Different functions for the lateral line in schooling behavior in three fish species

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ABSTRACT

Fish use vision and the lateral line in order to school together. Previous studies suggest that some species rely more than others on the lateral line for schooling, and no clear mechanistic reason for these differences has been identified. To address the question of how species-level differences in the lateral line might impact schooling behavior, we identified three schooling species (giant danios *Devario aequipinnatus*, cherry barbs *Puntius titteya*, and neon tetras *Paracheirodon innesi*) that come from different environments and have differently structured lateral line systems. We expected to see larger changes in schooling behavior in species of fish with more neuromasts. To test this hypothesis we imaged the lateral lines of these fishes, then quantified schooling behavior before and after lateral line ablation. Giant danios had the lowest density of neuromasts and, as expected, showed little response to ablation. Cherry barbs and neon tetras both had higher densities of neuromasts and schooled differently after ablation, but in opposite ways. Cherry barbs became a less cohesive school while neon tetras formed a more cohesive school. Both of these species had a greater density of neuromasts than giant danios, but the cherry barbs had a greater number of canal neuromasts. This difference in canal neuromasts in cherry barbs may explain the differences in schooling behavior after ablation if they depended more on them for schooling. Overall, these results shed light on the importance of the lateral line, and the diversity of roles it plays in fish schooling.

INTRODUCTION

Many fish spend their lives in schools (Shaw, 1978), a behavior that is driven by a need to avoid predators (Herbert-Read et al., 2017; Jeschke and Tollrian, 2007; Parrish, 1989; Rieucau et al., 2016; Ward et al., 2011), find food (Carrillo and McHenry, 2016; Schwalbe et al., 2012), and save energy while swimming (Ashraf et al., 2016; Reebs, 2000; Romenskyy et al., 2020; Zhang and Lauder, 2024). Fish rely primarily on two different senses, vision and the lateral line, to sense the other fish around them and maintain a school (Partridge and Pitcher, 1980; Tidswell et al., 2024).

Fish have a mechanosensory lateral line, a specialized sensory system that allows them to detect the movement of the water close to them. There are two main types of sensory receptors, called neuromasts, in the lateral line system: superficial and canal. Superficial neuromasts are found on the skin, while canal neuromasts are in canals under the scales that are open to the surrounding water via regularly spaced pores (Coombs et al., 2014). These two types of neuromasts have different roles in sensing the environment around a fish. Superficial neuromasts detect water velocity (McHenry et al., 2008), while canal neuromasts detect pressure differences between canal pores (Denton and Gray, 1989). This pressure differential allows them to sense spatial differences

in velocity such as wakes or vortex rings, allowing fish to pinpoint the movement of other creatures in the water with them Rapo et al. (2009).

There is a great deal of diversity among the lateral lines of fishes, but much of this variation is does not seem to correlate with functional or ecological differences among species (Coombs et al., 1988). In particular, it is unclear how much habitat or behavior drive evolutionary changes in lateral line structure. For a few, rather extreme morphologies, there is evidence that differences in neuromast density correlate with differences in environment. For example, blind cavefish (*Astyanax mexicanus*), have a much greater density of neruomasts as compared to their surface counterparts, indicating an evolutionary shift to favor the lateral line in absence of sight (Teyke, 1990). However, the results are less clear for non-cave dwelling species.

Work by Bleckmann (2009) studied twelve European Cypriniform fishes and found that there was significant variation in the density of superficial neuromasts both between and within species of fish. There were also significant differences in where on the body those neuromasts developed. While they had hypothesized a relationship between the number of neuromasts and whether these fish lived in flowing or still water, no such relationship was found (Bleckmann and Zelick, 2009). However, Spiller (2017) surveyed eight populations of the Australian western rainbowfish (*Melanotaenia australis*), and found a great deal of variation both between and within populations. In particular, the number of neuromasts was correlated with both the habitat of origin and availability of invertebrate prey. Additional work from the same lab (Kelley et al., 2017) found that Australian western rainbowfish reared in fast flowing water had more neuromasts on their tail fin, and fewer on their nasal region than fish reared in slower flow. A study by Nakae et al. (2022) found a 10% decrease in the number of lateral line neuromasts in thirteenth generation masu salmon (*Oncorhynchus masou masou*) as compared to wild masu salmon. These changes in the lateral line within a species indicates that environmental factors may be related to the development of the lateral line. However, the relationship between the structure of the lateral line and how fishes rely on it has not been established.

Moreover, it remains unclear how different fish species use their lateral line during schooling. Both rummy-nosed tetras (*Hemigrammus rhodostomus*) (Faucher et al., 2010; McKee et al., 2020) and yellow-eyed mullet (*Aldrichetta forsteri*) (Middlemiss, 2017) were unable to school with their lateral line ablated. However, work by Partridge and Pitcher (1980), Mekdara (2018), and Tidswell (2024) showed that saithe and giant danios could school without their lateral lines. This indicates that while the lateral line may be useful for schooling in some species, it may not be required for all species of fish.

Following our previous work (Tidswell et al., 2024), we hypothesized that these differences among species might be due to the natural environmental conditions of giant danios compared to the other species of fish. In a study with a similar setup to ours, McKee *et al.* (2020) found that rummy-nosed tetras schooled differently without their lateral lines. Rummy-nosed tetras are found in murky and slow-moving water where visibility is low (Lewis et al., 2000). Giant danios, on the other hand, are found in clear streams with higher visibility but with more natural interference from the flow and turbulence of rivers (Dey et al., 2014). This difference in habitat and flow structure might suggest that giant danios would rely less on their lateral line and more on vision, and would therefore be less affected behaviorally when their lateral line is ablated.

The goal of this current study was to examine the role of the lateral line in the schooling behavior of species with different lateral line structures. Specifically, we hypothesized species with a greater density of neuromasts would show larger changes to their schooling behavior when their lateral lines were ablated compared to species with fewer neuromasts. To this end we selected two species not previously tested in the lateral line ablation literature, cherry barbs (*Puntius titteya*), and neon tetras (*Paracheirodon innesi*). Both of these species are found in slow moving, turbid waters (Pethiyagoda, 1994; Saunders and Lewis, 1988); we therefore predicted that they would be more affected than giant danios by the ablation of their lateral lines. We expected that the cherry barbs and neon tetras would have more neuromasts than giant danios, and thus would exhibit less cohesive schooling kinematics (further apart and less polarized) after they were ablated. We used functional staining to measure the number of canal and superficial neuromasts along the body of these species to confirm that they showed differences from giant danios. We then recorded schooling behavior before and after lateral line ablation and measured the nearest neighbor distance (NND), polarization, speed, and number of schooling subunits. From these data we could determine if there were any behavioral changes caused by ablation of the lateral line.

MATERIALS AND METHODS

Animals

For this set of experiments, we used giant danios (*Devario aequipinnatus*), cherry barbs (*Puntius titteya*), and neon teras (*Paracheirodon innesi)*, which were purchased through LiveAquaria (LiveAquaria, Rhinelander, WI) and from PetSmart (PetSmart, Cambridge, MA). Before experiments, fish were kept in 40-liter aquarium tanks at 23°C and a conductivity of 400 µS, fed goldfish flakes daily (TetraFin, Blackburg, VA), and kept on a 12 hour night and day cycle. Cherry barbs and giant danios were only housed with conspecifics, but neon tetras were kept with zebrafish. Fish were selected and measured prior to experiments in order to determine body length for statistical analysis (Giant Danios, 8.32 ± 0.62 cm, n = 5; Cherry Barbs, 3.8 ± 0.47 cm, n = 5; Neon Tetras, 3.28 ± 0.23 cm, n = 5). All experiments followed an approved Tufts University IACUC protocol (M2021-99 and M2018-103).

Schooling Experimental Setup

Experiments were conducted under the same protocol as in our previous experiments with Giant Danios (Tidswell et al., 2024). Kinematics data from giant danios is the same as in (Tidswell et al., 2024) but plotted and analyzed differently.

For each day of the schooling experiments, eight individuals of one fish species were randomly selected from the pool of available fish. Experiments were conducted in a flow tank (Loligo Systems, Viborg, Denmark), and fish were constrained to a filming area that was 60 cm long, 25 cm wide, and filled to a water depth of 20 cm. Fish were given 10 minutes to adjust to the flow tank before filming began, and fish were filmed in 5 second bursts over a period of approximately an hour and a half. For the experiments with lateral line ablation, the fish were placed into gentamicin-treated water (0.001% in 5 liters, Sigma-Aldrich, St Louis, MO) for 24 h before the experiment began, following previously established protocols (Mekdara et al., 2018).

For each of these experiments, fish were filmed for five seconds at 60 frames per second (fps) using two high-speed cameras (Phantom M320, Vision Research, Wayne, NJ). This was done

multiple times for each set of experimental conditions. We used two synchronized cameras, which allowed us to reconstruct the 3D trajectories of all the fish in the school. The cameras were calibrated using open-source software DLTdv8 and the easyWand program (Hedrick, 2008; Theriault et al., 2014) running in Matlab R2020a (Mathworks, Natick, MA). We used the open-source software Deeplabcut (Lauer et al., 2021; Mathis et al., 2018; Nath et al., 2019) (version 2.2.3) as well as SLEAP (Pereira et al., 2022) (version 1.4.0) to train neural networks to track fish in each video. 3D fish positions were then triangulated with camera calibrations using DLT code written in Python (Python Software Foundation, version 3.9.12). For each species and ablated or non-ablated condition, four randomly chosen videos were processed, each at least 300 frames long, which corresponded to approximately 15 tailbeats.

Analysis of Schooling Behavior

School behavior was analyzed the same as in the previous paper (Tidswell et al., 2024) with the exception that we only digitized the head and midbody points on the fish. Due to the differences in fish size, we also calculated NND and number of subunits in centimeters instead of in body lengths. Subunits were determined in the same way as in (Tidswell et al., 2024), by creating a distance matrix between all the fish in the school and turning that into a node and edge graph connecting the fish that were within a given number of body lengths of each other. We then calculated the number of completely separate subnetworks using the Python package NetworkX (Hagberg et al., 2008) .

Specifically, we determined how many subunits the school was divided into for a given cutoff distance, and then calculated the percent of tailbeats for which the school was in a single group for a variety of distances. We could then see how aggregated the ablated and non-ablated schools were by comparing the percentage of time spent in a single group for each given cutoff.

Statistical Analysis

To determine if there was an effect of ablation on a the schooling metrics of a species we used preformed likelihood-ratio test from the package lmtest (Zeileis and Hothorn) (version0.9.40) to determine if the model that included ablation as a parameter was significantly better than the model without it.

Neuromast Staining

To visualize the lateral line system, fish were exposed to 4-(4-Diethylaminostyryl)-1-methylpyridinium iodide (4-di-2-asp; Sigma-Aldrich), a vital fluorescent dye. Live fish were immersed in water containing the dye for 10 minutes before being rinsed for 30 minutes and then anesthetized with buffered 0.02% tricaine methanesulfonate (MS-222, pH 7.4, Sigma-Aldrich). Fish were then imaged under a fluorescence microscope (Leica M165-FC, Leica Microsystems, Wetzlar, Germany) using a green fluorescent protein blue emission filter (γ=385 nm). We then captured images of the lateral line though multiple photos on a Nikon DSLR camera (Nikon Corporation, Tokyo, Japan), and the micrographic images were stitched together (using Procreate, Savage Interactive Hobart, Tasmania, Australia) to create a full view of the fish.

RESULTS

Neon tetras have the largest number of superficial neuromasts, while cherry barbs have the most canal neuromasts

Figure 1 shows images from 4-di-2-asp staining of a cherry barb, neon tetra, and giant danio, and Figure 2 shows the numbers of different types of neuromasts at different points along the body of the three species. Cherry barbs, giant danios, and neon tetras all differ from the others in terms of neuromast density, regardless of the type of neuromast, or where is was on the body ($p < 0.001$). The only exception was that the giant danios do not have a significantly different number of canal neuromasts than the neon tetras (Figure 2 B; $p = 0.075$). Overall, neon tetras had a greater density of neuromasts than both the cherry barbs and the giant danios over the whole body (Figure 2 A), as well as on just the head (Figure 2 C) and just the trunk (Figure 2 D). Cherry barbs, in turn, had a greater density of neuromasts than giant danios in all of these cases as well. The only place where this pattern reversed is for canal neuromasts, where cherry barbs have a greater density than the other two fish species (Figure 2 B).

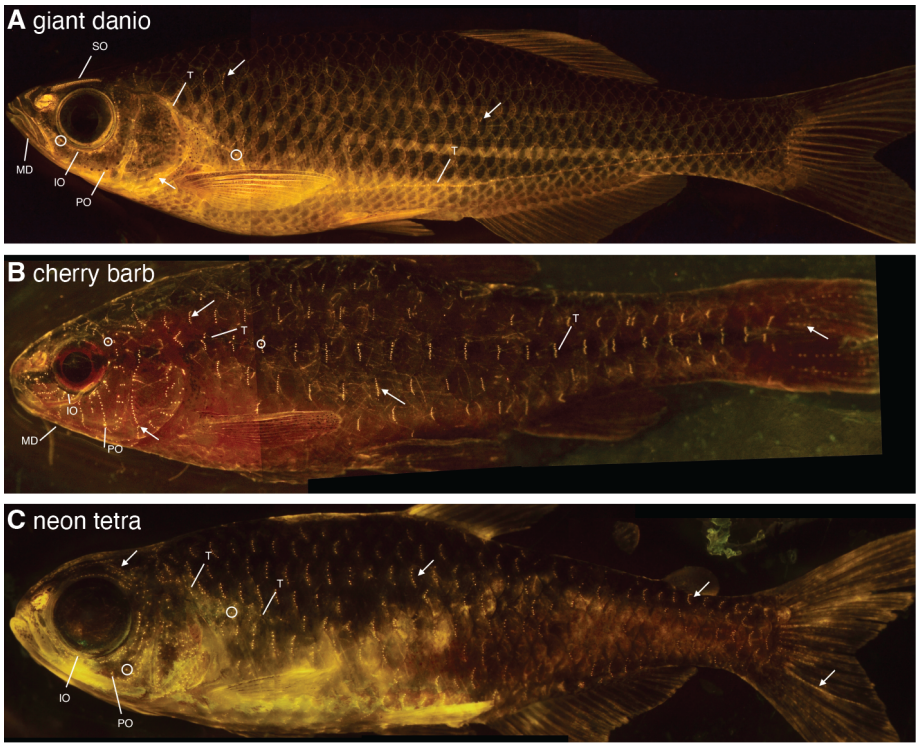

Figure 1: Adult giant danio (A), cherry barb (B), and neon tetra (C) stained with 4-di-2-asp, with neuromasts showing up as bright yellow dots. Examples of canal neuromasts are circled in white, while patches of superficial neuromasts are indicated with arrows. SO, supraorbital canal; IO, infraorbital canal; MD, mandibular canal; PO, pre-opercular canal; T, temporal canal, which extends laterally from head to tail along the trunk. The temporal canal runs the length of the trunk in the giant danio and cherry barb. In the neon tetra it only extends partially down through the trunk.

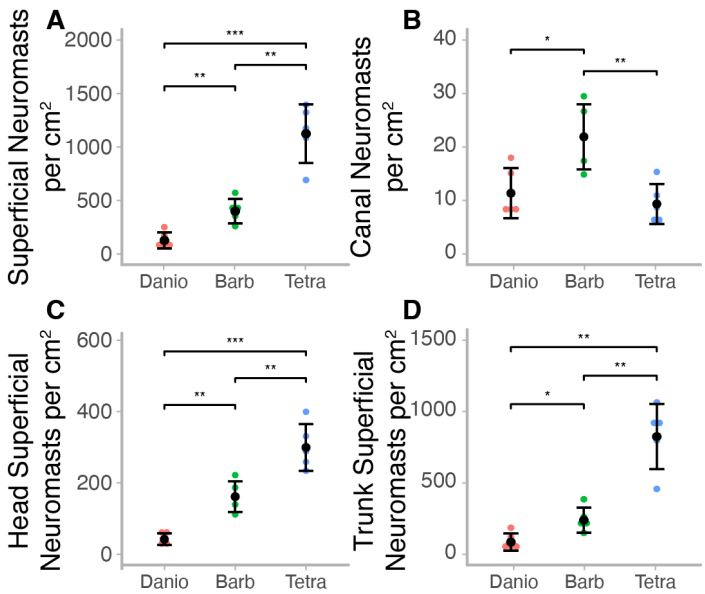

Figure 2: Giant danios had the smallest density of superficial neuromasts, while neon tetras had the most. The number of superfical (A) and canal (B) neuromasts per square centemeter was measured for 5 individuals of each species. The number of superficial neuromasts is subdivided into the density on the head (C) and trunk (D) of the fish. Each dot represents the count for a single fish with black dots and lines representing mean ± s.d. Significant differences ($p < 0.05$) between species conditions are indicated by asterisks (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

Without the lateral line, neon tetras improve at schooling while cherry barbs worsen, and giant danios stay the same

Performing ablation experiments on these three species of fish allowed us to address their reliance on the lateral line when schooling. Figure 3 shows the changes to these schools with and without their lateral lines. Most striking is that, in contrast to giant danios, both the cherry barbs and the neon tetras differed significantly after ablation for nearly every schooling metric we measured. However, barbs and tetras changed in opposite ways. When ablated, neon tetras became more polarized and grouped closer together, forming a tighter school, while cherry barbs became less polarized and more spread out, splitting into more subunits.

The nearest neighbor distance of giant danios did not change significantly (Figure 3 A and Table 1; $p = 0.331$) while cherry barbs (Figure 3 A and Table 1; $p = 0.013$) swam further away from their neighbors, and neon tetras (Figure 3 A and Table 1; $p = 0.048$) both swam closer together after being ablated. Giant danios did not change polarization after ablation, but cherry barbs became less polarized with their lateral line ablated (Figure 3 B and Table 1; $p = 0.048$) and neon tetras became more polarized (Figure 3 A and Table 1; $p = 0.002$). Abalated and control giant danios swam at the same speed (Figure 3 C and Table 1; $p = 0.663$) while both cherry barbs (Figure 3 C and Table 1; $p < 0.001$) and neon tetras (Figure 3 C and Table 1; $p < 0.001$) swam faster when their lateral line was ablated.

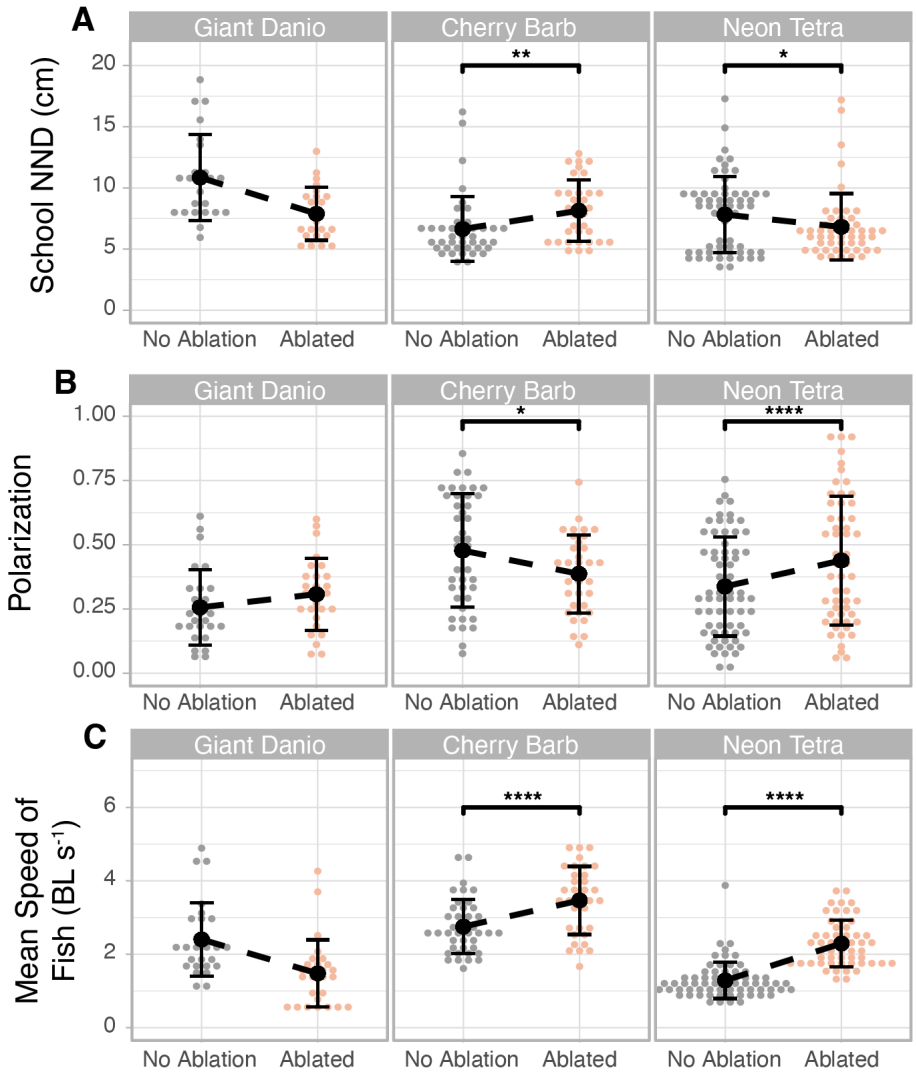

Figure 3: Giant danios showed no changes from lateral line ablation, while cherry barbs and neon tetras changed their behavior. The nearest neighbor distance (NND) (A), polarization (B), the mean speed of the fish (C), were measured with and without lateral lines ablated. Each dot represents the mean value for the school during one tailbeat, with black dots and lines representing mean ± s.d.. Significant differences ($p < 0.05$) between sensory conditions are indicated by asterisks (* $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$. Data for the giant danios is replotted from (Tidswell et al., 2024).

For each distance cutoff we calculated what percent of the time the school spent in a single group with individuals separated by less than that distance, instead of split off into two or more subunits. We saw that giant danios formed a single group the same amount of time both with and without their lateral line, regardless of the distance (Figure 4 and Table 1; $p = 0.822$). When they were ablated, cherry barbs were in a single group less often than the control cherry barbs (Figure 4 and Table 1; $p < 0.001$). In contrast, when they were ablated, neon tetras spent more time in a single group (Figure 4 and Table 1; $p < 0.001$), though there isn't a significant difference between the number of subgroups at a smaller connecting distance.

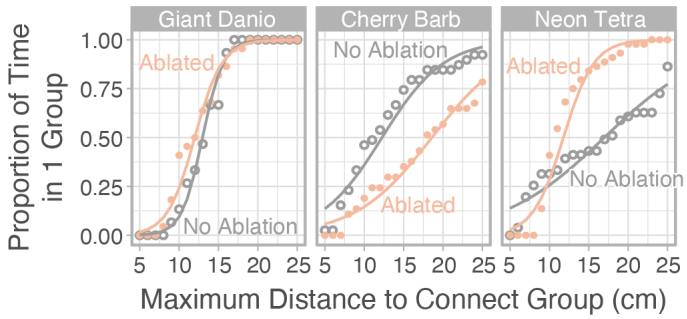

Figure 4: Giant danios showed no changes from lateral line ablation, while cherry barbs swam further apart and neon tetras swam closer together. Dots represent the mean proportion of time that fish schools of a given species, ablated or not, were in one single group for a given distance cutoff between groups. A larger proportion for a given distance indicates that the fish were all grouped closer together for that condition. The lines are from binomial GLMs fit to the data, and illustate the differnces between ablated and non-ablated schools for each species of fish.

Table 1: Results of the likelihood-ratio tests of linear models for each species with and without ablation. "Direction" refers to the change of the ablated fish relative to the control fish. P-values show differences relative control condition. Significant effects are shown in bold.

Parameter	Species	Direction	χ^2	P-Value
NND	Giant Danio	-	3.01	0.33
NND	**Cherry Barb**	↑	**9.67**	**0.01**
NND	**Neon Tetra**	↓	**7.04**	**0.05**
Polarization	Giant Danio	-	2.22	0.41
Polarization	**Cherry Barb**	↓	**7.02**	**0.05**
Polarization	**Neon Tetra**	↑	**13.06**	**<0.01**
Speed	Giant Danio	-	0.94	0.66
Speed	**Cherry Barb**	↑	**28.50**	**<0.01**
Speed	**Neon Tetra**	↑	**65.80**	**<0.01**
Groups	Giant Danio	-	0.05	0.82
Groups	**Cherry Barb**	↓	**326.90**	**<0.01**
Groups	**Neon Tetra**	↑	**129.87**	**<0.01**

DISCUSSION

This study addressed the role of the lateral line in the schooling behavior of different fishes. As in our previous paper (Tidswell et al., 2024) we found that the schooling of giant danios was not affected by the ablation of their lateral line neuromasts. However, both cherry barbs and neon tetras schooled substantially differently without their lateral line. Both species swam significantly faster without their lateral lines. But in terms of school structure, each species changed in a different way. Cherry barbs moved further from their neighbors, split into more groups, and became less polarized, while neon tetras did exactly the opposite. Overall, cherry barbs became a less cohesive school without their lateral lines, while neon tetras became a more cohesive school.

We selected neon tetras and cherry barbs, two species of fish from slow moving, turbid waters, expecting them to have a higher density of neuromasts compared to giant danios, and we therefore hypothesized there would be more drastic changes in behavior when their lateral lines were ablated. As expected, these two species both had a much high density of neuromasts than the giants danios and, as hypothesized, their behavior changed more than danios when their lateral lines were ablated.

Similar species-specific differences might help explain some of the differing reports in the literature on how much fish rely on the lateral line for schooling. Work by Partridge and Pitcher (1980) and Mekdara et al. (2018) found that saithe and giant danios could school without their lateral lines, but Faucher et al. (2010) and McKee et al. (2020) both found that rummy-nosed tetras were unable to school properly without their lateral lines. Chaput (2023) and Middlemiss et al. (2017) found similar results for zebrafish (*Danio rerio*) and yellow-eyed mullet (*A. forsteri*). It may be that the saithe and the giant danios have fewer neuromasts than the other species that did show significant effects from ablation.

However, our results from the cherry barbs and the neon tetras were not so clear-cut, as the neon tetras surprised us by schooling more cohesively after ablation, becoming more polarized and forming a closer aggregation. We speculate that lower nearest neighbor distances and greater polarization may be necessary for them to use visual information precisely enough to maintain the school. In contrast, the cherry barbs had the response we expected from ablation: a looser, less polarized school. We speculate that these behavioral effects may be due to differences in the structure of the lateral lines in the two species. Specifically, the cherry barbs had a mean density of canal neuromasts 2.8 times greater than than next closest species, the Cherry Barbs (Fig. 2B). In fact, neon tetras only have a partial trunk canal, which terminates halfway down the body. Canal neuromasts sense acceleration or pressure differences in surrounding water (Denton and Gray, 1989; Kalmijn, 1988), which may be more useful to pinpoint the movement of other fish around them, because the vortices in the wakes of other fish produce changes in pressure at a relatively high spatial and temporal scale, best sensed by canal neuromasts (Rapo et al., 2009). It may be that cherry barbs, with their more developed lateral line system, are also more affected by its absence. The neon tetras, with their shorter canal, may be less reliant on their neuromasts in order to school.

These differences between cherry barbs and neon tetras raise new lines of inquiry that could be addressed in three ways. First, how well do these species school in lower light levels, or even in darkness? Presumably, if the cherry barbs are more reliant on their lateral line for schooling, they might also be less reliant on vision, particularly at close distances, so they might be able to school better in darkness. It would also be informative to investigate the interactions between two

individual fish, similar to the work done by Calovi et al (2018), both with and without their lateral lines. Without all of the interactions of a school, this might allow us to better understand the attraction and alignment effects of the lateral line. Finally, it might also be informative to only ablate only a particular type of neuromast. Recently, Mekdara et al. (2022) showed that it may be possible to ablate superficial neuromasts without damaging canal neuromasts by immersing fish in streptomycin for a short amount of time. If the canal neuromasts are particularly important for schooling, we would expect that ablating only the superficial neuromasts in cherry barbs would not affect their schooling substantially.

Beyond looking a mechanistic explanations for the differences between cherry barbs and neon tetras, a better understanding of the differences in the environment and ecology around these fish might be revealing. It may be that their predators use different attack strategies, or they have to deal with different levels of flow and turbidity in the water around them. Due to this, the neon tetras may have a lateral line more focused on predator detection, while the cherry barb lateral line may be used more for schooling. Further study of into the natural habitats of these species could provide important context, even though we know that fish in captivity show changes to their lateral lines and behavior over generations (Nakae et al., 2022). Lab experiments focused on their schooling response to turbidity, predators, flow, and turbulence could reveal some important differences in their schooling behavior.

Overall, our results show that different species rely on their lateral lines differently during schooling. There is no one way to school, and future studies should account for the natural history and ecology of their study species before attempting to make generalizations across many species.

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Data and Resource Availability

Tracked data and processing code will be made available through Zenodo and Github. Original videos are available from the authers by request.

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