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RESEARCH ARTICLE

The effects of lateral line ablation and regeneration in schooling giant danios

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ABSTRACT

Fish use multiple sensory systems, including vision and their lateral line system, to maintain position and speed within a school. Although previous studies have shown that ablating the lateral line alters schooling behavior, no one has examined how the behavior recovers as the sensory system regenerates. We studied how schooling behavior changes in giant danios, *Devario aequipinnatus*, when their lateral line system is chemically ablated and after the sensory hair cells regenerate. We found that fish could school normally immediately after chemical ablation, but that they had trouble schooling 1–2 weeks after the chemical treatment, when the hair cells had fully regenerated. We filmed groups of giant danios with two high-speed cameras and reconstructed the three-dimensional positions of each fish within a group. One fish in the school was treated with gentamycin to ablate all hair cells. Both types of neuromasts (canal and superficial) were completely ablated after treatment, but fully regenerated after 1 week. We quantified the structure of the school using nearest neighbor distance, bearing, elevation, and the cross-correlation of velocity between each pair of fish. Treated fish maintained a normal position within the school immediately after the lateral line ablation, but could not school normally 1 or 2 weeks after treatment, even though the neuromasts had fully regenerated. By 4–8 weeks post-treatment, the treated fish could again school normally. These results demonstrate that the behavioral recovery after lateral line ablation is a longer process than the regeneration of the hair cells themselves.

KEY WORDS: Lateral line system, Neuromast, Hair cell, Schooling behavior, Fish

INTRODUCTION

Fishes make up about 50% of all existing vertebrate species, and most of them have a sensory system called the mechanosensory lateral line that detects flow in the water (Coombs and Van Netten, 2005; Mogdans and Bleckmann, 2012; Coombs et al., 2014; Webb et al., 2014). In fish, the lateral line system is involved in many behaviors such as detection of predators (McHenry et al., 2009; Stewart et al., 2013; Nair et al., 2017) and prey (Schwalbe et al., 2012; Carrillo and McHenry, 2016; Schwalbe et al., 2016), rheotaxis (Patton et al., 2010; Oteiza et al., 2017), obstacle avoidance (Teyke, 1985; Windsor et al., 2008), and local interactions within groups of fish (Partridge and Pitcher, 1980; Coombs and Van Netten, 2005; Chicoli et al., 2014; Coombs et al., 2014). In particular, most fishes and other aquatic amphibians seem to rely on the lateral line system, along with the visual system, for schooling behavior (Pitcher et al., 1976; Partridge and Pitcher, 1980; Mogdans and Bleckmann, 2012; Coombs et al., 2014).

Many species of fish school during some stages in their lives (Shaw, 1960, 1976). They benefit from schooling in many ways: it helps them detect and avoid predators, it increases foraging and spawning opportunities, and it reduces the energetic cost of swimming (Partridge and Pitcher, 1980; Bleckmann, 1986; Inada and Kawachi, 2002; Killen et al., 2012; Chicoli et al., 2014; Marras et al., 2015; Chivers et al., 2016; Daghooghi and Borazjani, 2016). A school also helps to position individuals so that they can most quickly respond to their neighbors. For most schooling species, individual fish prefer to maintain a distance of about one body length to their nearest neighbor (Partridge and Pitcher, 1980; Faucher et al., 2010; Middlemiss et al., 2017). Not only do fish swim together and maintain a specific individual distance, but the entire school can perform complicated maneuvers requiring individuals to respond quickly to changes in speed and directions of their neighbors (Partridge and Pitcher, 1980; Chicoli et al., 2014). For example, when schools of giant danios (*Devario aequipinnatus*) evade predators, the waves of response through the school can spread faster than the speed of the approaching predator (Chicoli et al., 2014). The fact that many species of fish form schools suggests that the behavior offers a clear evolutionary advantage (Greenwood et al., 2013; Kowalko et al., 2013). Yet, we are only just beginning to understand how fish form and maintain schools, and the roles of the lateral line system and vision in this behavior (Partridge and Pitcher, 1980; Pitcher et al., 1976; Faucher et al., 2010; Chicoli et al., 2014; Middlemiss et al., 2017).

The lateral line system is composed of a spatial array of neuromast receptor organs located on the head (anterior lateral line) and along the body (posterior lateral line). Neuromasts have two main types (Webb et al., 1995; Coombs et al., 2014; Webb and Ramsay, 2017): superficial neuromasts, which are located on the skin; and canal neuromasts, which are located within bony canals on the head or within specialized scales along the trunk. Each neuromast consists of hair cells with stereocilia and a kinocilium housed in gelatinous cupula, similar to the hair cell structures found in vertebrate auditory and vestibular systems (Kalmijn, 1988; Coombs et al., 2014). Superficial neuromasts respond to water velocity, while canal neuromasts, due to their location within canals, respond to pressure gradients or water acceleration (Kroese and Schellart, 1987; van Netten and Kroese, 1987; Denton and Gray, 1988; Kalmijn, 1988; Coombs et al., 2014). Superficial neuromasts also tend to contain fewer hair cells but are more abundant on the body and are more sensitive to lower frequency signals, while canal neuromasts may contain hundreds of hair cells and are more sensitive to higher frequencies (Kalmijn, 1988; Mogdans and Bleckmann, 2012; Coombs et al., 2014). These neuromasts are arranged in an array laterally from head to tail, and are generally innervated by two

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separate lateral line nerves, which separates the system along the anterior lateral line nerve and the posterior lateral line nerve (Coombs et al., 2014).

Researchers often study the role of the lateral line system in a behavior by ablating it and observing changes in the behavior (Partridge and Pitcher, 1980; Ayali et al., 2009; Faucher et al., 2010; Schwalbe et al., 2012). Heavy metal ions, such as cobalt chloride, and aminoglycoside antibiotics, such as gentamycin, are toxic to hair cells in the neuromasts (Karlsen and Sand, 1987; Van Trump et al., 2010). After these chemical treatments, the hair cells regrow over a few days to a few weeks (Harris et al., 2003; Faucher et al., 2010; Monroe et al., 2015). The posterior lateral line nerve can also be surgically severed to disable the trunk lateral line.

Disabling the lateral line system, either chemically or surgically, does not prevent fish from schooling, but it does change the position they maintain within a school (Pitcher et al., 1976; Partridge and Pitcher, 1980; Faucher et al., 2010; Greenwood et al., 2013; Kowalko et al., 2013; Chicoli et al., 2014; Middlemiss et al., 2017). Partridge and colleagues (Pitcher et al., 1976; Partridge and Pitcher, 1980) performed a set of experiments in the 1970s and 1980s to test the role of the lateral line and vision in schooling in saithe, *Pollachius virens*. They surgically disabled the posterior lateral line and found that saithe could still school, but they stayed closer to their neighbors (Partridge and Pitcher, 1980). In that study, Partridge and Pitcher (1980) severed the trunk lateral line nerve as it exited the skull, disabling the posterior lateral line system, but leaving a functional anterior lateral line system. After the trunk lateral line ablation, startling the saithe school caused collisions among the individuals within the group with strong enough forces to stun the fish (Partridge and Pitcher, 1980). They concluded that fish use the lateral line system to maintain their positions within the school, but that disabling it does not prevent the schooling behavior.

Partridge and Pitcher's studies (Pitcher et al., 1976; Partridge and Pitcher, 1980), although pioneering, did not inactivate the entire lateral line system. The anterior lateral line system, generally well developed in schooling species (Webb et al., 1995; Coombs et al., 2014), was fully functional. Furthermore, more recent studies have shown that the entire lateral line system plays a major role in determining the distance and direction of a stimulus (Janssen and Corcoran, 1998; Middlemiss et al., 2017). For example, when firehead tetras (*Hemigrammus bleheri*) are deprived of the entire lateral line system, they cannot maintain a school and swim at a greater distance from their nearest neighbor (Faucher et al., 2010).

These previous studies have established that the lateral line is important for maintaining a normal position within a school. In this study, we describe not only the effect of ablating the entire lateral line system, but also the process of recovering the ability to school. We examined three-dimensional (3D) schooling behavior in giant danios (*D. aequipinnatus*) immediately after ablation and during regeneration of the lateral line system, and we incorporated the use of velocity cross-correlations and auto-correlations to determine how treated fish behave in a group of normal fish. The schooling behavior may recover over a different time period than the regeneration of the hair cells themselves. If the behavior takes longer to recover than the hair cells, then it may have implications for how the sensory information is relayed to the brain or how it is processed in the brain.

MATERIALS AND METHODS

Animals

We studied schooling in giant danios, *Devario aequipinnatus* (McClelland 1839), because they are common river schooling fish native in tropical regions that are used in many aquariums, are commercially available (LiveAquaria, Rhinelander, WI, USA), are easy to maintain in the laboratory, and have been previously studied (Parrish et al., 2002; Viscido et al., 2004; Butail and Paley, 2012; Chicoli et al., 2014). Before experiments, fish were maintained and housed in four groups of 25 fish in 40 liter aquarium tanks at 23°C and a conductivity of 400 µS, fed goldfish flakes daily (TetraFin, Blackburg, VA, USA), and kept on a 12 h:12 h light:dark cycle. All experiments followed an approved Tufts University IACUC protocol (M2012-145 and M2015-149).

Experimental set-up

Twenty groups of giant danios (five fish per group) were filmed during schooling experiments conducted in a large circular tank (120 cm diameter, 85 cm height) filled to a depth of 60 cm of tank water that matched the water quality of their home tank. In each group, one individual was randomly selected for a gentamycin treatment (0.001% for 24 h, 5 liters; Sigma-Aldrich, St Louis, MO, USA) or a sham treatment (normal tank water for 24 h, 5 liters). To identify the treated fish, visible implant elastomers (Northwest Marine Technologies, Tumwater, WA, USA; Olsen and Vøllestad, 2001) were injected into the dorsal region of the treated fish for tracking 7 days prior to experiments. Trials were recorded at six time points relative to this treatment: 1 week before, immediately after, and 1, 2, 4 and 8 weeks following the treatment (referred to as weeks −1, 0, 1, 2, 4 and 8, respectively). In every schooling trial at each of the different time points, one treated fish (gentamycin or sham) schooled in a group with four normal, untreated fish. All fish completed a pre-treatment trial (week −1) to obtain a baseline of schooling behavior. For each time point and treatment type, ten groups of fish were filmed for a total of 5 min with three to five replicate trials.

The schooling behavior was recorded at 50 frames per second using two high-speed cameras (Phantom M120, Vision Research, Wayne, NJ, USA; PCO.edge, PCO AG, Kelheim, Germany). The two cameras allowed for reconstruction of 3D trajectories of each fish in the school and were synchronized by a common trigger. The camera's focal lengths, distortions and working distance were calibrated using standard techniques in open source software (easyWand; Theriault et al., 2014). Open source 3D tracking software (DLTdv5; Hedrick, 2008) was used to track the snout of the fish and compute its 3D position.

Visualization of the lateral line system

To visualize the entire lateral line system and determine the viability of hair cells within neuromasts, fish were exposed to a vital fluorescent dye in tank water at each of the six time points immediately after behavioral trials. Treatment and sham fish were stained with 63 mmol l^{-1} 4-(4-diethtylaminostyryl)-1-methylpyridinium iodide (4-di-2-asp; Sigma-Aldrich) solution for 5 min immediately following the removal from the experimental tank. 4-di-2-asp is a non-toxic mitochondrial fluorescent stain that can be used to target nerve terminals (Magrassi et al., 1987). At week −1, one gentamycin-treated fish and one sham-treated fish were randomly selected for fluorescent staining to confirm normal, positive neuromast staining. During the following weeks, one gentamycin-treated fish and one sham-treated fish were stained each week to assess the regeneration of the neuromasts. All stained fish were anesthetized with buffered 0.02% tricaine methanesulfonate (MS-222, pH 7.4, Sigma-Aldrich) and quickly imaged under a fluorescence microscope (Leica M165-FC, Leica Microsystems,

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Wetzlar, Germany) using a green fluorescent protein blue emission filter (γ=385 nm). Some fish (N=9) were euthanized (overdose of buffered MS-222) for high-quality images at the different time points. Multiple images of each fish were captured using a Nikon DSLR camera (Nikon Corporation, Tokyo, Japan), and the micrographic images were stitched together (using Adobe Photoshop CS, Ottawa, Ontario, Canada) to create a complete view of the fish.

Analysis of schooling behavior

Distance between each fish and all other fish within in the group was calculated from the 3D coordinates determined by computer-tracked videos. Fig. 1 shows a schematic of the values we calculated. Following Partridge and Pitcher (1980), we calculated bearing (θ) and elevation (ϕ) angles, and nearest neighbor distance (NND). The bearing is the angle on the horizontal plane between two fish, and the elevation is the angle above or below the horizontal plane (Fig. 1A):

$$\theta = \tan^{-1}[(y_2 - y_1)/(x_2 - x_1)] \quad (1)$$

and

$$\phi = \tan^{-1}[(z_2 - z_1)/((x_2 - x_1)^2 + (y_2 - y_1)^2)^{1/2}], \quad (2)$$

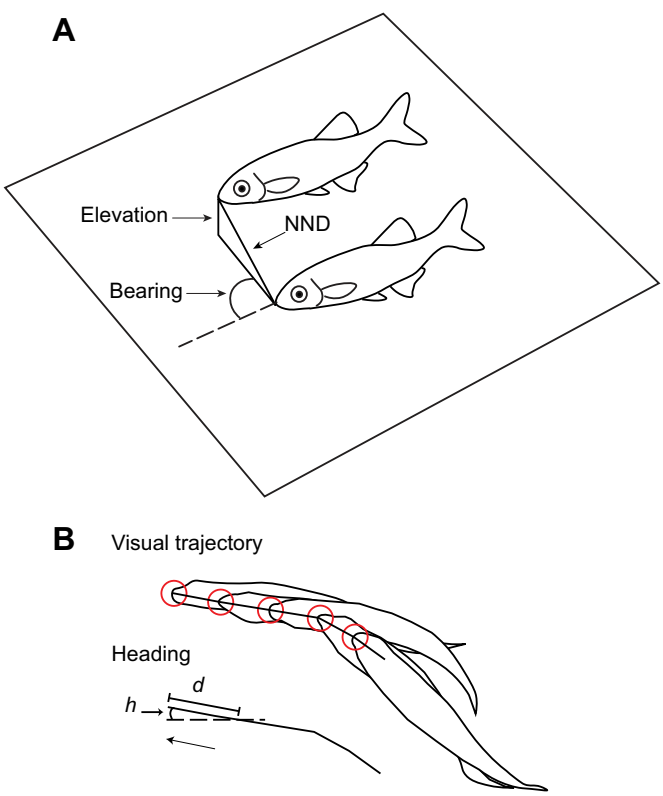

Fig. 1. Quantification of schooling behavior in giant danios (*Devario aequipinnatus*). (A) Representation of two neighboring fish in a school. The schematic shows the nearest neighbor distance (NND), bearing angle in the horizontal plane (θ), and elevation (ϕ) measured above or below the horizontal plane between two neighboring fish. (B) Calculation of the fish heading or swimming direction. The *visual trajectory* shows a single giant danio swimming through five consecutive frames (20 ms per frame) where swimming position of the fish was determined by tracking the snout position. The *heading* was determined by measuring successive position of the snout to calculate *d* (distance moved per frame) and *h* (heading angle with respect to the *x*-axis).

where (x_1, y_1, z_1) and (x_2, y_2, z_2) are the positions of a pair of fish. The nearest neighbor distance (NND) was calculated using the Euclidean distance:

$$\mathrm{NND} = \sqrt{(x_1 - x_2)^2 + (y_1 - y_2)^2 + (z_1 - z_2)^2}. \quad (3)$$

Schooling tendency, or time spent swimming in the main group of fish in a trial, was determined by calculating the distance of the treated fish from the geometric center of the 3D volume of the school. We chose a threshold distance of 1.5 times the mean distance of an untreated fish from the center of the school. The mean distance was 0.72±0.13 total body lengths. We quantified the fraction of time that the fish spent swimming within the threshold distance as a measure of its tendency to school.

Statistical analysis

Our experiment had a repeated measure design. The NND, schooling tendency, volume and distance from the school were tested with various statistical tests to find significant differences among the treatment weeks. All data were tested for normality and uniformity using JMP Pro 13 (SAS Institute, Cary, NC, USA). A generalized linear mixed model (GLMM) (SPSS version 19, IBM, Armonk, NY, USA) was used to analyse the NND, schooling tendency, volume and distance from the school where the weeks were considered as a fixed factor variable and the trial number as a random factor. This approach allowed for the selection of random (trials) and fixed effects (pre-treatment versus post-treatment) while addressing the time or repeated measures for each individual. As we were mainly interested in the comparison between the pre-treatment group (week −1), and the treated and post-treated groups, we used a Dunnett's multiple comparison test (Dunnett, 1955) to test the significant differences of the post-treatment trials (weeks 0, 1, 2, 4 and 8), relative to the results from the pre-treatment trials (week −1).

For angular data (bearing and elevation), we used circular statistics to compare the pre-treatment group (week −1) to all other weeks. For bearing and elevation, we first computed a mean vector that corresponds to the direction:

$$\bar{C} = \frac{1}{n}\sum_{k=1}^{n} \cos\theta_k \quad (4)$$

and

$$\bar{S} = \frac{1}{n}\sum_{k=1}^{n} \sin\theta_k, \quad (5)$$

so that the mean angle is:

$$\bar{\theta} = \tan^{-1}(\bar{S}, \bar{C}). \quad (6)$$

The mean vector length, $R = \sqrt{\bar{C}^2 + \bar{S}^2}$ is a measure of concentration of angles in the given range, and ranges from 0 to 1, from completely random to a highly concentrated distribution. From the mean vector length, Batschelet (1981) defines an angular standard deviation as:

$$s = [2(1 - R)]^{1/2}. \quad (7)$$

The angular deviation, s, increases as the mean vector length decreases.

We used custom MATLAB code (The MathWorks, Natick, MA, USA) to calculate the mean, standard deviation (s.d.) and mean vector length (R) for the data distribution. We calculated R for each

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trial and used a Rayleigh test for circular uniformity. Significance of the Rayleigh test shows both one-sidedness and concentration of the directions around the angular mean (Batschelet, 1981). To test the differences between two samples, we used a Mardia–Watson–Wheeler *F*-test, which tests for differences in the mean and angular variance (Batschelet, 1981). We also used a χ^2 test for binned angular data to compare the overall angular distribution between each treatment week.

All statistical tests were considered significant at $P<0.05$. Values are reported as means±s.d., with sample size, and the *P*-value from the statistical test. Angular data are reported as angular means±standard error of mean (s.e.m.), with sample size *n*, *R* and the *P*-value from the statistical test.

RESULTS

All giant danios actively swam around the experimental tank in every schooling trial. Furthermore, all treated fish survived the gentamycin or sham treatments. Individuals used for rapid fluorescent staining to confirm lateral line viability after schooling trials also quickly recovered from this procedure. Fig. 2 shows examples of schooling behavior before, and 1 week and 4 weeks after treatment.

To make sure that any effects observed were not due to handling or stress caused by the treatment or by transferring the fish from tank to tank, we collected data from schools containing either a gentamycin- or a sham-treated fish. The pre-treatment week (week −1) was considered the control group because they were

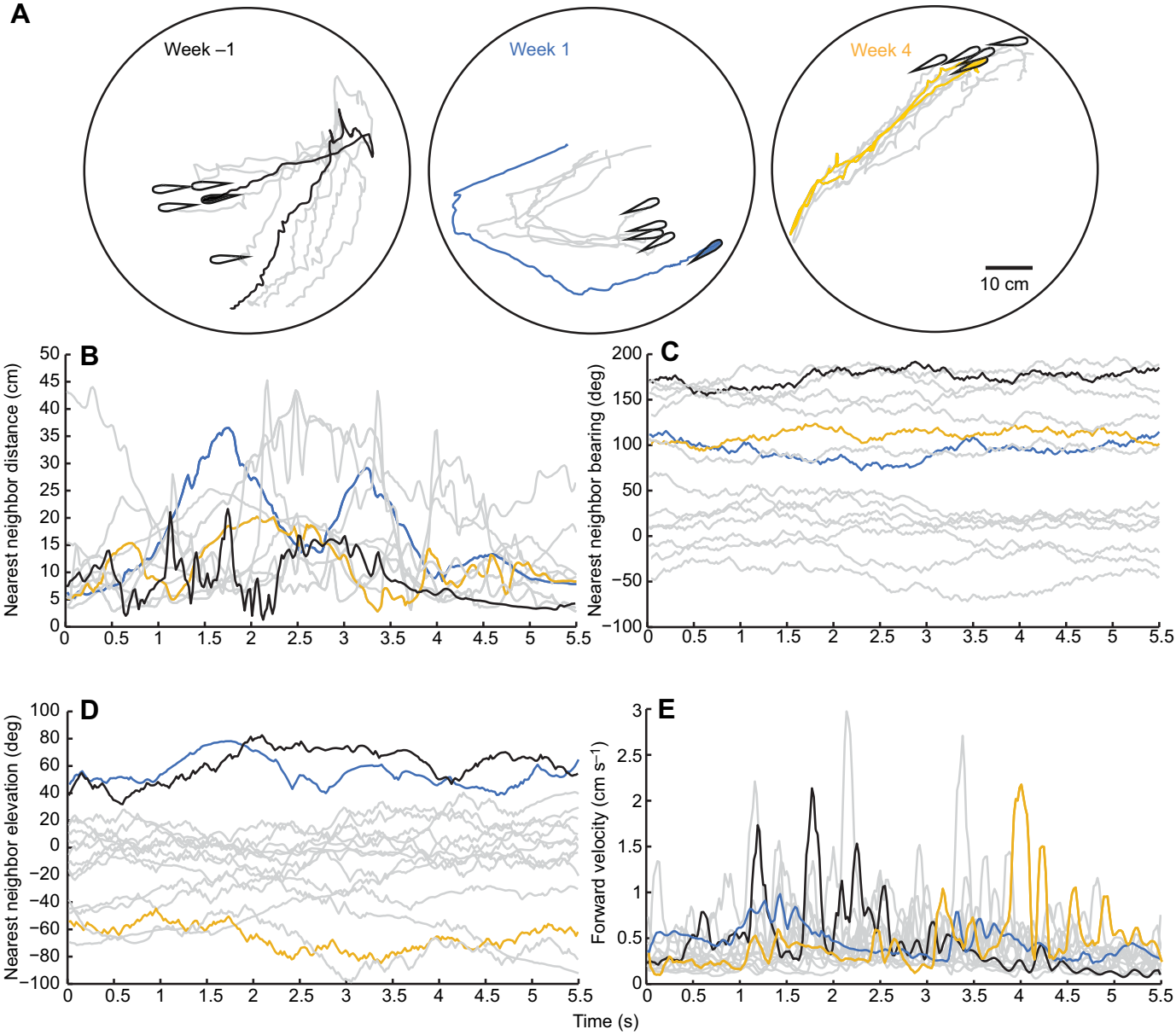

Fig. 2. Example of swimming trajectories from three groups of fish from three different weeks along with the extracted behavioral parameters. Colored and gray paths indicate examples from the treated and untreated fish, respectively, where black is week −1, blue is week 1, and orange is week 4. (A) Swimming trajectories of each treated fish swimming with a group of normal untreated fish from the top view of the 3D constructed paths (scale bar, 10 cm). The circle indicates the edge of the experimental tank. (B) The nearest neighbor distance of each fish over time extracted from the trajectories shown in A. (C) The bearing angle with the nearest neighbor in the horizontal plane. (D) The elevation above or below the nearest neighbor. (E) The forward velocity of each fish in the group.

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not treated with any chemicals or transferred to a specific treatment tank. The sham-treated fish were subjected to the same handling as the gentamycin-treated fish, but did not have their lateral line system inactivated.

Ablation and regeneration of hair cells

To confirm the effectiveness of the gentamycin treatment to disable the lateral line system, we use a fluorescent stain (4-di-2-asp) to visualize functional neuromasts. The stain is only taken up by metabolically active cells. On the head, canal neuromasts were located in the supraorbital, infraorbital, mandible, pre-opercular, otic, post-otic, temporal canals and supratemporal commissure (Fig. 3). Large clusters of superficial neuromasts were located on each side of the head between the supraorbital canal and the eye socket and anterior to the naris. Superficial neuromasts were also distributed in a line on the ventral edge of the operculum above the mandibular canal (Fig. 3). Along the body, the trunk lateral line system is complete and the ventrally placed trunk canal originated at the dorsal edge of the operculum near the temporal canals and extended to the base of the tail (Fig. 4A). Accessory superficial neuromasts were located on trunk canal scales above and below the canal, and other lines of superficial neuromasts were located in the dorsal region between the eye and dorsal fin and on the caudal fin (Fig. 4A).

Gentamycin ablated hair cells in the giant danio lateral line system, as demonstrated by the lack of fluorescence staining in treated fish (Fig. 4B). Immediately after treatment, neuromasts were not visible (Fig. 4B). However, olfactory tissue in the naris was still highly visible, indicating that the stain is functional. One week after the treatment, the neuromasts were again visible across the whole body (Fig. 4C).

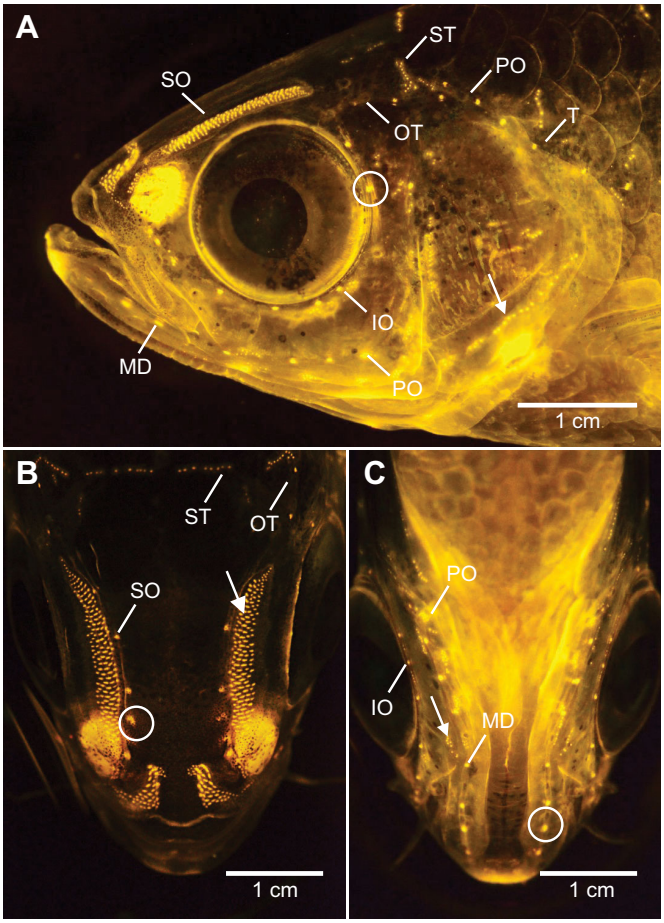

Fig. 3. The mechanosensory lateral line system of *Devario aequipinnatus*. Adult giant danio (total body length, 55 mm) stained with 4-di-2-asp, showing metabolically active neuromasts as bright yellow dots. An example of one canal neuromast is circled in white, while patches of smaller superficial neuromasts are indicated with arrows. SO, supraorbital canal; IO, infraorbital canal; MD, mandibular canal; PO, pre-opercular canal; OT, otic canal; PO, post-otic canal; ST, supratemporal canal; T, temporal canal, which can extend laterally from head to tail along the full length of the trunk. (A) Lateral view; (B) dorsal view; (C) ventral view. Olfactory tissue in the naris is also visible as a large circular structure in front of the eye in A. Scale bars, 1 cm.

Percent time spent in school

Immediately after the treatment, fish with ablated lateral lines were able to remain in a school with other fish. One and 2 weeks after treatment, however, they spent much less time in the school (Fig. 5). Over the course of a trial, gentamycin-treated fish often swam away from the school, but would quickly correct their trajectory to return to the school, especially in weeks 1 and 2 (Fig. 2, blue traces). The amount of time gentamycin-treated fish stayed in a school significantly varied before and after treatment (Fig. 5B, Table 1). Immediately after treatment (week 0), gentamycin-treated fish appeared to spend less time in school, but this was not statistically different from the control (Fig. 5B, Table S1). One and 2 weeks after treatment, when the hair cells in neuromasts had regenerated (Fig. 4C), gentamycin-treated fish spent significantly less time in the school than control fish (Fig. 5B, Table S1). By 4 weeks after treatment, the normal schooling behavior returned to baseline levels, not significantly different from the control group. Sham-treated fish were not statistically different from the control at any time (Table S1).

Nearest neighbor distance

Even when treated fish stayed in the school, they also swam further from their neighbors than other fish (Fig. 6, Table 1). Control fish (week −1) tended to swim very close to one another (Fig. 6, Movie 1). Immediately after treatment, the mean NND for treated fish increased but was not significantly different from the control (Movie 2, Table S1). The sham-treated fish also had similar mean distance to the gentamycin-treated fish at week 0 and were not statistically different from each other or the control group (Table S1). During regeneration, 1–2 weeks after treatment with gentamycin, NND increased significantly (Fig. 6, Movie 3, Table S1). By weeks 4 and 8, the NND of the treated fish returned to similar values with respect to the control group. The NNDs of the sham-treated fish did not vary substantially throughout the experiment, and were always statistically indistinguishable from the controls (Table S1).

Position of nearest neighbors

Ablating the entire lateral line system also affected the angular position of fish with respect to their nearest neighbors, causing them

Table 1. Results of overall statistical tests for the treatment week effect

Metric	$d.f._1$, $d.f._2$	*F*	*P*-value
Schooling tendency (%)	5, 11	18.278[a]	<0.001
Nearest neighbor distance (BL)	5, 11	9.613[a]	<0.001
Bearing (deg)	5, 11	1.877[b]	0.043
Elevation (deg)	5, 11	0.491[b]	0.908
Velocity (BL s^{-1})	5, 11	11.210[a]	<0.001

Results of overall statistical test using (a) the generalized linear mixed models approach or (b) the Mardi–Watson–Wheeler *F* statistic. BL, body length; $d.f._1$, degrees of freedom 1; $d.f._2$, degrees of freedom 2.

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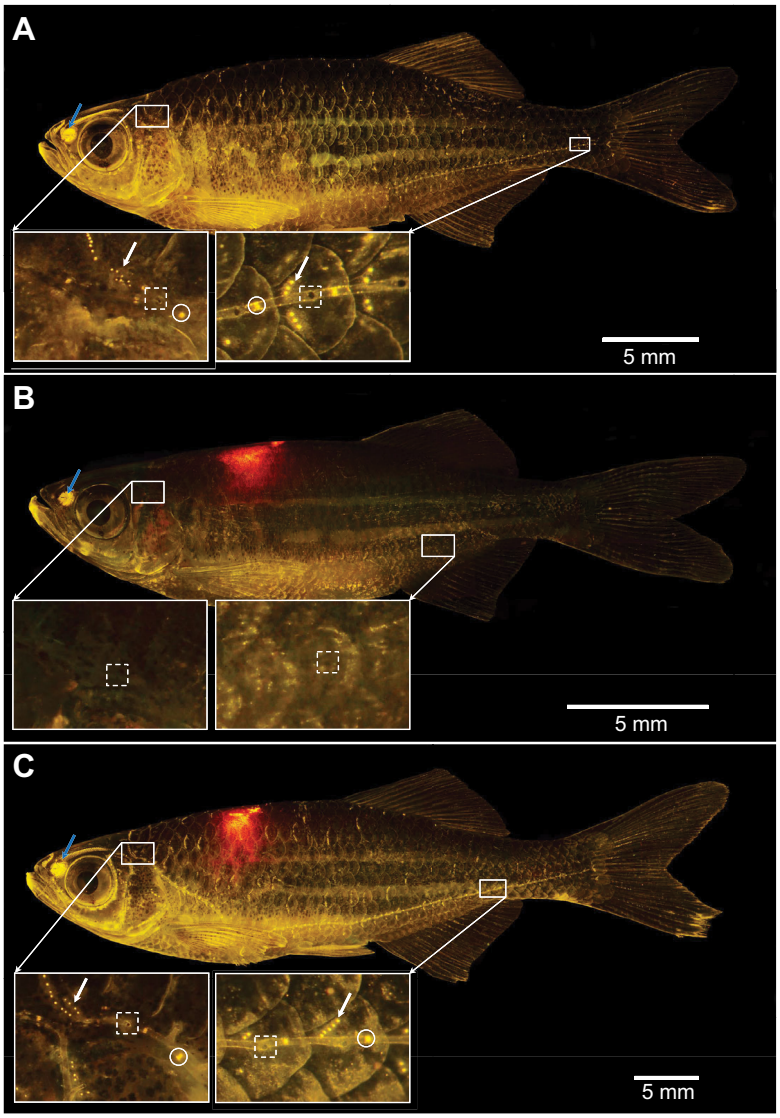

Fig. 4. Fluorescent staining of the giant danio lateral line system before, immediately after and 1 week after treatment with gentamycin. Neuromasts are stained as in Fig. 3 and can be seen as bright yellow points. In each panel, the inset figures show close-ups (35× magnification) of the lateral line system in approximately the same two regions on the fish: head (left) and trunk (right), showing superficial and canal neuromasts (indicated with arrows and open circles, respectively). The white dashed boxes indicate canal pores found between canal neuromasts. The red fluorescence on the back is from the fluorescent visible implant elastomer used to track the treated fish. (A) Before treatment (week −1). (B) Immediately after gentamycin treatment (week 0). Olfactory tissue in the naris (indicated with a blue arrow) is visible because of the active nerve terminal regions, and serves as a positive control for functional 4-di-2-asp stains. (C) Post-gentamycin treatment (week 1). Scale bars, 5 mm.

to swim more often directly beside their neighbors (Fig. 7, Table 1). Without treatment, giant danios mostly adopted a diamond school formation, with each fish following diagonally behind and to the side of their nearest neighbor (Fig. 7A). Immediately after gentamycin treatment and up to 4 weeks later, the treated fish tended to swim in a box formation, directly beside or directly behind their neighbors (Fig. 7). We quantified the mean bearing, $\bar{\theta}$, of each nearest neighbor fish relative to the treated fish (Figs 1A and 7, Table S1). A bearing of 45 or 135 deg indicates the diamond formation, while a bearing of 0, 90 or 180 deg indicates the box formation (Fig. 7A).

Control fish (week −1) followed their nearest neighbor in a diamond pattern (Fig. 7, Table S1). Immediately after treatment and in weeks 1 and 2, the treated fish swam alongside its nearest neighbor. There is a shift in position in week 4 as the nearest neighbor fish began to swim slightly behind the treated fish (Fig. 7, Table S1). The bearing returned to pre-treatment angular position by week 8 (Fig. 7, Table S1). Sham-treated fish swam at all bearings and did not vary substantially from control fish (Table S1).

To specifically examine changes in school formation from diamond to box patterns, we binned the range of bearings into three ranges with equal areas and compared the distribution (left and right side combined) between the control and all treatment weeks. The three ranges were as follows: (i) fish that swam either directly ahead or behind their neighbors ($\bar{\theta}$=15±15 or 165±15 deg), (ii) fish that swam in a diamond formation ($\bar{\theta}$=45±15 or 135±15 deg) and (iii) fish that swam directly beside their neighbors ($\bar{\theta}$=90±30 deg) (Fig. 7). We found significant differences between binned bearing angles (χ^2=129.06, d.f.=15, P<0.001). Control fish spent the most time directly ahead or behind each other (box formation) or in a diagonal formation (Fig. 7B). Immediately after treatment with gentamycin (week 0) and in week 1, fish substantially increased the amount of time spent in a box formation when compared with the control group (Fig. 7B; week 0, chi-squared test, χ^2=16.436, P<0.001; week 1, χ^2=11.060, P=0.004). In week 2, fish spent more time in other patterns, but still mainly stayed in the box formation, a pattern that significantly differed from control (χ^2=11.183, P=0.004). In week 4, treated fish continued to swim parallel to their nearest neighbor, but moved closer to forming a diamond formation (χ^2=16.436, P<0.001). In week 8, the treated fish moved back into a diamond formation and spent most of their time swimming directly diagonally to their nearest neighbor, a pattern that did not differ from the control group (χ^2=4.220, P=0.121).

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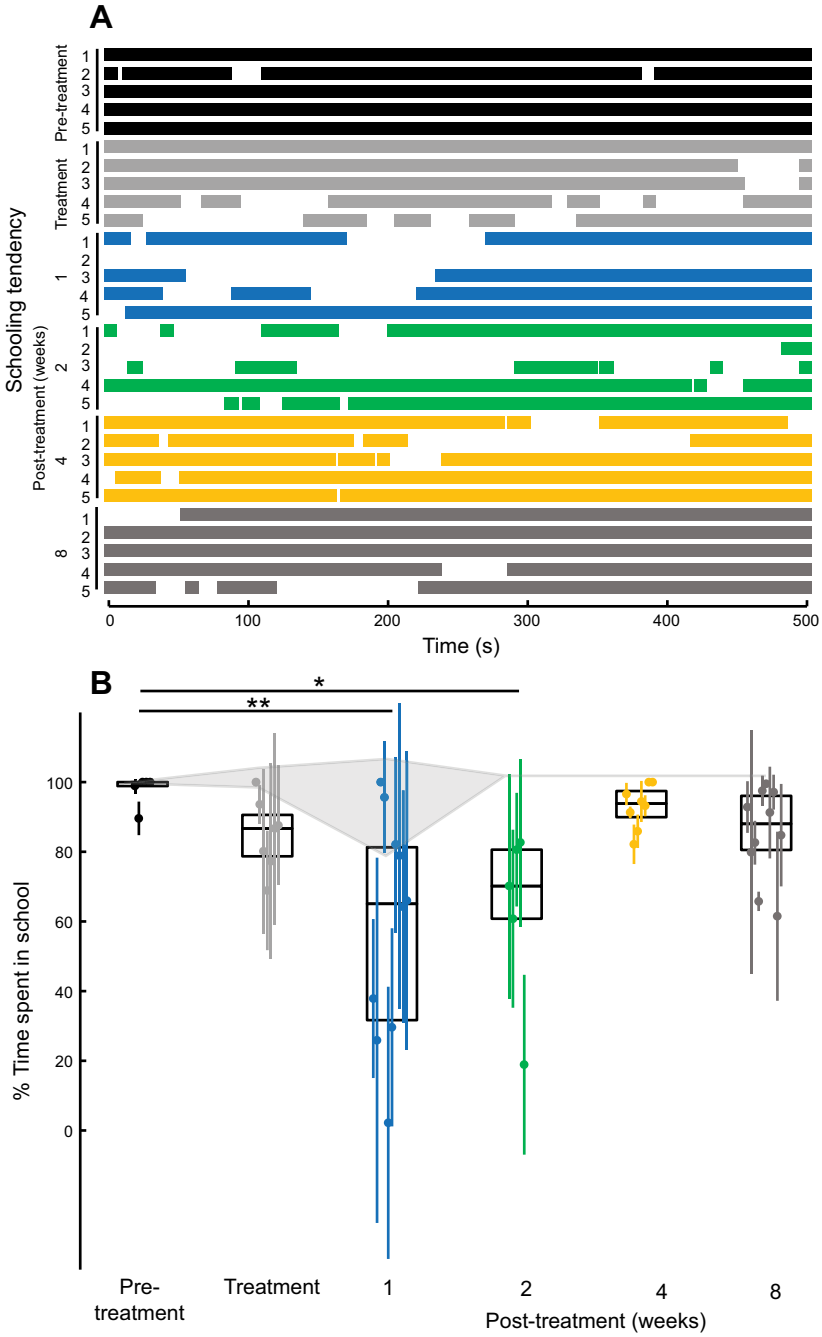

Fig. 5. Lateral line inactivation decreases schooling tendency 1 and 2 weeks post-treatment. (A) Raster plots depicting schooling tendency. Continuous horizontal bars represent schooling bouts for five gentamycin-treated fish per week. (B) Box plots showing the percentage of time spent in a school for gentamycin-treated fish across all time points. Each point represents the mean and s.d. for an individual fish. The gray shaded region represents the mean±s.d. for the sham-treated fish. Statistical differences are denoted by asterisks (*P<0.05, **P<0.001).

The mean elevation between gentamycin-treated fish and their neighbors was not affected by the treatment (Table 1, Table S1), but the distribution of elevations did change significantly (Fig. 8, Table 1). Mean elevation between treated fish and controls did not differ at any week (Table 1). We then categorized the range of elevations observed in our schooling fish into the following bins: (i) fish that swam directly above their neighbors (60±30 deg), (ii) fish that swam in the same plane as their neighbors (0±30 deg) or (iii) fish that swam directly below their neighbors (−60±30 deg). Before treatment, fish spent time in all regions (Fig. 8). We found significant differences between binned elevation angles (χ^2=30.213, d.f.=15, P<0.011). Immediately after treatment with gentamycin, fish spent nearly all their time side by side with their neighbors, a significant difference in distribution when compared with control (chi-squared test, χ^2=41.329, P<0.001, Fig. 7). One and 2 weeks after treatment, fish also spent more time side by side when compared with the control (χ^2=26.268, P<0.001; χ^2=38.628, P<0.001; Fig. 8). In weeks 4 and 8, the elevation of treated fish returned to the normal distribution, with more time spent above or below their neighbors (χ^2=4.176, P=0.124; χ^2=5.944, P=0.051; Fig. 8).

Forward velocity and velocity correlation

Immediately after ablating the lateral line system, fish swam at the same mean speed and with the same correlation with neighbors (Fig. 9), but during the regeneration period (weeks 1 and 2), the mean speed increased significantly and the correlation structure changed (Table 1, Table S1). Immediately after the gentamycin treatment, treated fish appeared to swim faster, but the difference was not significant relative to control fish (Table S1, Fig. 9A), probably because of the high variability in swimming speeds. After

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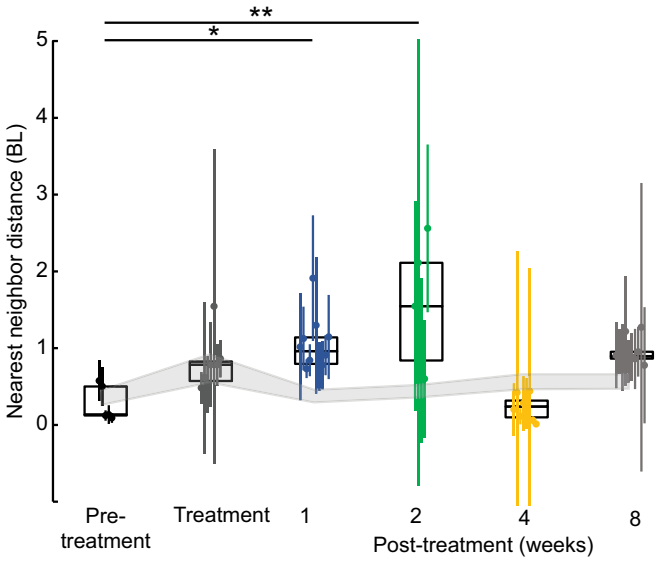

Fig. 6. Distance between the treated fish and its nearest neighbor increases 1 and 2 weeks after lateral line inactivation. The box plots show the mean distance in total body lengths (BL). Colored points and error bars show the mean±s.d. for each individual fish. The gray shaded region represents the mean±s.d. for the sham-treated fish. The statistical differences are noted by asterisks (**P*<0.05, ***P*<0.001).

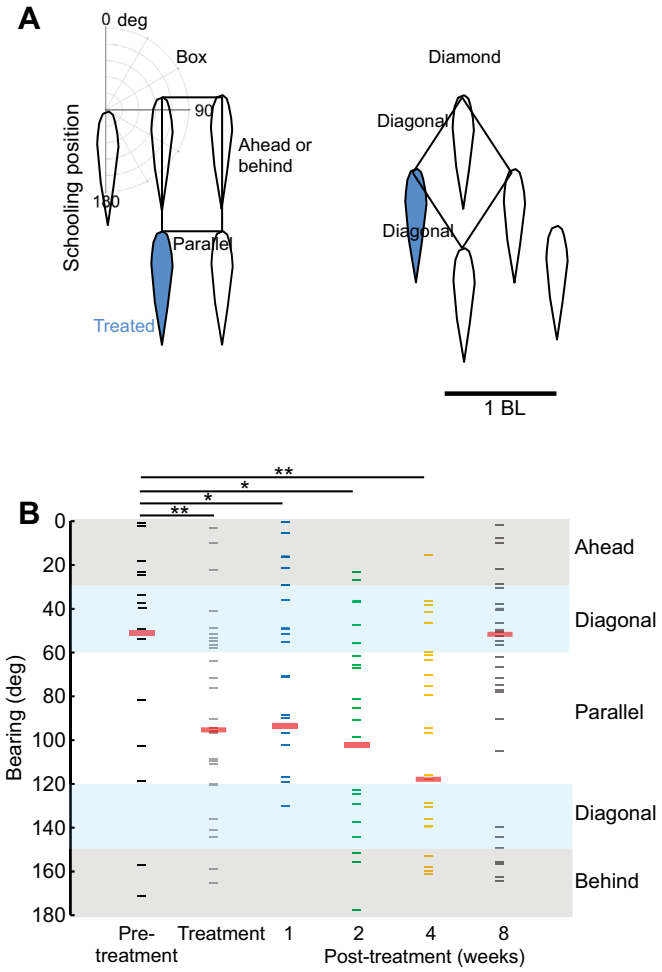

Fig. 7. The bearing of the nearest neighbor fish changes after lateral line inactivation. (A) Schematic diagram of schooling formations. *Box* formations consist of fish swimming at ranges near 0, 90 or 180 deg next to its nearest neighbors. *Diamond* formations consist of fish swimming at ranges near 45 or 135 deg next to its nearest neighbors. The blue silhouette represents the treated fish in the horizontal plane swimming with a group of four normal untreated fish. BL, body length. (B) The mean bearing of each nearest neighbor fish with respect to the treated fish. Tick marks represent the mean bearing for each trial. The red bar shows the mean bearing for each treatment group. The shaded regions represent ranges that correspond to the schooling formations shown in A. The statistical differences are noted by asterisks (**P*<0.05, ***P*<0.001).

regeneration of the lateral line system in weeks 1 and 2, forward velocity increased significantly (Table S1, Fig. 9A). The swimming speed returned to normal by week 4.

During the regeneration period, fish also changed velocity more slowly and more similarly to their neighbors (Fig. 9B,C). We measured the correlation of the treated fish's forward swimming velocity with itself (the autocorrelation) at a range of different time lags. For control fish before treatment, the velocity autocorrelation is very low for lags greater than 20 ms (Fig. 9B). This means that the swimming velocity varies rapidly and relatively unpredictably. During the regeneration period, autocorrelation increased substantially, remaining as high as 0.67 for lags up to 300 ms. These high values show that treated fish change their swimming speed more slowly. By 8 weeks post-treatment, the velocity autocorrelation returned to the control pattern (Fig. 9B). Similarly, we assessed the cross-correlation of the treated fish's velocity with its nearest neighbor. For this metric, a positive lag means that the treated fish is following changes in its neighbor's velocity: its response comes after a change in the neighbor fish. A negative lag means that the treated fish is leading the neighbor: changes in its velocity come before changes in its neighbor's. Like the autocorrelations, the cross-correlations (Fig. 9C) are low for control fish and immediately after treatment, but are higher at all lags for treated fish during regeneration. Correlations at both negative and positive lags are high, indicating that there is not a clear 'leader' or 'follower' fish, until week 8, when the correlation structure returns to the same pattern as control.

DISCUSSION

In this study, we showed that giant danios (*D. aequipinnatus*) can school without their lateral line system, but that schooling behavior is disrupted for several weeks after regeneration of the hair cells in the neuromast receptors. Several previous studies have shown that disruption of the fish lateral line system does not cause schools to disperse, but leads to changes in distances between neighbors. For example, saithe (*P. virens*) and yellow-eyed mullet (*Aldrichetta forsteri*) swim closer to their neighbors (Partridge and Pitcher, 1980; Middlemiss et al., 2017), while firehead tetras (*H. bleheri*) swim further away from their neighbors (Faucher et al., 2010). In our study, immediately after lateral line ablation, *D. aequipinnatus* maintained a similar distance within the normal school of fish (Movie 2), but swam more side by side and at the same elevation as their neighbors. For several weeks after the hair cells regenerated, treated giant danios swam away from the main group. When they were in the school, they swam further from their neighbors but more parallel and more in the same elevation. Treated fish also swam faster on average. By 4–8 weeks after the treatment, schooling behavior returned to the pre-treatment control. Our results demonstrate that danios can compensate for the complete lack of lateral line input, probably using vision (as shown previously; Pitcher et al., 1976), or other sensory cues from the acoustic or olfactory systems. As the hair cells regenerate, the behavior degrades. We hypothesize that this result indicates that the signal

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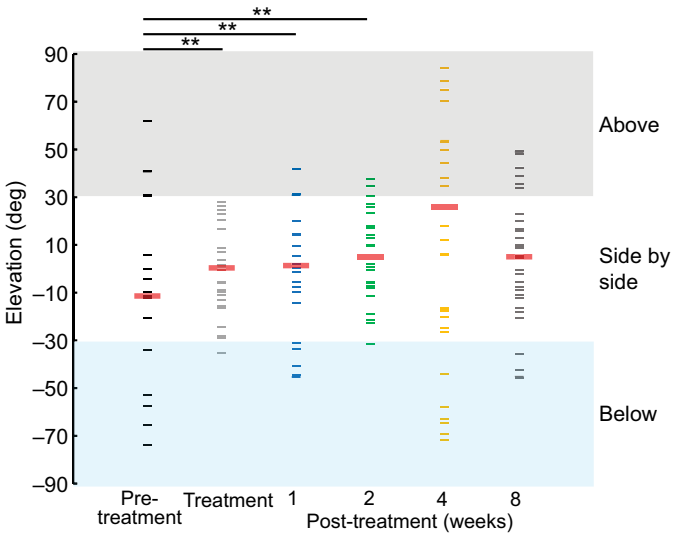

Fig. 8. Most treated fish swam at the same elevation relative to their nearest neighbor. Tick marks indicate the mean angular elevation for treated fish relative to their nearest neighbor for each trial within the specific weeks. Elevations were grouped into the following ranges: above (gray), side by side (white) or on the same plane, and below (blue). The statistical differences are noted by asterisks (**P<0.001).

from the lateral line system is different or altered during regeneration, and that this difference causes the degradation in schooling behavior.

Initially, ablating the lateral line system of giant danios did not have a very large effect on its schooling behavior (Movie 2). Unlike Faucher et al. (2010), we saw relatively little change immediately after treatment. In general, the schooling behavior immediately after treatment was not significantly different from the pre-treatment control or the sham-treated individuals. Statistically, compared with their behavior before the gentamycin treatment, the treated fish at first maintained the same schooling tendency (Fig. 5), the same distance to their nearest neighbor (Fig. 6), and the same mean swimming speed (Fig. 9A). They did change their position within the school, spending more time directly beside and at the same elevation as their neighbors (Figs 8 and 9).

Unexpectedly, 1 week after the treatment, the schooling behavior changed dramatically, even though the hair cells in the lateral line system had regenerated. Other studies have shown that the hair cells are fully regenerated by 1 week after treatment (Harris et al., 2003; Schwalbe et al., 2012; Pinto-Teixeira et al., 2015; Schwalbe et al., 2016), and we observed the same time course of recovery in our fluorescent staining (Fig. 4C). Even though the neuromasts had regenerated, treated fish had difficulty staying within a school (Fig. 5), and they altered both the distance (Fig. 6) and bearing (Fig. 7) they maintained relative to their nearest neighbor. Fish were able to recover their normal schooling behavior within a month after lateral line system inactivation, similar to the firehead tetras studied by Faucher et al. (2010).

This long recovery duration suggests that fish need additional time to readjust to their newly regenerated lateral line system before they can school normally again. Although hair cells are known to regrow (Harris et al., 2003; Schwalbe et al., 2012; Pinto-Teixeira et al., 2015; Schwalbe et al., 2016), it is not known what happens to the afferent neurons during regeneration. The afferents may degrade over some distance, as is common in peripheral nerve damage (Devor et al., 1979; Easter and Nicola, 1996; Navarro et al., 2007; Faucherre et al., 2009), and then would have to regrow after the hair cells regenerate.

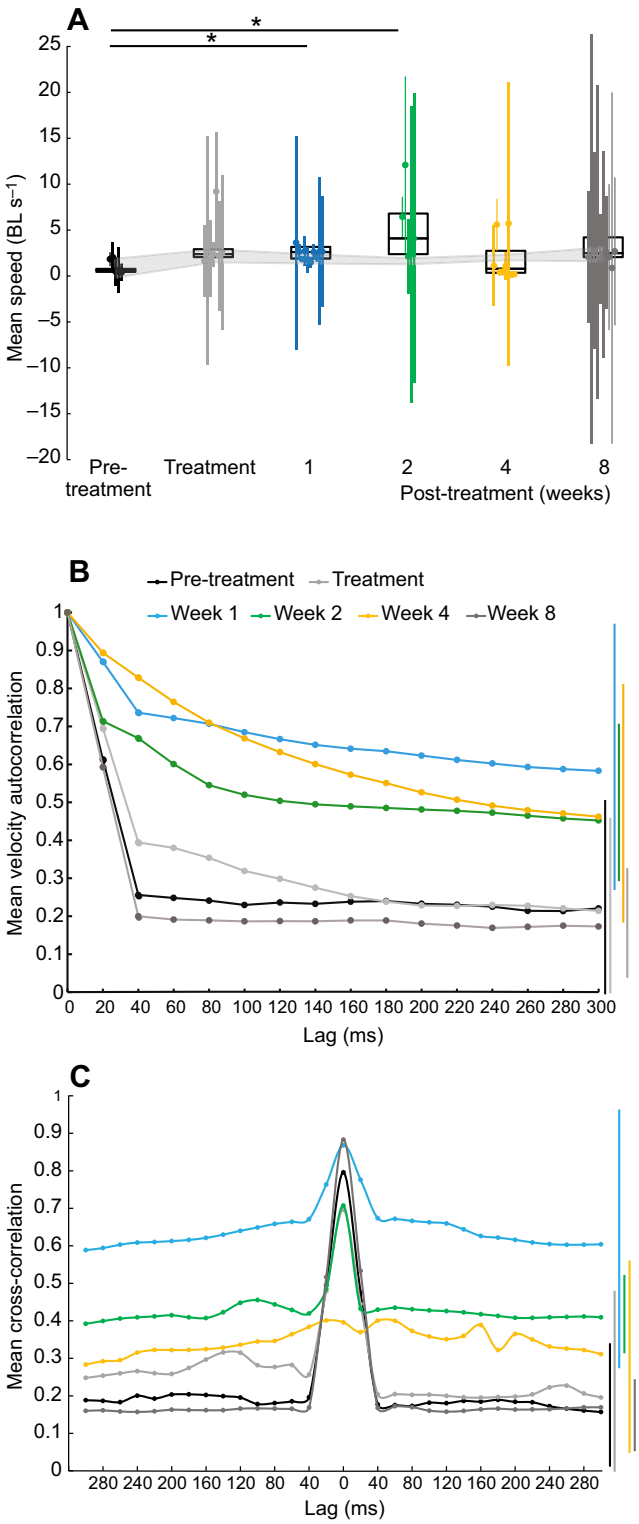

Fig. 9. Fish in post-treatment weeks 1, 2 and 4 swam faster and more smoothly than fish in earlier and later weeks. (A) Swimming speed at each treatment week. The statistical differences are noted by asterisks (*P<0.05). (B) Mean velocity autocorrelations. Standard deviations were consistent through each lag interval and are shown as color coded bars on the right. (C) Mean velocity cross-correlations between a fish's velocity at a particular instant and its neighbor fish at a different time. Positive lags indicate that the treated fish is following changes in its neighbor's speed, while negative lags indicate that the neighbor is following the treated fish.

The afferents may make new connections to different hair cells as they regrow, which could cause the brain to receive very different inputs from the newly regenerated lateral line system than it did before the gentamycin treatment (Monroe et al., 2015). Neuronal regeneration of the afferents does not require intact lateral line hair cells, but without the hair cells there can be deviation and errors in pathfinding during re-innervation (Villegas et al., 2012). In addition, neurons deprived of input can become hypersensitized (e.g. Hoffman and Parker, 2010). This hypersensitization could also happen with the lateral line afferents, so that the newly regenerated afferents could initially produce much stronger signals from the same flow patterns. This could be an effect similar to tinnitus in the auditory system, which is sometimes thought to be due to the deprivation of input to auditory afferents (Møller, 2003; Eggermont and Roberts, 2004). Lastly, although the hair cells themselves are metabolically active, given evidence by fluorescent staining (Fig. 3), the newly regenerated microvilli might not be fully functional, as suggested by Faucher et al. (2010). The dye is taken up through mechanosensitive channels (Harris et al., 2003; Chiu et al., 2008; Van Trump et al., 2010), but, even if the channels are present, the microvilli may need time to regenerate and mature to effectively transduce the flow patterns during normal behavior of the fish (Faucher et al., 2010).

Our results are broadly consistent with the few other studies that also investigated the role of the lateral line system in schooling, but there are some important differences. The pattern of behavior we observed is similar to what Faucher et al. (2010) observed, although the time course of lateral line recovery was different. They observed similarly increased distances in later days after treatment (Faucher et al., 2010). These results are different from those in Partridge and Pitcher (1980), who found that disruption of the lateral line system led saithe to swim closer to their neighbors. Like Partridge and Pitcher (1980), however, we found that treated fish tended to swim more parallel to their neighbors (Fig. 6), particularly 1–2 weeks after treatment. At this bearing, fish deprived of their lateral line system would be best able to visually monitor changes in their neighbors' velocities (Partridge and Pitcher, 1980; Middlemiss et al., 2017). Treated fish were able to return to a more staggered formation, similar to a diamond formation, within the school 4 weeks after treatment. Finally, throughout our study, fish tended to swim more side by side with their nearest neighbor, rather than above or below elevation, while control fish and fish 4 weeks after treatment swam at all elevations (Fig. 7). In contrast, Partridge and Pitcher (1980) observed that treated saithe swam above their nearest neighboring fish. These differences could be related to differences in the visual field of saithe and giant danio. It might be that saithe can see better below themselves than danios, but little is known about the optic field in saithe (Sadler, 1973; Fernald, 1988). Because their lateral line system was inactivated, fish might have to rely more on their vision to track other fish in the school and choose a more advantageous position by swimming parallel (e.g. bearing, 90 deg) and side by side (elevation, 0 deg) to their nearest neighbor.

Vision is good for detecting changes in relative velocity, but may be worse at detecting changes in distance. This may explain some of the changes in school structure that we observed. Like previous studies, our results indicate that vision can be used to maintain the schooling structure, even with a completely inactivated lateral line system. Both vision and the lateral line system can be used to regulate distances between neighbors or maintain a preferred NND (Partridge and Pitcher, 1980; Faucher et al., 2010; Middlemiss et al., 2017). In our case, vision was probably used to observe small changes in velocity, but was a poor indicator for perceived distances. Our results show that fish change their relative positions when they rely on vision, and hence alter the structure of their schools (Figs 6 and 7). Partridge and Pitcher (1980) and Pitcher et al. (1976) have suggested that marine schooling fish in a circular channel could more or less maintain a normal position within the school when they were blinded, and compensated for their visual loss by more closely matching the direction and speed of the neighboring fish. However, fish without their lateral lines but with vision intact changed only the distance to their neighbors, not their velocity or heading angles, and were still able to maintain a specific position with the school (Partridge and Pitcher, 1980).

Normal fish must integrate the information from both their eyes and their lateral line systems, but there may be times when the two systems conflict with each other. For example, a conflict could arise from when a school of fish swimming side by side perform a turn. During a turn, if the fish maintain their relative positions, then those on the outside of the school must swim faster than those on the inside. Visually, neighbors would stay in the same relative positions, indicating that they are moving at the same speed. However, the lateral line would sense a difference in the speeds due to the turn. All animals naturally integrate these different sources of information and can give one more weight than another depending on its reliability and importance to the task at hand (Zupanc, 2010).

In our study, after the lateral line regenerated, we observed that treated fish lost track of their position in the school (Movie 3), which may have been caused by conflicting information between the visual and lateral line systems. During this period, the velocity of the treated fish was moderately correlated with its neighbors, but this correlation was maintained even over long lags (Fig. 9C). In contrast, the velocity of control fish was much more strongly correlated with their neighbors, but only at very short lags. This difference indicates that treated fish were not able to match their neighbors' rapid fluctuations in speed, but instead were essentially filtering out high frequency changes in speed of their neighbors. For example, we observed that some treated fish lost their position in a school by swimming ahead of the school or while the rest of the school performed a quick turn, the fish lost sight of the school (Movie 3). This suggests that these treated fish may not have been able to sense the flows shed by their neighbors or received conflicting information from their newly regenerated hair cells, and thus momentarily strayed away from the school. In both situations, fish quickly rejoined their school once they visually located it. Moreover, the fact that correlations were relatively high at both positive and negative lags (Fig. 9C) suggests that, for the treated fish, there was not a clear 'leader' or 'follower' fish. It may be that the treated fish were following the overall trajectory of the school, not one specific individual.

Conclusions

Our major finding was that giant danio can school normally without their lateral line system, but that the behavior degrades after the hair cells regenerate. Based on this finding, we suggest that the signal from the lateral line system is altered after regeneration, possibly because the afferents change their sensitivity or their connections. When the hair cells were ablated, the afferents or central lateral line brain regions may have become hypersensitized, a pattern somewhat like tinnitus in human hearing (Eggermont and Roberts, 2004). Re-innervation, or re-establishing functional nervous system connection with target hair cells, coincides with regeneration, and is necessary to establish a functional lateral line system. If neurons are unable to establish functional communication with the newly regenerated hair cells, signaling and information delivery to the brain will be variable or not occur. Afferent neurons in the lateral

line system use polarity guidance cues to target and establish connections with regenerated hair cells with a specific orientation (Faucherre et al., 2009). It is not known what happens to the afferent neurons in giant danios during regeneration. Studies have shown that innervation can take place before hair cell regeneration, which can disrupt signaling (for review, see Monroe et al., 2015). Fish that have non-functioning mechanotransduction channels have afferent neurons that show increased arborization, instability and pathfinding errors (Faucherre et al., 2009; Villegas et al., 2012). Currently, additional studies are needed to highlight the mechanisms that modulate the neuronal re-innervation with target hair cells. Future directions will include examining the functionality of the lateral line system through fluorescent staining, tracing and imaging of the afferent nerves through ablation and regeneration. Electrophysiological recordings of the lateral line nerve through ablation and regeneration could be helpful to understand when giant danios are able to transduce mechanical stimuli during different stages of the regeneration, and if the sensory response to mechanical stimuli changes during regeneration. Ultimately, these studies will help to illuminate how fish and other vertebrates use the lateral line system for schooling and other complex behaviors.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: P.J.M., M.A.B.S., E.D.T.; Methodology: P.J.M., M.A.B.S., E.D.T.; Software: P.J.M., E.D.T.; Validation: P.J.M., E.D.T.; Formal analysis: P.J.M., L.L.C.; Investigation: P.J.M., L.L.C.; Resources: E.D.T.; Data curation: P.J.M.; Writing - original draft: P.J.M.; Writing - review & editing: P.J.M., M.A.B.S., E.D.T.; Visualization: P.J.M., M.A.B.S., E.D.T.; Supervision: E.D.T.; Project administration: P.J.M.; Funding acquisition: P.J.M., E.D.T.

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Data availability
Data are available from the Dryad Digital Repository (Mekdara et al., 2018): https://doi.org/10.5061/dryad.t2hh6tj

Supplementary information
Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.175166.supplemental

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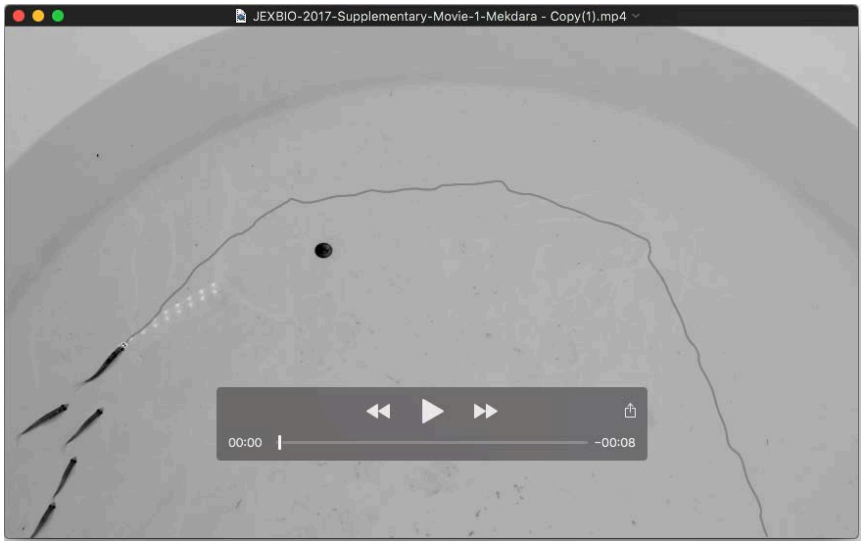
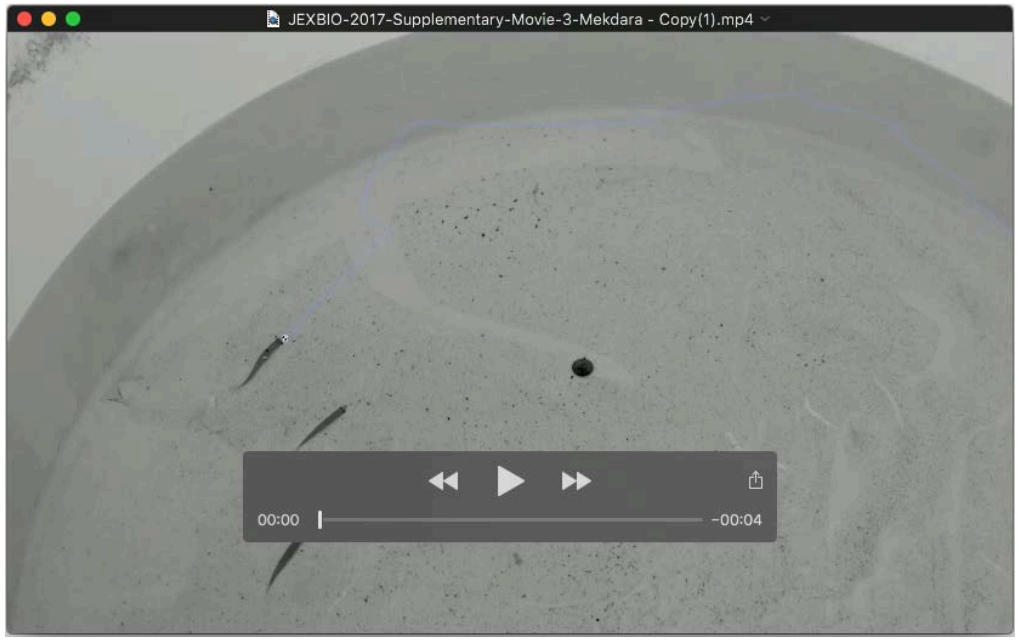
Supplementary Information



Movie 1. Example of a pretreatment filming trial in week -1. Control fish is shown with black trajectory swimming alongside four other fish. Recording is from one camera view shown in real time at 50 frames s^{-1}.

Movie 2. Treated giant danio in a school of normal fish immediately after lateral line system ablation swims normally. Treated fish (week 0) is indicated with gray trajectory and appears to swim with the school in the same direction while maintaining a normal distance from its nearest neighbor fish. The hair cells are ablated with gentamycin seen in Fig. 4B. The schooling behavior appears to be similar to the pretreatment group in Mov. S1. Recording is from one camera view shown in real time at 50 frames s^{-1}.



Movie 3. Treated giant danio in a school of normal fish two weeks after treatment. Complete hair cell regeneration is supported by fluorescent staining in Fig. 4C. The treated fish is indicated in blue trajectory. Treated fish appears to lose track of the group briefly and swims at a further distance from its nearest neighbor once it rejoins the group. Recording is from one camera view shown in real time at 50 frames s^{-1}.

Table S1. Mean values and statistical comparisons for all swimming variables.

Metric	Control Week -1	Treatment Week 0	Week 1	Week 2	Week 4	Week 8
Schooling tendency (%)	98±5 (5)	85±10 (10)	**58±33 (10)**	**63±26 (5)**	93±6 (10)	85±13 (10)
		p = 0.871	**0.009**	**0.054**	0.998	0.855
Sham	100±0 (5)	96±2 (5)	91±11 (5)	100±0 (5)	100±0 (5)	100±0 (5)
	1.000	*p* = 0.853	0.991	1.000	1.000	1.000
Nearest neighbor distance (BL)	0.28±0.17 (5)	0.82±0.65 (10)	**1.50±0.33 (10)**	**1.54±0.82 (5)**	0.22±0.16 (10)	0.85±0.16 (10)
		p = 0.184	**0.006**	**<0.001**	1.000	0.957
Sham	0.28±0.23 (5)	0.60±0.33 (5)	0.21±0.26 (5)	0.56±0.08 (5)	0.61±0.17 (5)	0.71±0.12 (5)
	1.000	*p* = 0.772	1.000	0.354	0.328	0.093
Bearing (degrees)	51±15 (5)	**95±15 (10)**	**94±10 (10)**	102±18 (5)	**127±18 (10)**	52±12 (10)
	R = 0.92	***R* = 0.86**	**0.90**	0.89	**0.77**	0.85
		***p* = 0.023**	**0.048**	0.059	**0.036**	0.201
Sham	133±17 (5)	89±17 (5)	102±17 (5)	82±16 (5)	86±15 (5)	87±15 (5)
	R = 0.63	0.74	0.63	0.61	0.63	0.63
	p = 0.061	0.247	0.132	0.456	0.375	0.326
Elevation (degrees)	-11±14 (5)	0±3 (10)	1±8 (10)	5±7 (5)	26±21 (10)	5±4 (5)
	R = 0.83	*R* = 0.99	0.93	0.98	0.71	0.87
		p = 0.241	0.340	0.207	0.067	0.103
Sham	4±6 (5)	6±13 (5)	0±8 (5)	-3±5 (5)	-2±6 (5)	-2±4 (5)
	R = 0.86	0.83	0.84	0.91	0.88	0.95
	p = 0.653	0.263	0.702	0.412	0.522	0.204
Velocity (BL s^{-1})	1.1±0.8 (5)	3.2±2.7 (10)	**3.4±0.6 (10)**	**5.4±4.1 (5)**	1.8±2.4 (10)	2.5±0.9 (10)
		p = 0.475	**0.049**	**0.020**	0.986	0.801
Sham	1.2±1.0 (5)	1.95±0.4 (5)	1.95±0.5 (5)	1.5±0.3 (5)	2.0±0.3 (5)	2.4±0.4 (5)
	0.995	1.000	0.998	0.999	0.999	1.000

Values are shown as mean ± standard deviation, followed by number of individuals in parentheses, and the *p* value for comparisons to control. For angular data, the Rayleigh *R* statistic is also given. Significant differences relative to control are shown in bold.