

RESEARCH ARTICLE

Sedentary behavior as a factor in determining lateral line contributions to rheotaxis

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ABSTRACT

Rheotaxis is a robust, multisensory behavior with many potential benefits for fish and other aquatic animals. Visual (optic flow) cues appear to be sufficient for rheotaxis, but other sensory cues can clearly compensate for the loss of vision. The role of various non-visual sensory systems, in particular the flow-sensing lateral line, is poorly understood, largely because of widely varying methods and sensory conditions for studying rheotaxis. Here, we examine how sedentary behavior under visually deprived conditions affects the relative importance of lateral line cues in two species: one that is normally sedentary (the three-lined corydoras, *Corydoras trilineatus*) and one that normally swims continuously along the substrate (the blind cavefish, *Astyanax mexicanus*). No effect of lateral line disruption on rheotactic performance was found in blind cavefish, which were significantly more mobile than three-lined corydoras. By contrast, rheotaxis was significantly impaired at low, but not high, flow speeds in lateral-line-disabled corydoras. In addition, lateral-line-enabled corydoras were characterized by decreased mobility and increased rheotactic performance relative to lateral-line-disabled fish. Taken together, these results suggest that sedentary behavior is an important factor in promoting reliance on lateral line cues.

KEY WORDS: Benthic, Flow, Lateral line, Mobile, Rheotaxis, Sedentary

INTRODUCTION

Fish placed in moving water often exhibit an unconditioned orienting response, typically orienting either upstream (positive rheotaxis) or downstream (negative rheotaxis) (Lyon, 1904; Arnold, 1974). Rheotaxis is of strong ecological significance to fish as it facilitates interception of downstream-drifting prey and odors (Kleerekoper, 1978; Gardiner and Atema, 2007), directional guidance of migratory behaviors (Thorpe et al., 1981; Tytler et al., 1978), and energetic cost savings for fish resisting downstream displacement (Liao, 2007; Montgomery et al., 1995). Rheotaxis is a taxonomically widespread behavior that occurs throughout the life cycle of fish under widely varying behavioral, flow and sensory conditions (Arnold, 1974). For example, rheotaxis has been observed in the diurnal, stream-dwelling giant danio (*Devario aequipinnatus*) (Bak-Coleman et al., 2013; McClure et al., 2006) and in blind cavefish [*Astyanax mexicanus* (De Filippi 1853)], which inhabit lightless, subterranean cave pools (Baker and Montgomery, 1999b).

The diversity of ecological and sensory conditions under which rheotaxis occurs is indicative of the robustness of this behavior and its reliance on multiple sensory cues. Visual cues arise from the

downstream displacement of fish, which causes apparent movement of objects across the retina, known as optic flow. Optic flow appears to be a very important cue that can be sufficient, if not dominant, as fish exhibit rheotactic responses to a fictive optic flow stimulus in the absence of any true water movement or body movement caused by downstream displacement (Lyon, 1904). When visual cues are absent or degraded (e.g. at night, or in turbid or deep water), fish can successfully orient to currents using non-visual sensory modalities such as the vestibular system, touch and the flow-sensing lateral line (Lyon, 1904; Montgomery et al., 1997; Baker and Montgomery, 1999a; Baker and Montgomery 1999b).

Although each of these non-visual sensory modalities could theoretically be used for rheotaxis, their actual contribution to this behavior is poorly understood for several reasons. One reason is that there is presently no good way to block the vestibular system without disrupting the ability of fish to maintain an upright posture, making it difficult, if not impossible, to assess the unique contribution of the vestibular system. A second reason is that there is conflicting evidence for (Montgomery et al., 1997; Baker and Montgomery, 1999a; Baker and Montgomery, 1999b; Suli et al., 2012; Olszewski et al., 2012) and against (Lyon, 1904; Dijkgraaf, 1933; Dijkgraaf, 1963; Van Trump and McHenry, 2013; Bak-Coleman et al., 2013) a substantial role of the lateral line in rheotaxis. All of these studies together raise questions about the precise circumstances under which the lateral line may or may not play a role in rheotaxis.

There are at least two frequently overlooked factors that may influence the utility of the lateral line and other non-visual senses in rheotaxis. One is behavior, in particular sedentary versus mobile behaviors. Sedentary benthic fish tend to be more negatively buoyant and may additionally have morphological and behavioral adaptations for coupling themselves to the substrate (Hart and Finelli, 1999; Blake, 2006) to resist downstream displacement. Sedentary behaviors thus tend to minimize vestibular and optic flow cues, while maximizing lateral line cues (water flowing across the skin surface). Furthermore, sedentary behaviors may provide additional non-visual information through touch, due to constant contact with the substrate. By contrast, more neutrally buoyant fish that swim in the water column tend to be coupled to the surrounding water, and thus are more readily swept downstream, presumably generating useful vestibular and optic flow cues while reducing the strength of lateral line cues. A second factor is the extent to which flow is spatially uniform (flow speed and direction everywhere the same) or non-uniform. From a biophysical point of view, the lateral line system is designed to respond to spatial non-uniformities in flow (Kalmijn, 1989). If the fish and surrounding water move together at the same speed and direction, as might be expected in a globally uniform flow of sufficient speed to displace the fish, non-uniformities would not arise and there would be no stimulus to the lateral line. However, if the spatial scale of a non-uniform flow can be registered within the width or length of the fish, the lateral line will theoretically be stimulated.

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List of abbreviations

BCF	blind cavefish
BL	boundary layer
CFD	cumulative frequency distribution
GLMM	generalized linear mixed model
LDA	linear discriminant analysis
LL-	lateral line disabled
LL+	lateral line enabled
RI	rheotactic index
RT	rheotactic threshold
TLC	three-lined corydorass

Methodological differences that may give rise to indirect rather than direct lateral line effects on rheotactic performance have also been overlooked. For example, several studies tested groups of fish (five to 20) simultaneously in a small, rectangular space (Montgomery et al., 1997; Baker and Montgomery, 1999a; Baker and Montgomery, 1999b; Suli et al., 2012). Under these circumstances, the packing density of multiple fish in a confined area might arguably lead to an orientation bias in the streamwise direction. Any disruption of this orientation preference in lateral-line-deprived fish could thus be due to an indirect impairment in their ability to maintain distance and orientation with respect to each other (rather than the current), as has been observed in lateral-line-deprived schools of fish (Partridge and Pitcher, 1980). As a second example, various lateral line blocking techniques may have had unintended effects – e.g. global toxic effects (Janssen, 2000) or incomplete blocking of intended receptors (Van Trump et al., 2010; Brown et al., 2011).

This study is designed to examine the effect of lateral line deprivation on rheotaxis while eliminating factors that may have given rise to indirect effects. We measure rheotactic performance and lateral line deprivation effects in two species, the three-lined corydorass (*Corydorass trilineatus* Cope 1872) and the blind cavefish (*Astyanax mexicanus*), under identical test conditions. The three-lined corydorass was chosen for its sedentary, benthic behavior, whereas the blind cavefish was chosen for its propensity to swim continuously near the substrate and its heavy reliance on non-visual senses, in particular the lateral line (reviewed in Montgomery et al., 2001). Given that blind cavefish have previously been shown to

exhibit lateral-line-dependent rheotaxis (Baker and Montgomery, 1999b), we also thought it was important to retest this species under conditions that minimize indirect effects. The results of the present study show that lateral line deprivation leads to a decrement in rheotactic performance in sedentary three-lined corydorass, but not in mobile blind cavefish. Furthermore, the rheotactic performance of lateral-line-enabled and -disabled corydorass could be distinguished on the basis of mobility and other metrics designed to quantify the sedentary–mobile continuum of activity. The results of the present study, in conjunction with those from past studies, suggest that sedentary behavior is an important factor in lateral-line-mediated rheotaxis at low flow speeds.

RESULTS**DASPEI verification of lateral line treatments**

The utility of DASPEI as a means of verifying the efficacy of streptomycin in blocking the lateral line was determined to be highly species-specific. Blind cavefish represented a ‘best-case’ scenario showing strong DASPEI labeling of both canal and superficial neuromasts in all but one sham-treated fish, but only a handful of faintly labeled neuromasts (<1%) in all streptomycin-treated fish (Fig. 1). Data from one sham-treated fish with sparse labeling were excluded from the behavioral analysis. By contrast, DASPEI labeling of three-lined corydorass was difficult to interpret for two reasons. First, DASPEI was sometimes taken up by numerous small structures, presumed to be chemosensory organs on the skin surface, making it difficult to discern small superficial neuromasts. Second, the more heavily pigmented skin of corydorass (compared with the pigment-free skin of blind cavefish) made it more difficult to see canal neuromasts. Despite these difficulties, it was always possible to identify DASPEI-labeled canal neuromasts in both species because of their smaller number, larger size and more stereotyped locations. In contrast, DASPEI-labeled superficial neuromasts were much more difficult to identify in corydorass. Thus, the presence or absence of DASPEI-labeled canal neuromasts (on both head and trunk) was our sole criterion for assessing lateral line functionality in corydorass. Based on this criterion, the lateral line system was judged to be impaired in all streptomycin-treated catfish and unimpaired in all sham-treated catfish.

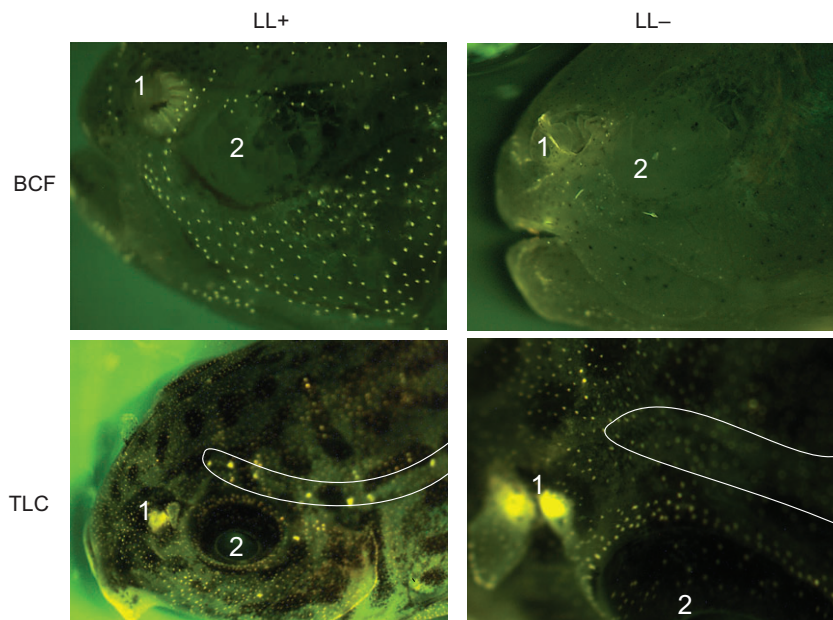


Fig. 1. DASPEI-labeled neuromasts in blind cavefish (BCF) and three-lined corydorass (TLC). Labeling of superficial and canal neuromasts in blind cavefish was strong for sham-treated (LL+, left panels) individuals, but undetectable for streptomycin-treated (LL-, right panels) individuals. In corydorass, numerous small structures, believed to be chemosensory in nature, were labeled in some individuals, making it difficult to distinguish these small structures from superficial neuromasts. Nevertheless, DASPEI labeling of canal neuromasts above the eye and along the trunk (outlined area) was observed in sham-treated, but not streptomycin-treated, individuals. 1, nares; 2, eye socket.

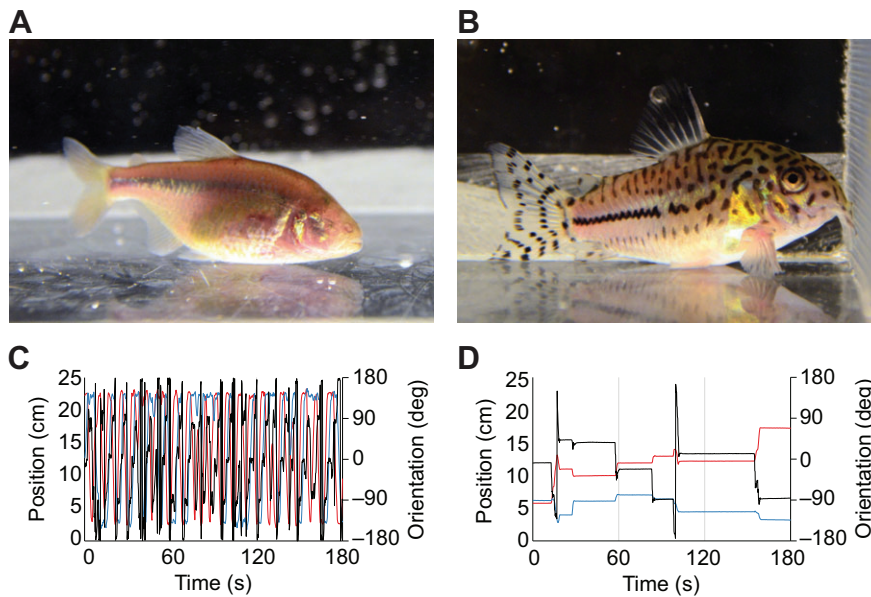


Fig. 2. Characteristic body posture and spatiotemporal dynamics. Typical postures (A,B) and temporal changes in the fish's orientation relative to upstream (black lines), crosswise (blue lines) and streamwise (red lines) position (C,D) of blind cavefish (A,C) and three-lined corydoras (B,D) near the substrate at flow speeds of 1 cm s^{-1} . Three-lined corydoras move infrequently, tending to rest motionless on the substrate for tens of seconds, while propped up on their pectoral and/or pelvic fins. Blind cavefish make transient contacts with the substrate while swimming in a burst and coast fashion, either parallel to the substrate or in a head-down posture.

Behavioral postures and movements of blind cavefish and three-lined corydoras

Casual observations of both blind cavefish and three-lined corydoras indicate that both species maintained positions close to the substrate, but that their movements and postures relative to the substrate were quite different (Fig. 2A,B). As previously documented, blind cavefish were observed to swim in a burst and coast fashion (Windsor et al., 2008; Tan et al., 2011), with the ventral surface of their body or head making light contact with the substrate and paired pectoral fins being kept largely off of the substrate (Fig. 2A). By contrast, corydoras were more likely to remain stationary on the substrate, with their head propped upwards by their paired pectoral and pelvic fins (Fig. 2B). Corydoras moved in a saltatory fashion, remaining motionless on the substrate for long periods of time (Fig. 2D), whereas blind cavefish swam continuously (Fig. 2C). GLMM of mobility indicated a significant interaction between flow speed and species ($F_{7,167}=2.387$, $P=0.024$). While mobility remained somewhat constant across flow speeds in blind cavefish, in corydoras it was very low at low flow speeds and increased at speeds above 3 cm s^{-1} (Fig. 3). Overall, blind cavefish were confirmed to be much more mobile than corydoras ($F_{1,25}=30.479$, $P<0.0001$). Finally, an effect of flow speed was observed, suggesting that mobility is flow-speed dependent, even after accounting for species and treatment effects ($F_{1,167}=2.725$, $P=0.011$).

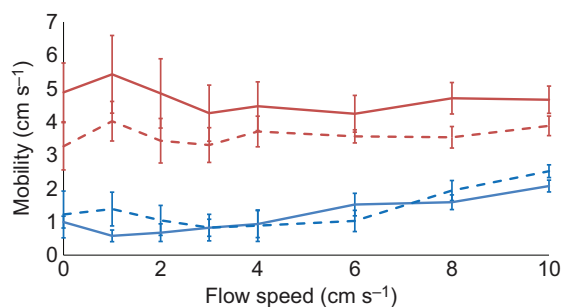


Fig. 3. Mean (±s.e.m.) mobility as a function of flow speed for sham-treated, lateral-line-enabled (solid line) and streptomycin-treated, lateral-line-deprived (dashed line) individuals of blind cavefish (red) and three-lined corydoras (blue).

Rheotactic performance

We begin this section with an overall description of rheotactic performance in each species as a function of flow speed and lateral line treatment. This is followed by a summary of (1) the main effects, as determined from the GLMM analysis and (2) the factors that best explain treatment effects in three-lined corydoras, as determined from the discriminant analysis.

The rheotactic index (RI) (Bak-Coleman et al., 2013) increased as a function flow speed for each treatment group in both species (Fig. 4). Furthermore, the increase in RI was much steeper and the overall magnitude of this index was much higher in three-lined corydoras compared with blind cavefish. The RI of fish in no flow did not differ significantly between lateral-line-enabled (LL+) and lateral-line-disabled (LL-) individuals for either blind cavefish (unpaired t -test: $t_{14}=-0.893$, $P=0.1931$) or three-lined corydoras (unpaired t -test: $t_{10}=-0.617$, $P=0.551$; Fig. 4). Therefore, RIs in no flow were pooled across treatment groups in each species to yield a mean (±s.e.m.) RI that was very close to zero (0.0018 ± 0.011 , $N=16$ for blind cavefish and -0.016 ± 0.0548 , $N=12$ for three-lined corydoras), suggesting no underlying directional bias. The criterion RI for determining the rheotactic threshold (RT) was two standard deviations above the no-flow pooled mean (0.09 for blind cavefish and 0.37 for three-lined corydoras). RT was significantly lower in LL+ corydoras (mean ± s.e.m.= 0.852 ± 0.189 , $N=6$) than LL-

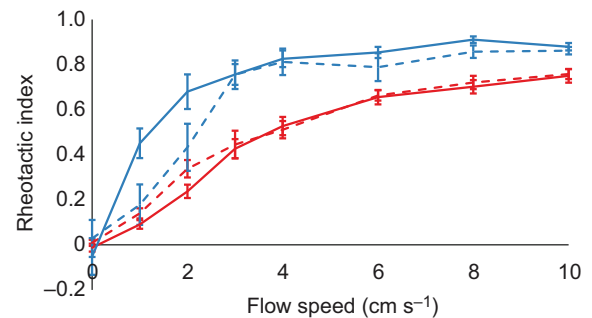


Fig. 4. Mean (±s.e.m.) rheotactic index as a function of flow speed for lateral-line-enabled (solid lines) and -disabled (dashed lines) blind cavefish (red) and three-lined corydoras (blue). While no effect of lateral line deprivation was observed in blind cavefish, a significant effect was observed in corydoras at 1 and 2 cm s^{-1} flow speeds.

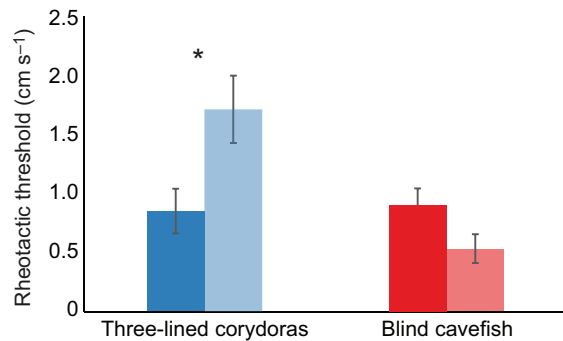


Fig. 5. Rheotactic threshold for sham-treated, lateral-line-enabled (LL+, darker shade) and streptomycin-treated, lateral-line-deprived (LL-, lighter shade) individuals of three-lined corydorass (blue) and blind cavefish (red). Rheotactic thresholds were significantly elevated ($*P < 0.05$) in LL- compared with LL+ individuals of three-lined corydorass but not blind cavefish.

corydorass (mean \pm s.e.m. = 1.86 ± 0.285 , $N=6$) (two-tailed Welch's unpaired t -test: $t_{8,69} = -2.506$, $P=0.034$; Fig. 5). In contrast, RT was slightly higher, though not quite significantly so (two-tailed unpaired t -test: $t_{14} = 2.00$, $P=0.06$), in LL+ blind cavefish (mean \pm s.e.m. = 0.90 ± 0.137 , $N=8$) than in LL- blind cavefish (mean \pm s.e.m. = 0.540 ± 0.120 , $N=8$; Fig. 5).

A GLMM analysis of RI indicated a significant interaction between flow, species and lateral line condition ($F_{32,41} = 3.966$, $P < 0.0001$). This interaction was characterized by a significant decrease in performance in LL- corydorass under the 1 cm s^{-1} ($t_{38} = -3.727$, $P=0.001$) and 2 cm s^{-1} conditions ($t_{35} = -2.948$, $P=0.001$; Fig. 4). At no other flow speeds was an effect of lateral line disruption observed (supplementary material Table S1). Additionally, a significant main effect of flow was observed ($F_{8,50} = 213.083$, $P < 0.0001$) and characterized by increased rheotaxis as a function of flow speed (Fig. 4). Finally, three-lined corydorass exhibited significantly higher overall rheotactic performance than blind cavefish, regardless of flow speed ($F_{1,23} = 43.236$, $P < 0.0001$) (Fig. 4).

To examine the lateral line effects in three-lined corydorass in greater detail, we performed a linear discriminant analysis (LDA) on the 1 cm s^{-1} dataset. This speed was chosen because it lies between the rheotactic threshold speeds for LL+ and LL- groups, and would thus provide information regarding behavioral differences at a flow speed where the effect resides. The model generated by the LDA was able to successfully discriminate 12 out of 12 fish (100%). The LDA (Table 1, Fig. 6) indicated that the strongest discriminating factors were mean vector strength (Batschelet, 1981) of fish orientations and streamwise positional

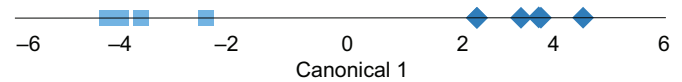


Fig. 6. Canonical plot showing the separation of lateral-line-enabled (dark blue diamonds) and -disabled (light blue squares) three-lined corydorass at the 1 cm s^{-1} flow speed on the basis of characteristics identified by the discriminant analysis (see Results, 'Rheotactic performance'). All six fish in each treatment group are plotted here, but nearly identical canonical values for pairs of fish obscure some of the markers.

variability. Higher vector strength (orientations narrowly distributed) and lower streamwise positional variability were more associated with LL+ fish. LL+ fish were also associated with lower levels of mobility, as well as mean angles closer to the upstream direction. Taken together, LL+ fish were characterized by decreased movement and increased rheotactic performance. In fact, RI is nearly a linear function of mobility in LL+ fish at low flow speeds (Fig. 7).

Space utilization

Spatial density plots of the combined frequency with which different fish spend time in different regions of the flow tank indicate that blind cavefish (Fig. 8A) and three-lined corydorass (Fig. 8B) utilized space differently, especially at the lower flow speeds. For example, blind cavefish spent more time near all four of the walls of the arena than corydorass (Fig. 8A). This behavior, known as wall-following, has been well documented in blind cavefish (reviewed in Sharma et al., 2009). GLMM indicated a significant decrease in wall proximity (distance to nearest wall) as a function of flow speed for blind cavefish ($F_{7,98} = 2.341$, $P=0.030$). Though non-significant, GLMM indicated that the wall-proximity of cavefish was affected by an interaction between flow and lateral line condition ($F_{7,98} = 1.945$, $P=0.070$). This is likely due to the wall-hugging tendencies of LL+ individuals at low flow speeds ($1-3 \text{ cm s}^{-1}$), which appear to be slightly stronger than those of LL- individuals (Fig. 8). That is, LL+ fish exhibit strong wall-following around the entire perimeter of the arena for flow speeds up to $\sim 3 \text{ cm s}^{-1}$, whereas LL- individuals stop wall-following at speeds above 1 cm s^{-1} , spending a higher percentage of their time near the upstream barrier. Finally, although not significant, GLMM indicated that LL+ individuals may be closer to walls overall than LL- individuals ($F_{1,14} = 3.313$, $P=0.090$). Finally, spatial density plots show that both species move further upstream as flow speed increases (Fig. 8). Conversely, corydorass (especially LL+ fish) appear to have a distinct preference for the downstream end of the tank at low flow speeds.

Table 1. Descriptive discriminant analysis

Variable	Coefficient		Mean	
	Standardized	Correlation	LL+	LL-
Mean streamwise position (cm)	2.72	-0.13	7.99	9.21
Mean cross-stream position (cm)	1.36	0.46	13.65	9.32
Streamwise s.d. (cm)	-5.25	-0.48	2.42	4.86
Cross-stream s.d. (cm)	-1.18	-0.08	3.42	3.73
r	-5.46	0.38	0.57	0.38
Orientation (deg)	-2.60	-0.71	8.74	46.29
Mobility (cm s^{-1})	-2.39	-0.48	0.58	1.38

r , Vector strength of orientation distribution; orientation, mean angle relative to upstream. Magnitude of standardized coefficients indicates relative importance in the discriminant function. Correlation coefficients indicate correlation between individual scores for a given variable and the corresponding score on canonical 1. LL+, lateral line enabled; LL-, lateral line disabled.

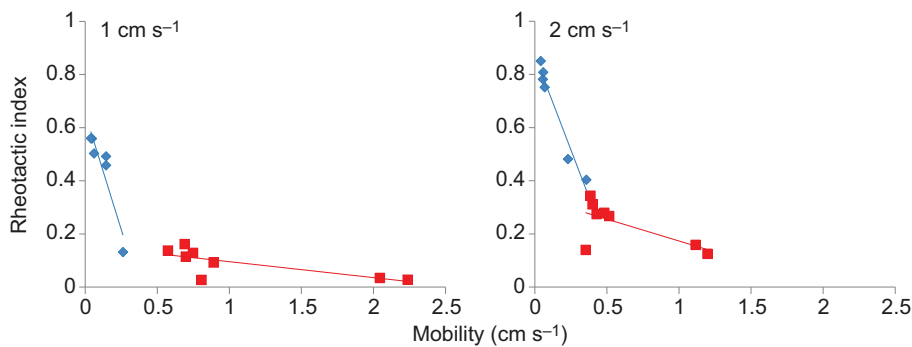


Fig. 7. The relationship between rheotactic index and mobility in lateral-line-enabled three-lined corydoras (blue diamonds) and blind cavefish (red squares). For corydoras, there is a strong relationship between rheotaxis and mobility at both 1 cm s⁻¹ ($r=0.93$) and 2 cm s⁻¹ ($r=0.97$). A similar relationship exists for blind cavefish at both 1 cm s⁻¹ ($r=0.73$) and 2 cm s⁻¹ ($r=0.67$), though the correlation is not quite as strong and the slope is much less steep.

DISCUSSION

Our study is the first to compare rheotactic behavior of a sedentary and a non-sedentary species under identical test conditions that eliminate some of the confounding factors that might give rise to indirect lateral line effects. This study provides several lines of evidence that immobility and, by implication, the degree to which fish are coupled to the substrate are important factors in determining the sensory basis of rheotaxis at low flow speeds. Lateral line deprivation caused a decrease in the rheotactic performance of visually deprived three-lined corydoras, especially at low flow speeds. Furthermore, the rheotactic performance of LL+ and LL- corydoras could be distinguished by mobility and similar metrics that measured 'sedentariness'. In contrast, lateral line deprivation caused no effect in blind cavefish at any flow speed, suggesting that these fish rely more heavily on other non-visual sensory modalities, namely tactile and vestibular cues.

Context dependency of multisensory cues

These findings can be interpreted in terms of the sensory cues available under different flow and behavioral conditions. As both vestibular and optic (when available) flow cues are generated during downstream displacement, fish that exhibit substrate coupling will not have access to these cues at flow speeds insufficient to cause

displacement. Moreover, substrate coupling should increase the signal to the lateral line as it maximizes flow across the skin surface, which, in turn, can provide fish with information about current speed and direction (Chaugnaud and Coombs, 2014). Conversely, fish that are not tightly coupled to the substrate will be more readily displaced downstream, thus generating useful optic flow and vestibular cues at the cost of a reduced stimulation of the lateral line. Thus, sedentary behavior may promote reliance on the lateral line by increasing the stimulus to the lateral line while reducing sensory redundancy provided by other modalities. The results of our discriminant analysis (Table 1, Fig. 6) on three-lined corydoras are largely consistent with this overall idea in that LL+ fish were characterized by increased positional and directional stability compared with LL- fish.

For fish that are mobile, other factors may also reduce the utility of the lateral line. For example, the octavolateralis efferent system is known to inhibit the lateral line during active swimming, presumably to reduce the effects of self-generated noise (Flock and Russell, 1976). For blind cavefish, which swim in a burst and coast fashion, the information to the lateral line is thus intermittent, being theoretically suppressed during the burst but not coast phase of the swim cycle, as evidenced by decreased obstacle avoidance ability during the burst phase of the swim cycle (Windsor et al., 2008). In

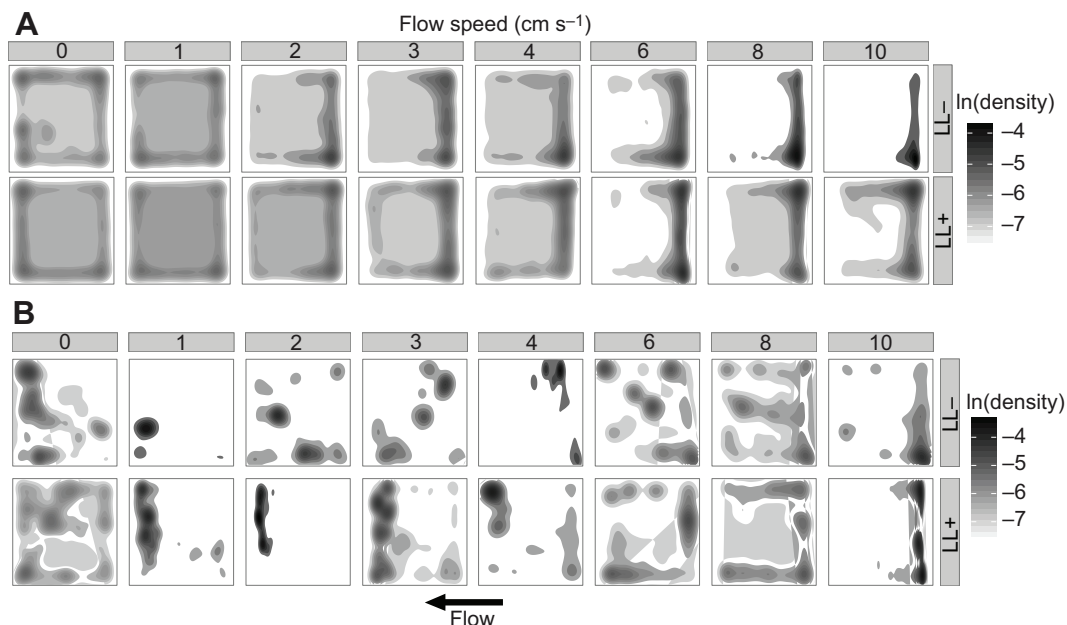


Fig. 8. Spatial density as a function of flow speed, species and lateral-line condition. Spatial density plots for blind cavefish (A) and three-lined corydoras (B). Each plot represents the combined positional data across all individuals within a treatment group (LL+, lateral line enabled; LL-, lateral line disabled) and for each flow speed.

the context of rheotaxis, the absence of continuous lateral line sensory feedback could cause heading estimation errors to accumulate at a faster rate. By contrast, three-lined corydoras would be expected to have continuous access to lateral line feedback during periods of immobility. Corydoras likely face a simpler sensory challenge, as the net movement between the fish and the surrounding water (the stimulus to the lateral line) is always caused by the movement of the surrounding water alone. In contrast, the net motion for mobile fish such as blind cavefish involves relative movements between the water and the fish, making the assessment of ambient flow direction more difficult.

Comparisons with other species

As predicted, the corydoras results of this study are consistent with lateral line deprivation effects reported in other species with similar benthic/sedentary behaviors (Montgomery et al., 1997; Baker and Montgomery, 1999a). In addition, Arnold (Arnold, 1969) provides some evidence that benthic flatfish (*Pleuronectes platessa*) also rely on lateral line information, as rheotactic responses were observed in the dark at flow speeds that did not displace the fish downstream. Similarly, epaulette sharks (*Hemiscyllium ocellatum*) exhibit rheotaxis when they are located on the benthos, but not when they are higher in the water column (Peach, 2001). Finally, our blind cavefish results are consistent with the absence of lateral line effects observed in other species with non-sedentary behaviors, such as the surface-dwelling giant danio (Bak-Coleman et al., 2013). In giant danio, lateral line deprivation did not cause a significant reduction in the rheotactic performance of visually deprived giant danio; however, it did alter the spatiotemporal dynamics of rheotactic behavior. Thus, while these fish can obviously compensate for the loss of lateral line information, they rely on different sensorimotor strategies to do so.

Although the lateral-line-dependent effect observed in the present study is similar to that observed in other benthic or sedentary species (Montgomery et al., 1997), the size of the effect in three-lined corydoras ($\sim 1 \text{ cm s}^{-1}$ shift in threshold) was relatively small compared with that reported for other species ($\sim 6 \text{ cm s}^{-1}$ shift for bald notothen, *Pagothenia borchgrevinki*, and a 2 cm s^{-1} shift for torrentfish, *Cheimarrichthys fosteri*) (Montgomery et al., 1997; Baker and Montgomery, 1999a). This may be accounted for by study-specific differences in how rheotactic performance was measured (see discussion of methodological differences below) or species-specific differences in the ability of fish to hold station (stay coupled to the substrate) (Webb, 1989; Blake, 2006). Resisting downstream displacement likely depends on a number of factors, including the mass and specific gravity of the fish, whether a gas-filled swimbladder is present, and other morphological and behavioral adaptations (e.g. body shape, fin angle, oral suckers, etc.) (Blake, 2006). Although measures of displacement resistance (e.g. slip speed and critical swim speed) (Webb, 1989; Pavlov et al., 2000) are unavailable for these species, it is noteworthy that the species with the largest threshold shift, the bald notothen, was considerably larger (14–21 cm in length) than the other two species (3–5 cm in length). Based on total mass alone, the bald notothen might be predicted to better withstand downstream displacement than the other species, and thus to rely more heavily on lateral line information over a wider range of flow speeds.

Given that blind cavefish (Characiformes) and three-lined corydoras (Siluriformes) are in different orders, other factors besides sedentary behavior could account for the observed differences in the importance of the lateral line to rheotaxis. For example, morphological or physiological differences in the lateral line system could lead to differences in sensitivity to flow. Indeed, corydoras

have lower RTs and higher RIs compared with blind cavefish (Fig. 4). However, an examination of inter-individual variation in RI and RT among and between treatment groups shows that: (1) RI is negatively correlated with mobility in both species and (2) mobility (and other metrics to quantify the sedentary–mobile continuum) can explain performance differences between LL+ and LL– populations of three-lined corydoras (Table 1, Fig. 6). Finally, lateral-line-mediated rheotaxis has been demonstrated in sedentary species from widely divergent taxa and ecological contexts: an Antarctic notothenioid marine species (bald notothen) that lives under the ice (Montgomery et al., 1997; Baker and Montgomery, 1999b), a freshwater New Zealand perciform species (torrentfish) that lives in torrential rivers (Montgomery et al., 1997) and a South American siluriform catfish (three-lined corydoras) that lives in tropical waters (present study). Likewise, the absence of lateral-line-mediated rheotaxis has been demonstrated in two unrelated non-sedentary species: a tropical diurnal cypriniform species (giant danio) from Southeast Asia (Bak-Coleman et al., 2013) and a characiform species (blind cavefish) that lives in lightless limestone caves of northeastern Mexico (present study). Thus, there is accumulating evidence to suggest that lateral-line-mediated rheotaxis is convergent across taxonomically diverse groups of sedentary species. This is not to say that sedentary behavior is the only factor that governs whether the lateral line is involved in rheotaxis.

Methodological differences impacting observed lateral line effects

Inconsistent with the predicted lateral line effects along the sedentary/non-sedentary dichotomy are three studies on two non-sedentary species (zebrafish and blind cavefish), all of which showed decrements in rheotactic performance after blocking the lateral line (Baker and Montgomery, 1999b; Suli et al., 2012; Olszewski et al., 2012). However, these studies differed from the present study in several important ways, including how flow was generated, the number of fish tested simultaneously, the shape of the flow tank, how the lateral line was blocked and the developmental stage of the fish.

In terms of flow generation, the present study utilized an impeller to circulate water in a closed-system circuit of nearly constant diameter throughout to minimize spatial heterogeneities in the flow (Vogel and LaBarbera, 1978). Under such spatially uniform flow conditions, fish displaced downstream are more likely to move at the same speed as the surrounding water, thus minimizing lateral line cues, while maximizing vestibular, tactile and optic flow cues (if visual conditions permit).

In contrast, previous studies on zebrafish (Suli et al., 2012) and blind cavefish (Baker and Montgomery, 1999b) used pumps to generate flow, which likely involved small-diameter inlet and outlet connections to the main flow chamber. Unpublished particle image velocimetry data from our lab (M. Kulpa and S.C., unpublished data) suggests that such an arrangement (even with collimators present) causes a high-velocity stream down the center of the tank, low flow in surrounding regions and steep velocity gradients in between. Furthermore, the third study (Olszewski et al., 2012), which employed a circular tank with a drain in the center, produced measurable velocity gradients, with velocity increasing in a radial direction towards the center of the tank. Velocity gradients may thus have facilitated lateral-line dependence in these cases.

Tank shape and the simultaneous testing of groups of fish may also have affected the probability of lateral line effects in previous studies (Baker and Montgomery, 1999a; Suli et al., 2012). These studies simultaneously tested five (Baker and Montgomery, 1999b) to 20 (Suli et al., 2012) fish in a tank that was much longer in the

streamwise direction than in the crosswise direction. Packing density effects may thus have caused fish to align along the streamwise axis of the tank. Under these circumstances, lateral line effects could be interpreted as a disruption in the abilities of fish to maintain their orientation and distance with respect to other fish in a confined area. Indeed, the lateral line has been shown to play a role in maintaining inter-fish distances (Partridge and Pitcher, 1980) and orientations (Faucher et al., 2010) in schooling fish. In this regard, it is worth noting that Olszewski et al. (Olszewski et al., 2012) tested solitary fish in a circular arena and in this case, tank shape and group effects can be ruled out as potential explanations for observed lateral line effects.

Another key difference between studies is the way in which the lateral line system was blocked. Blocking techniques used in rheotaxis studies fall into two general categories: (1) pharmacological techniques (typically streptomycin, gentamicin or CoCl_2) to block the transduction channels of lateral line hair cells and (2) physical ablation techniques in which the skin surface of the fish is scraped. Both CoCl_2 and streptomycin have been used in numerous studies to block the entire lateral line system (i.e. both canal and superficial neuromasts) (Karlsen and Sand, 1987; Blaxter and Fuiman, 1989; present study), whereas gentamicin and skin scrapes have been used to block canal and superficial neuromasts, respectively, while leaving the remaining submodality intact (Song et al., 1995; Montgomery et al., 1997; Baker and Montgomery, 1999a; Baker and Montgomery, 1999b). Recent studies utilizing DASPEI verification techniques have questioned the validity of gentamicin as a selective blocker of canal neuromasts, as DASPEI uptake was reduced in both submodalities of two species (zebrafish and blind cavefish) (Van Trump et al., 2010). Assuming that both submodalities are blocked by gentamicin and CoCl_2 alike, it is difficult to reconcile results from different treatment groups in the blind cavefish study by Baker and Montgomery (Baker and Montgomery, 1999b). That is, gentamicin treatments caused no decline in rheotactic performance [also confirmed by Van Trump and McHenry (Van Trump and McHenry, 2013)], whereas CoCl_2 and skin scrape treatments did. One possible explanation of what appears to be an irreconcilable set of results is that both CoCl_2 and skin scrape treatments caused more than just sensory deprivation effects – i.e. more global effects at the behavioral level – perhaps because of CoCl_2 toxicity (Janssen, 2000) and/or the repeated handling and anesthesia required for the skin scrapes (Baker and Montgomery, 1999b).

As a final point of difference between studies, zebrafish studies showing lateral-line-dependent rheotaxis were performed on larval fish (Suli et al., 2012; Olszewski et al., 2012), and it is unclear how the relative importance of various sensory modalities changes throughout the life history of fishes. In this regard, it is interesting to point out that the specific gravity of larval zebrafish decreases at approximately the same time as the swimbladder inflates (Robertson et al., 2007). According to Stewart et al. (Stewart, 2013), this should decrease the flow stimulus to the lateral line from a suction predator by as much as 80% (Stewart et al., 2013), suggesting that adult zebrafish may be less dependent on their lateral line than larval fish. The fact that adults of a closely related species (giant danio) do not rely on their lateral line for rheotaxis (Bak-Coleman et al., 2013) is consistent with this idea. Future studies of how the sensory basis of rheotaxis changes during development, especially during critical transition phases such as the inflation of the swimbladder, are needed.

Space utilization during rheotactic behaviors

As noted previously, both blind cavefish and three-lined corydoras spend more time upstream than downstream at the higher flow

speeds, when both species are presumably relying on vestibular and/or tactile cues instead of lateral line cues. At low flow speeds, when lateral line information is most likely to be available, LL+ corydoras show a distinct preference to stay near the downstream end of the tank. In this respect, it is well known that boundary layers are thicker at low than high flow speeds and that furthermore, boundary layer (BL) thickness increases as a function of increasing distance from the leading (upstream) edge of a flat plate in flow (Vogel, 1996) (supplementary material Table S2). Thus, if BLs along the walls and floor of our flow tank increase in thickness in the downstream direction, as predicted, then BL thickness may very well play a role in shaping the spatial preference of corydoras. Given that BL thickness (1–2 cm) is within the same range as the height or width of corydoras, it is reasonable to assume that vertical and horizontal velocity gradients within the BL of the tank floor and walls, respectively, are within range of detection by the lateral line of these fish.

In contrast to corydoras, which exhibit downstream spatial preferences in slow flows, blind cavefish exhibit distinct wall-following behavior – not only for slow flows ($<4 \text{ cm s}^{-1}$), but also in no flow (Fig. 8A). Wall-following is a well-documented behavior that occurs in visually deprived animals and is believed to be exploratory in nature (reviewed in Sharma et al., 2009). When fish are exposed to a novel environment in flow, they may be forced to choose between wall-following (thigmotaxis) and rheotaxis. Thigmotaxis appears to be slightly more distinct in LL+ blind cavefish, and this is consistent with evidence that wall-following behavior is mediated, at least in part, by the lateral line in blind cavefish (Patton et al., 2010; Windsor et al., 2011).

Potential trade-offs between thigmotaxis and rheotaxis are not well understood, and it remains possible that rheotactic performance was suppressed at these low flow speeds in lateral-line-enabled individuals. While this may account for the slight (but statistically insignificant) increase in rheotactic performance in lateral-line-disabled individuals (Figs 4, 5), the effect is subtle and unlikely to account for the large shift ($\sim 4 \text{ cm s}^{-1}$) in rheotactic thresholds previously noted for blind cavefish (Montgomery et al., 1997; Baker and Montgomery, 1999a). Furthermore, lateral-line-disabled fish in previous studies exhibited no evidence of rheotactic behavior below 16 cm s^{-1} , whereas the present study clearly shows that lateral-line-disabled fish exhibit some level of rheotaxis over most of the flow speed range (Fig. 4).

Conclusions

This paper presents strong evidence that rheotaxis in slow flows ($<3 \text{ cm s}^{-1}$) depends upon the lateral line under conditions unlikely to provide useable vestibular, optic flow or tactile slippage cues – i.e. in a species (three-lined corydoras) that is sedentary and coupled to the substrate. Moreover, rheotactic performance in this species was negatively correlated with the degree of mobility and, correspondingly, mobility was found to explain performance differences between LL+ and LL– individuals. In contrast, rheotaxis was found to be independent of the lateral line in a species (blind cavefish) that likely minimizes useable lateral line cues by exhibiting high levels of mobility near the substrate. By eliminating group testing, tank shape and blocking techniques as possible sources of indirect lateral line effects, the results of this study also help to resolve some of the discrepancy that has persisted in the literature. Finally, while this study establishes that the lateral line is utilized by benthic fish that rest motionless on the substrate, other factors besides sedentary behavior may govern the role of the lateral line in rheotaxis in other species and in other circumstances.

MATERIALS AND METHODS

Experimental overview

We compared the rheotactic performance of a benthic siluriform catfish (*Corydoras trilineatus*) with that of the epibenthic characid blind cavefish under identical test conditions. Fish of both species were evenly divided into a lateral-line-disabled and sham treatment groups. To rule out possible streamwise biases and other effects caused by simultaneous testing of multiple fish, both species were tested individually in an enclosed area of equal width, length and height (25 cm). A repeated-measures design was used in each treatment group to determine each fish's response to an ascending series of flow speeds from 0 to 10 cm s⁻¹ in a spatially uniform flow field. Both species were also tested in the dark under infrared light to eliminate visual cues and to maximize the potential for reliance on non-visual senses. Their behavior was video recorded and automatically tracked to document differences in mobility and to determine the effects of lateral line deprivation on rheotactic performance, both in terms of the degree to which they oriented upstream at different flow speeds and the minimum (threshold) flow speed required to elicit a rheotactic response.

Experimental animals

All fish were obtained from commercial aquarium suppliers, and housed in 76-l tanks (up to eight fish per tank) at ambient temperature (21–23°C) on a 12 h:12 h light:dark cycle. Fish were fed daily with commercially available TetraMin (Tetra, Melle, Germany) flakes and pellets. In total, 16 blind cavefish (42–50 mm in total length) and 13 three-lined corydoras (34–44 mm in total length) were used. All protocols for the maintenance, care and experimental use of the animals in this study were approved by the Bowling Green State University Institutional Animal Care and Use Committee.

Experimental setup

Fish were tested in the working area (25×25×25 cm) of a flow tank designed to produce spatially uniform flows (Vogel and LaBarbera, 1978) and used in previous rheotactic studies (Bak-Coleman et al., 2013). The working area was constructed out of translucent Plexiglas with flow-through dividers (Penn Plax, Hauppauge, NY, USA) on the upstream and downstream ends. The main body of the flow tank was a rectangular channel (154×28×35 cm). Water was cycled via a polyvinyl chloride tube (20.3 cm in diameter). Unidirectional flow was produced using a chem-stirrer (IKA Labortechnik RW 20DZM, Staufen, Germany) attached to a 12.7 cm aluminum impeller blade. Turbulence created by the impellers was reduced with three collimators: one coarse and one fine collimator at the upstream end and one coarse collimator at the downstream end. Coarse and fine collimators were constructed of large (1×3 cm) and small (0.5×3 cm) soda straws, respectively. Experiments were conducted in the dark (vision disabled) under diffuse, upwelling infrared light ($\lambda < 970$ nm).

The flow speed associated with a range of impeller speeds (60–600 rpm) was measured by videotaping traces of Methylene Blue dye delivered simultaneously via an array of three equally spaced, 20 gauge syringe needles at three different elevations (6.25, 12.5 and 18.75 cm) above the tank floor. The speed of each dye streak was calculated and averaged across the working area, yielding an average flow speed. A regression line was fitted to the flow speed versus motor speed data points across all elevations ($R^2=0.98$), and the motor speed required for each of the flow speed conditions was computed from the regression line. Dye streaks and particle imaging velocimetry, as described elsewhere (Elder and Coombs, 2013), confirmed that flow speed was spatially uniform in both horizontal and vertical planes with the exception of boundary layer (BL) effects near tank surfaces. BL velocity profiles were not measured but BL thickness was estimated from equations to describe BL development along a flat plate [eqns 8.2 and 8.3 in Vogel (Vogel, 1996)] (supplementary material Table S2). For these estimates, the characteristic length is given as the distance from the upstream collimator to the upstream (25 cm) and downstream (50 cm) edge of the working arena.

Disabling the lateral line system

The lateral line was disabled (LL- group) by immersing fish in a 5.5 l treatment tank containing 0.5 g l⁻¹ streptomycin sulfate for 3 h prior to

testing (Baker and Montgomery, 1999b). The lateral-line-enabled (LL+) group of fish was also immersed in a sham treatment tank without streptomycin for the same period of time. Lateral line blockage was verified using DASPEI after behavioral testing of each experimental animal in both treatment groups. DASPEI is a vital dye that is taken up by the transduction channels of lateral line hair cells (Meyers et al., 2003; Van Trump et al., 2010). Absence of dye uptake is an indication that the transduction channels, and thus the functional viability of hair cells, have been effectively blocked by the streptomycin treatment. Fish were immersed in a 0.008% DASPEI solution for 10 min. Fish were then anesthetized in a 0.01% buffered solution of MS-222 and observed under an epi-fluorescent stereomicroscope to determine whether lateral line neuromasts were labeled.

Experimental procedures

Fish were transferred from the sham or streptomycin treatment tank using a plastic-lined net to avoid damage to the lateral line. Fish were given a 30 min period (in the dark under infrared light) to acclimate before testing (video recording) began. A series of eight (0, 1, 2, 3, 4, 7, 8 and 10 cm s⁻¹) flow speeds were then presented to the fish in ascending order to reduce carryover effects resulting from prior exposure to a higher flow. For the no-flow control, the impeller motor was turned on with the gear disengaged to control for effects of motor noise. For each flow speed, 2 min were allotted for the flow to ramp up to the desired speed before behavior was recorded for 3 min. The flow speed was then accelerated to the next speed and the process was repeated until all speeds had been tested.

Data collection and analysis

Fish behavior in the flow tank was recorded at a rate of 5 frames s⁻¹, using a Sony Handicam (Sony, Tokyo, Japan) mounted above the center of the working area. The video was captured using video capture software (Winnov, Version 3.3, Santa Clara, CA, USA) in a nearby room. Video was then broken down into images and analyzed using custom-written tracking software (Butail and Paley, 2012; Bak-Coleman et al., 2013) as well as the Circular Statistics Toolbox for MATLAB (Berens, 2009) to determine the orientation of the fish relative to the upstream direction as well as the fish's location with respect to the walls of the tank. Because the elevation of the fish in the tank was not measured, there were potential depth-of-field errors in the estimated streamwise and crosswise position of fish. Maximum possible errors were measured to be less than 4% of the streamwise/crosswise length of the test arena. Finally, data were occasionally lost when the identifying characteristics of the fish were lost for several frames, e.g. when a fish swam in a vertical direction or when it rolled sideways. Thus the number of video frames per each flow speed trial ranged from a minimum of 777 to a maximum of 900 with an average of 898.

Rheotactic and mobility metrics

Rheotactic performance was measured with two metrics: the rheotactic index (RI) and the rheotactic threshold (RT). RI is an overall measure of the degree to which fish head upstream or downstream and is derived from the cumulative frequency distribution (CFD) of all observed fish headings (Bak-Coleman et al., 2013). RI is defined as the signed area between the theoretical CFD for uniformly distributed headings and the observed CFD, normalized so that it ranges between positive and negative 1. A value of ± 1 indicates that all fish headings are either directly upstream (+1) or downstream (-1), whereas a value of 0 indicates no upstream or downstream preference. Values between 0 and ± 1 indicate varying degrees in the dispersion of fish headings around the mean and/or in the deviation of the mean from the upstream or downstream direction. For a more complete description and evaluation of this metric, see Bak-Coleman et al. (Bak-Coleman et al., 2013).

The RT is based on the relationship between RI and flow speed and was defined as the lowest flow speed that causes the RI to rise two standard deviations above the mean RI measured for the no-flow condition. No-flow RI values were first compared across treatment groups within each species to ascertain that there were no orientation biases. In the absence of any orientation biases (RI ~ 0), these values were then pooled for each species across treatment groups to determine the mean no-flow RI and threshold

criteria. If the threshold value fell between two measured flow speeds, linear interpolation was used to estimate the threshold flow speed.

Finally, to quantify the mobility of fish, we measured the fish's displacement (positional change in the horizontal plane) between each consecutive video frame in units of cm s^{-1} . For a continuously swimming fish, the mean value of this metric is equivalent to the average swim speed, but for a sedentary fish, it represents an average of mobile and immobile (sedentary) periods of time, and thus is not an accurate measure of swim speed.

Data analysis

All statistical analyses were performed using R (R Development Core Team, 2013). Generalized linear mixed models (GLMM) were used for the bulk of the analysis, as they enable both fixed (flow speed, lateral line condition, species) and random (individual) factors to be examined under the repeated-measures design of this study. Full-factorial models looked for the effects of flow speed, lateral line condition and species on various dependent variables. *Post hoc* tests used sequential Dunn–Sidak to control for experiment-wise error arising from multiple comparisons. To test whether RI varied significantly between LL+ and LL– treatment groups in the no-flow condition, an unpaired *t*-test was performed for each species. The no-flow RI data were then pooled to calculate the arithmetic mean and associated standard deviation for each species. A two-tailed unpaired *t*-test was used to determine the effect of lateral line deprivation on rheotactic threshold. Rheotactic thresholds in corydoras appeared to have unequal variance, so a one-tailed unpaired Welch's *t*-test was employed. Upon finding a clear effect of lateral line deprivation in corydoras, a stepwise descriptive discriminant analysis was performed to determine the factors that best explained the LL+/LL– differences.

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Competing interests

The authors declare no competing financial interests.

Author contributions

S.C. and J.B.-C. designed this study, performed anatomical work, interpreted the findings, and participated equally in writing the manuscript. J.B.-C. performed the behavioral experiments, video analysis and statistical analysis.

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Supplementary material

Supplementary material available online at <http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.102574/-DC1>

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Table S1. *Post hoc* comparison of rheotaxis between LL+ and LL- fish

Flow (cm s ⁻¹)	Species	Contrast Estimate (RI _{LL+} - RI _{LL-}) ± 95% C.I.	S.E.	t(df)	Adj. Sig.
0	TLC	-0.056 ± 0.183	0.090	-0.625(31)	0.537
	BCF	-0.021 ± 0.159	0.078	-0.274(32)	0.786
1	TLC	0.269 ± 0.146	0.072	3.727(38)	0.001
	BCF	-0.049 ± 0.127	0.063	-0.781(38)	0.439
2	TLC	0.245 ± 0.169	0.083	2.948(35)	0.006
	BCF	-0.100 ± 0.147	0.072	-1.384(35)	0.175
3	TLC	-0.001 ± 0.138	0.068	-0.010(41)	0.992
	BCF	-0.019 ± 0.121	0.060	-0.315(41)	0.754
4	TLC	0.012 ± 0.113	0.056	0.206(41)	0.838
	BCF	0.016 ± 0.100	0.050	0.325(41)	0.747
6	TLC	0.074 ± 0.104	0.052	1.424(38)	0.862
	BCF	-0.008 ± 0.090	0.044	-0.176(35)	0.862
0	TLC	0.069 ± 0.104	0.050	1.374(22)	0.183
	BCF	-0.019 ± 0.092	0.044	-0.431(21)	0.671
10	TLC	0.033 ± 0.107	0.052	0.638(20)	0.530
	BCF	-0.008 ± 0.094	0.045	-0.172(20)	0.865

Pairwise comparisons of rheotactic performance (RI) between LL+ and LL- individuals for both corydoras (TLC) and blind cavefish (BCF). Dunn-sidak corrections were used to control for experimentwise error. Bold rows indicate significance. Contrast estimates represent the actual difference between the two groups (i.e. RI_{LL+} - RI_{LL-})

Table S2. Boundary layer thickness as a function of flow speed, characteristic length and associated local Reynolds number

Flow Speed (cm s⁻¹)	Characteristic Length (cm)	Local 'Re'	BL (cm)
1	25	2500	2.5
2	25	5000	1.8
4	25	10000	1.25
8	25	20000	0.9
10	25	25000	0.8
1	50	5000	3.5
2	50	10000	2.5
4	50	20000	1.8
8	50	40000	1.25
10	50	50000	1.1