of turbulence on rheotactic performance.

The reasons for these differences are unclear, but the two previous studies differed in a number of important ways from the present study, including the type of turbulence generating structure and the metric for rheotactic performance. Pavlov and colleagues (2000) used a hydraulic jump to produce turbulence, whereas the present study used a cylinder array. A hydraulic jump is the transition between a supercritical and subcritical flow regime, i.e. a transition from a flow in which the current is fast enough to push all the wave energy downstream to a flow in which wave energy can move upstream as well, which leads to the formation of a standing wave (Murzyn and Chanson, 2009). Although the intensity and spatial
characteristics of turbulence in the Pavlov et al. (2000) study were not reported, common
c characteristics of hydraulic jumps include an abrupt increase in water height and turbulence
level, entrapment of air bubbles, and the formation of a standing wave (Chachereau and
Chanson, 2011; Murzyn and Chanson, 2007). Thus, the turbulence expected from the hydraulic
jump is likely to differ quite a bit from the more predictable von Karman vortex streets produced
by the cylinder array of this study. It might be expected, for example, that turbulence levels were
higher in the Pavlov et al. (2000) study and if so, this might explain the larger threshold shifts
reported by Pavlov and colleagues (2000) compared to the present study.

Van Trump and McHenry (2013), in contrast, used two methods to produce turbulence.
First, they used a single cylinder (diameter=3 cm), which should produce a single von Karman
vortex street. In addition, they used an angled grate to produce “asymmetrical” turbulence.
Maximum TI (0.2) in each case was near that reported for the same flow speed in the present
study (Figure 1). In both turbulence conditions, they reported no significant change in either
orientational (vector strength) or positional preferences. Unfortunately, spatial density plots
indicate that fish spent little time in the turbulent regions near the TGS. Thus, measures of
rheotactic performance may have been based largely on the fish’s behavior outside of the
turbulent region. Furthermore, given that only one flow speed was tested, it is impossible to
know whether there was any turbulence-induced shift in rheotactic threshold. It is conceivable
that this single, relatively low flow speed may have been suboptimal for revealing turbulence
effects.

Pavlov and colleagues (2000) measured rheotactic performance in a substantially
different way from that used by van Trump and McHenry (2013) and the present study. That is,
they tested groups of five fish and recorded the proportion of fish facing within 90° of upstream
every 30 seconds. This type of group measure is common in rheotactic studies, but as a variable, it has several drawbacks, including the fact that it has discrete values (0, 0.2, 0.4, 0.6 etc.) with large step sizes. By comparison, the PRI used in this study for solitary fish is a continuous variable making it is more sensitive to changes in rheotactic performance. Thus, the less sensitive metric employed by Pavlov and colleagues (2000) may have missed the more subtle overall enhancement of rheotactic performance observed in this study. A second important difference is that the group metric is likely confounded by the influence of group dynamics on rheotactic performance, whereas the solitary metric of this study is not.

*Why and how does turbulence increase rheotactic performance?*

Given that turbulence introduces spatiotemporal fluctuations in the both the direction and magnitude of the current, one could argue that the sensory task of determining overall flow direction, as well as the motor task of withstanding the destabilizing influences of turbulence would be more challenging. Thus, one might expect turbulence to degrade rather than enhance the ability of fish to orient directly upstream. There are at least two possible (not necessarily mutually exclusive) explanations for the enhancement effect. One is that turbulence affects the motivation to rheotactt and the other is that it changes the sensory task and thus, perhaps the relative contribution of different senses.

The motivation to rheotact may be increased by turbulence in several ways. Since turbulence can be energetically costly, fish may use rheotactic strategies to lower overall drag, thereby conserving energy. Additionally, increased turbulence may signify food abundance, for example by stirring up edible sediment (Cotel et al., 2006), in which case rheotactic strategies are likely to enhance the probability of encountering and obtaining food. Upstream orientation to large-scale flow is already known to be a crucial component of the search strategies used by fish.
and sharks for locating odorous food sources (Gardiner and Atema, 2007). In addition, sharks (and likely other fish as well) use a strategy known as eddy chemotaxis to track small-scale ‘packets’ of odors that are dispersed by turbulent vortex eddies caused by e.g. cobbled substrates or bluff bodies in a current (Gardiner and Atema, 2007). Under circumstances like these, turbulence may function as a trigger to release rheotactic behavior, in much the same way as food odors reportedly release rheotactic behaviors in many sharks and fish. In fact, both food odors (Baker and Montgomery, 1999b) and turbulence (present study and Pavlov et al., 2000) produce similar effects in that they reduce the threshold speed at which rheotaxis is first initiated.

Alternatively (or additionally), rheotactic performance may be stronger in turbulent flow because the sensory information needed to determine flow direction and/or to counteract the destabilizing influences of turbulence has changed, requiring different sensory capacities. A sensory capacity for detecting turbulence is certainly consistent with experimental findings indicating that fish exhibit preferences for certain turbulence regimes (Cotel et al., 2006) and can distinguish between environments based on turbulence levels (Smith, 2003).

In this respect, the flow-sensing lateral line would appear to be ideal for detecting the hydrodynamic disturbances associated with turbulence. Vortex eddies, which are shed by swimming fish and stationary obstacles in a current, clearly elicit neural responses from lateral line afferents that are capable of encoding various vortex features, such as shedding frequency and vortex spin direction (Chagnaud et al., 2006). Moreover, the lateral line appears to be important for tracking the turbulent wake of swimming fish (Gardiner and Atema, 2007; Pohlmann et al., 2004). The role of the lateral line in rheotaxis is less clear, although there is evidence that in some species and under some circumstances (at low flow speeds < ~ 1 body length/s, when fish are in tightly-spaced groups, and when fish are coupled to the substrate), the
lateral line plays a role in mediating rheotaxis (Bak-Coleman and Coombs, in press; Montgomery et al., 1997; Olszewski et al., 2012; Suli et al., 2012) and may also influence the spatiotemporal dynamics of rheotaxis (Bak-Coleman et al., 2013).

Despite the ability of the lateral line to detect turbulent wakes and to mediate rheotaxis (at least under some circumstances), it does not seem to play any role in the turbulence-induced enhancement of rheotaxis observed in this study. The turbulence-induced lowering of rheotactic threshold persists in the face of unimodal deprivation of either visual or lateral line information, suggesting that when visual information is unavailable, fish can rely on lateral line information to detect turbulence and likewise, when lateral line information is unavailable, they can rely on vision.

Effects of visual deprivation on rheotaxis

One of the most striking findings of the present study is the effect of visual deprivation on rheotactic performance. In particular, fish without visual cues exhibited cross stream sweeping behaviors similar to those observed in visually-deprived giant danio (*Devario aequipinnatus*) (Bak-Coleman et al., 2013). This behavior drastically reduced the abilities of these fish to maintain spatial position in a current and was accompanied by a decrease in rheotactic accuracy (Figure 5a,b), but little if any change in the rheotactic threshold (Figure 6) or overall rheotactic performance, as measured by PRI (Figure 4c,d). This effect was largely independent of whether the turbulence generating structure or lateral line information was present or absent. Taken together, these results suggest that vision is important for maintaining spatial position, but relatively unimportant for maintaining a general upstream direction. In this light, a reduction in rheotactic accuracy can be interpreted as an indirect effect caused by the loss of positional stability. That rheotactic accuracy may be linked to positional stability is further
reinforced by the results of the principal component analysis, which combined orientation and stability metrics into a single principal component that explained 55% of the total variance (Table 2).

The Pavlov et al. (2000) study also reported a decrease in rheotactic performance for groups of visually-deprived roach: a substantial increase (~ 5 cm/s) in the rheotactic threshold (Figure 12). The absence of any threshold shift for solitary Mexican tetra in this study could be explained by species-specific differences. On the other hand, the fact that vision is important for maintaining the relative position and orientation of schooling fish (Partridge and Pitcher, 1980; Pitcher et al., 1976), suggests that the large threshold shift observed by Pavlov and colleagues (2000) may have been caused by a disruption in the ability of the fish to maintain position and orientation with respect to each other, rather than with respect to the current per se.

Summary and conclusions

The present study finds strong evidence that turbulence increases rheotactic and station-holding performance in fish; turbulence also lowers the threshold speed of rheotaxis. Rheotactic performance is also increased by the availability of visual information in a flow speed-dependent manner, an effect that is likely indirectly caused by an increase in positional stability. The lateral line system, however, was found to influence neither overall rheotactic performance nor rheotactic threshold in the conditions tested. Future work testing the role of the vestibular system in responding to turbulence, as well as the effect of different types of turbulence generation (e.g. hydraulic jumps vs. bluff bodies vs. jet streams) on rheotactic performance may help resolve some of the discrepancies between this and previous studies.
REFERENCES


Bak-Coleman, J., Coombs, S. (In press). Sedentary behavior as a factor in determining lateral line contributions to rheotaxis. *Journal of Experimental Biology*


### APPENDIX A: TABLE 1

Table 1: Correlation matrix of original dependent variables. Values are 1-tailed p-values for the null hypothesis that the variables are uncorrelated.

<table>
<thead>
<tr>
<th></th>
<th>Streamwise Position</th>
<th>Rheotactic Strength</th>
<th>Rheotactic Accuracy</th>
<th>Streamwise Variability</th>
<th>Crosswise Variability</th>
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<tr>
<td>Streamwise Position</td>
<td>.000</td>
<td>.000</td>
<td>.025</td>
<td>.005</td>
<td>.497</td>
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<td>.000</td>
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<td>.000</td>
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<tr>
<td>Rheotactic Accuracy</td>
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<td>.000</td>
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<td>.000</td>
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<tr>
<td>Crosswise Variability</td>
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**APPENDIX B: TABLE 2**

Table 2: Initial Eigenvalues of the five components extracted (orientation and position metrics). Components 1 and 2 were chosen for further analysis.

<table>
<thead>
<tr>
<th>Component</th>
<th>Total</th>
<th>% Variance Explained</th>
<th>Cumulative %</th>
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<td>2</td>
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<td>.675</td>
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<td>4</td>
<td>.298</td>
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<td>97.951</td>
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<tr>
<td>5</td>
<td>.102</td>
<td>2.049</td>
<td>100.000</td>
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</table>
APPENDIX C: TABLE 3

Table 3: Rotated (varimax) component matrix

<table>
<thead>
<tr>
<th>Original Dependent Variable</th>
<th>PC-1</th>
<th>PC-2</th>
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</thead>
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<tr>
<td>Streamwise Position</td>
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<td>Rheotactic Strength</td>
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<td>Streamwise Variability</td>
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<tr>
<td>Crosswise Variability</td>
<td>-.768</td>
<td>.164</td>
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</tbody>
</table>
APPENDIX D: TABLE 4

Table 4: Initial Eigenvalues of the three components extracted (orientation metrics). Only component 1 was chosen for further analysis.

<table>
<thead>
<tr>
<th>Component</th>
<th>Total</th>
<th>% Variance Explained</th>
<th>Cumulative %</th>
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</thead>
<tbody>
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<td>1</td>
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<td>0.212</td>
<td>7.060</td>
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<td>3</td>
<td>0.018</td>
<td>0.602</td>
<td>100.000</td>
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APPENDIX E: FIGURE 1

Figure 1: Effects of the turbulence generating structure (TGS) on flow speed (A), turbulence intensity (B) and absolute vorticity (C). TGS absent (TGS -, black) and TGS present (TGS +, red). In A, each symbol represents the flow speed at one of three elevations, whereas red dashed and black solid lines are linear regressions through each data set. In B and C, solid lines indicate best power (B) and exponential (C) fits to the data with each data point representing values obtained at different elevations and flow speeds.
APPENDIX F: FIGURE 2

Figure 2: Photomicrographs of DASPEI-labeled *Astyanax mexicanus* with intact lateral line system (sham-treated, A) and disabled lateral line system (streptomycin-treated, B). Note strongly-labeled canal neuromasts (large, oval spots) below the eye and superficial neuromasts (small, circular spots) across the skin’s surface in A and the lack of labelled neuromasts in B.
Figure 3: Values of PC1 (A,B) and PC2 (C,D) as a function of flow speed for fish with (1) enabled (LL+)(A,C) and disabled (LL-)(B,D) lateral line systems, (2) enabled (V+, red functions) and disabled (V-, black functions) visual systems and (3) in the presence (TGS+, dashed lines) or absence (TGS-, solid lines) of turbulence generating structures. High PC1 values correspond to low variability in streamwise and crosswise positions, as well as upstream headings (i.e. greater upstream accuracy), whereas higher values of PC2 correspond to streamwise positions that are biased more towards the upstream than downstream end of the tank. Turbulence level significantly (MANOVA, df=2,13, Wilk’s Lambda p=.049) increased rheotactic stability (PC1) overall. There was a significant visual condition by flow speed interaction (the availability of visual cues increased rheotactic stability more at higher than lower flow speeds). No condition had a significant effect on PC2, although there is a trend toward upstream movement in the absence of visual information when the lateral line is disabled. Error bars represent 95% confidence intervals.
Figure 4: Rheotactic performance as a function of flow speed under different experimental conditions and using three metrics of rheotactic performance: (1) proportion of observations in which fish are within +/10° of upstream (A,B), (2) Positive rheotactic index (RI90) (C,D) and (3) principal component 1 of the orientation PCA (E,F). Experimental conditions: turbulence present (dashed lines); turbulence absent (solid lines); vision enabled (V+, red) and disabled (V-, black); lateral line enabled (LL+, left panels) and disabled (LL-, right panels). Straight dotted lines in each panel represent the upper bound (mean + 2 standard deviations) of chance levels of performance (calculated from 0 cm/s condition) for each metric. Error bars represent 95% confidence intervals.
Figure 5: Mean rheotactic thresholds for different sensory and turbulence conditions. Vision enabled: V+, red solid or hatched bars; Vision disabled: V-, black solid or hatched bars; Lateral line enabled: LL+, solid red or black bars; Lateral line disabled: LL-, hatched red or black bars; Turbulence generating structure absent: TGS-, left; Turbulence generating structure present: TGS+, right. The presence of the TGS significantly (F(1,56)=7.007, p=0.011) reduced rheotactic threshold speed. Error bars represent 95% confidence intervals.
APPENDIX J: FIGURE 6

Figure 6: Graphic displays of (A) time waveforms of the fish’s orientation re: upstream (black line re: right-hand Y axis) and crosswise (blue line) and streamwise (red line) position (re: left-hand Y axis), (B) spatial density plot of the fish’s position in the horizontal plane of the tank and (C) frequency distribution of the fish’s orientation re: upstream. This particular individual shows very little temporal variation in either position or orientation (A), holds station near a corner of the working arena for the duration of the trial (B) and maintains a strongly upstream orientation (C). These results are from a LL+ fish in the light exposed to the TGS+ condition at 7 cm/s current speed. PRI=0.91
Figure 7: Graphic display of time waveforms (A), spatial density plots (B) and frequency distributions of orientation (C) as described in greater detail in Fig. 4. This individual shows wide fluctuations in spatial position and orientation over time (A), no preference for any particular location and no station holding behaviors (B) and little orientation preference (C). These results are from a LL- fish in the dark exposed to the TGS- condition at 1 cm/s current speed. PRI = 0.317
APPENDIX L: FIGURE 8

Figure 8: Graphic display of time waveforms (A), spatial density plots (B) and frequency distributions of orientation (C) as described in greater detail in Fig. 6. This individual exhibits periodic cycles of wide-ranging motion in the cross-stream direction, but small irregular changes in streamwise position and orientation (A). The fish’s position in the horizontal plane varies much more in the crosswise than streamwise direction (A,B). The leftward and rightward sweeping motions in the cross stream direction result in a nearly bimodal distribution of orientations centered on the upstream direction (C). These results are from a LL+ fish in the dark exposed to the TGS+ condition at 4 cm/s current speed.
Figure 9: Crosswise sweeping frequency (where a sweep is a cross-stream excursion of greater than 5 cm) as a function of flow speed and sensory condition. As flow speed increases from 1 to 12 cm/s, sweeping frequency decreases (fish maintain more stable cross-stream positions) when visual information is available ($V^+$, red lines), but not when it is unavailable ($V^-$, black lines). The presence or absence of the turbulence generating structure ($T^+$, dashed lines/$T^-$, solid lines) and lateral line information ($LL^+$, open symbols/$LL^-$, filled symbols) has no effect.
APPENDIX N: FIGURE 10

Figure 10: Example of a spatial density plot of fish positions in the horizontal plane of the test arena for a single flow speed (4 cm/s) and set of conditions (V-/T-) over a 3-min trial for the entire population of LL+ fish (N=8). Flow direction is from right to left as indicated by the arrow. Dark blue regions represent areas where fish spend less time than expected, based upon the null condition of a uniform distribution. Light blue to red regions represent areas where fish spend more time than expected by a uniform distribution. This particular plot shows the propensity of fish to exhibit cross-stream sweeping behaviors in the dark. The red hot spot near one of the tank walls is largely due to the anomalous behavior of a single individual. See Fig. 11 for overall trends across conditions and flow speeds.
Figure 11: A: Spatial density plots of fish positions for lateral line enabled (LL+, left 6 columns) and disabled (LL-, right 6 columns) populations of fish for each of the different visual (V+/V-) and turbulence (T+/T-) conditions and flow speeds. See figure 10 for further details. Fish show a trend of “site fidelity” when visual information is available (top two rows), whereas they tend to utilize more of the available space when visual information is unavailable (bottom two rows). At 0 and 1 cm/s flow speeds, fish tend to spend more time along all 4 walls of the tank, whereas at higher flow speeds, they spend more time moving in the crossstream direction than the streamwise direction. In addition, there is a slight trend for fish to move further upstream in the dark as flow speed increases.
Figure 12: Rheotactic threshold speeds reported by Pavlov and colleagues (2000) in the roach (*Rutilus rutilus*). Values correspond to the average proportion of five fish that were facing upstream (±90°). Dashed lines indicate the presence of added turbulence (T+), solid indicate no added turbulence (T-). Blue and black lines correspond to fish tested in the dark (V-), while red indicate fish tested in the light (V+). The straight dotted line represents the chance level of performance (based on 50% of fish facing upstream and 50% facing downstream).
Dr. Sheryl Coombs  
Biological Sciences  
Bowling Green State University

Re: Annual Renewal of IACUC Protocol 10-018

Title:  
*Information Processing by the Auditory and Lateral Line Systems of Fish*

Dear Dr. Coombs:

On November 3, 2011 the annual renewal for the above referenced protocol received **final approval** after review of the requested modifications by the IACUC. This renewal is in effect for one calendar year and expires on November 2, 2012. Please see the next page for a summary of the approved clarifications.

Please consult with the staff of the Animal Facility about your requirements to continue with this project.

Sincerely,

[Signature]

Hillary Harms, Ph.D.
IACUC Administrator
Summary of approved clarification

1. In item 6, a more detailed description of the mortality experienced during the past year was provided.
Dr. Sheryl Coombs  
Biological Sciences  
Bowling Green State University  

Re: Annual Renewal of IACUC Protocol 10-018

Title:  
Information Processing by the Auditory and Lateral Line Systems of Fish

Dear Dr. Coombs:

On October 17, 2012 the annual renewal for the above referenced protocol was **approved** after review by the IACUC. This renewal expires on October 16, 2013. Please consult with the staff of the Animal Facility about any special needs you might have to continue with this project.

**Comments:**

Sincerely,

Hillary Harms, Ph.D.  
IACUC Administrator
August 22, 2013

Dr. Sheryl Coombs  
Biological Sciences  
Bowling Green State University  

Re: IACUC Protocol 10-018  

Title:  
Information Processing by the Auditory and Lateral Line Systems of Fish  

Dear Dr. Coombs:  

On August 21, 2013 the final renewal for the above referenced protocol was approved after review by the IACUC. This renewal is in effect only for the 107 days between the approval date and the December 6, 2013 expiration of the three year "life" of the protocol.  

If you wish to continue the work beyond December 6, 2013, you must submit a new protocol. The current protocol form and associated instructions can be found at www.bgsu.edu/offices/orc/iacuc/page44956.html. The IACUC Meeting Calendar (www.bgsu.edu/offices/orc/iacuc/page44981.html) has upcoming meeting dates and associated submission deadlines.  

Please consult with the staff of the Animal Facility about any special needs you might have to continue with this project.  

Sincerely,  

[Signature]  

Hillary Harms, Ph.D.  
IACUC Administrator  

Comments: