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RESEARCH ARTICLE

Zebrafish learn to forage in the dark

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ABSTRACT

A large diversity of fishes struggle early in life to forage on zooplankton while under the threat of predation. Some species, such as zebrafish (*Danio rerio*), acquire an ability to forage in the dark during growth as larvae, but it is unclear how this is achieved. We investigated the functional basis of this foraging by video-recording larval and juvenile zebrafish as they preyed on zooplankton (*Artemia* sp.) under infrared illumination. We found that foraging improved with age, to the extent that 1-month-old juveniles exhibited a capture rate that was an order of magnitude greater than that of hatchlings. At all ages, the ability to forage in the dark was diminished when we used a chemical treatment to compromise the cranial superficial neuromasts, which facilitate flow sensing. However, a morphological analysis showed no developmental changes in these receptors that could enhance sensitivity. We tested whether the improvement in foraging with age could instead be a consequence of learning by raising fish that were naïve to the flow of prey. After 1 month of growth, both groups foraged with a capture rate that was significantly less than that of fish that had the opportunity to learn and indistinguishable from that of fish with no ability to sense flow. This suggests that larval fish learn to use water flow to forage in the dark. This ability could enhance resource acquisition under reduced competition and predation. Furthermore, our findings offer an example of learning in a model system that offers promise for understanding its neurophysiological basis.

KEY WORDS: Behavior, Flow, Foraging, Larvae, Lateral line, Learning

INTRODUCTION

The ability to sense prey during foraging is critical to the early growth and survival of fishes. Although vision is essential, larval zebrafish (*Danio rerio*) can forage on zooplankton in the dark by sensing water flow with the lateral-line system (LLS) (Westphal and O'Malley, 2013). The LLS serves this function with a series of mechanoreceptors in the skin called superficial neuromasts (Dijkgraaf, 1963). These neuromasts presumably allow a fish to detect the perturbations created by the propulsion of prey. However, it is not clear how this is achieved and it is therefore unknown what properties of the LLS matter to foraging performance. The present study examined how zebrafish acquire the ability to forage in the dark over their first month of age.

A number of studies have examined the foraging behavior of larval zebrafish. Larvae begin foraging after the swim bladder inflates, which occurs 1 day after hatching and at 5 days post fertilization (dpf). Foraging is characterized by spontaneous intermittent swimming that is interrupted by targeted movement toward prey upon detection (Fuiman and Webb, 1988; Budick and O'Malley, 2000). This targeted swimming includes a series of pectoral fin and tail motions that serve to align the rostrum with the prey (McElligott and O'Malley, 2005; Patterson et al., 2013). Once in a close position (<0.5 mm), the larva attempts to capture the prey with a suction-feeding strike (Patterson et al., 2013).

Although foraging proceeds similarly in the dark, vision greatly enhances a larva's ability to detect prey. Under illumination, larvae respond to zooplankton at a greater distance (<3 mm) than in the dark (<1 mm) (Gahtan et al., 2005; Bianco et al., 2011; Patterson et al., 2013). Larvae strike at prey in the dark with a relatively low frequency and each strike is less successful than when they can see (Westphal and O'Malley, 2013). Whether mediated by vision or other sensory systems, the strike rate and capture probability increase with age, perhaps through a combination of changes in sensory perception, neuromuscular control, jaw development, and hydrodynamics (Easter and Nicola, 1996; Hernandez, 2000; Hernandez et al., 2005; McHenry and Lauder, 2006; Danos and Lauder, 2007; Green and Hale, 2012; Staab and Hernández, 2010; China and Holzman, 2014).

It is not clear whether improvements in foraging with age are related to the development of the LLS. Upon hatching, the LLS includes approximately 24 superficial neuromasts on each side of the body, with nearly half positioned on the head at high density. Superficial neuromasts on the trunk may increase in number by a factor of two or three in the first month of age (Metcalfe et al., 1985; Ghysen and Dambly-Chaudière, 2007; Nuñez et al., 2009). After this period, many of the cranial superficial neuromasts transform into canal neuromasts by enlarging and becoming enclosed in bony canals with pores that open at the body's surface (Webb and Shirey, 2003). These morphological changes alter the frequency response of the neuromasts such that they become sensitive to pressure gradients in the flow field (van Netten, 2006). These changes are likely to be important to foraging because larval fish appear to be responsive to prey motion around the head (Bianco et al., 2011; Patterson et al., 2013). The present study included a morphological analysis of the superficial neuromasts during larval growth to test whether changes in morphology might account for the improvement of foraging in the dark with age.

Zebrafish larvae may alternatively enhance their foraging by learning to identify the flow that prey generate. This would appear to be possible because zebrafish are capable of associative learning. For example, adults can be trained to exhibit feeding behavior in response to a conditioned olfactory stimulus under classical conditioning (Braubach et al., 2009). From an early age, zebrafish are also responsive to operant conditioning. For example, larvae can be trained to orient their swimming to visual cues by negative reinforcement with an electric shock, and they improve in this task with age to the extent that larvae perform almost flawlessly by 4 weeks of age (Valente et al., 2012). The present study tested whether improvement in foraging in the dark is a consequence of associative learning by measuring the foraging of fish that were experimentally restricted from exposure to the flow of prey.

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MATERIALS AND METHODS

We tested the role of the LLS in the foraging of zebrafish by comparing rates of prey strikes and captures between fish with a compromised lateral line system and control fish. These experiments were performed on fish that varied in age, from hatchling larvae to 1-month-old juveniles. We tested whether the role of flow sensing differs when aided by vision by performing these experiments both in the dark and under illumination visible to the fish. Upon confirming reports finding that foraging improves with age and that the LLS is necessary for improved foraging, we tested hypotheses about this improvement. We addressed whether foraging is mediated primarily by the cranial lateral line by compromising only that region. Morphometrics of superficial neuromasts tested whether flow sensing is augmented by heightened sensitivity to flow (McHenry et al., 2008; Van Trump and McHenry, 2008). Finally, we raised fish that were naïve to the flow of *Artemia* to test the effects of associative learning.

Animal husbandry

Adult zebrafish, *Danio rerio* (Hamilton, 1922), were bred from wild-type (AB line) colonies that were housed in a flow-through aquarium system (Aquatic Habitats, Apopka, FL, USA) and maintained at 27°C on a 14 h:10 h light:dark cycle. Fertilized eggs from random mating pairs were cultured using standard rearing techniques (Westerfield, 1993). Larvae were raised in 3-liter tanks and were fed pellets (Larval AP100, Ziegler Bros, Gardens, PA, USA) from ages 5 to 30 dpf. This diet was enriched daily by feeding larvae live rotifers, *Brachionus plicatillis*, from 5 to 10 dpf, and *Artemia franciscana* nauplii from 7 to 30 dpf. Under these conditions, our fish attained a body length of 9.02±0.13 mm (N=15) by the age of 30 dpf. This period spans the duration of larval growth, with the oldest fish perhaps initiating juvenile development (Schilling, 2002; Brand et al., 2002). We therefore will refer to the younger (<25 dpf) fish in the present study as 'larvae' and the older fish (25 and 30 dpf) as 'juveniles'. With the exception of the experimental groups used to test the effects of learning (described below), all fish were fed under illumination throughout the rearing period and were therefore provided the opportunity to associate the appearance of prey with their water flow. All rearing and experimental protocols were conducted with the approval of the Institutional Animal Care and Use Committee at the University of California, Irvine.

Foraging experiments

Foraging experiments with *Artemia* nauplii were video-recorded. A single fish and approximately 15 nauplii (1 day post-hatch) were placed in a cylindrical arena (watch glass, 40 mm diameter×3 mm max. depth) with a glass cover to reduce distortions at the surface. A camera (Marlin, Allied Vision Technologies, Stadtroda, Germany) was positioned above the arena to record feeding experiments (760×480 pixels, 5×5 cm field of view at 14.7 frames s^{-1}). Infrared panels (850 nm) were positioned below the arena with a diffuser (Parafilm sheet, 'M' Film, Pechiney Plastic Packaging, Chicago, IL, USA) to generate transmitted illumination for high-contrast videos with light not visible to the larvae. A 25 W fluorescent bulb was directed away from the arena to provide visible light for experiments under illuminated conditions. Prior to the experiments, all fish were fasted for 24 h to ensure motivation to feed during experiments.

We video-recorded foraging over a 10 min period. When reviewing these videos, we noted the number of instances that the fish visibly struck at prey and the number of those strikes that succeeded in capturing prey. These measurements provided the basis for three metrics of foraging performance: (1) the strike rate (the number of strikes per minute) served as an indication of the foraging activity of a larva; (2) the capture rate (the number of prey successfully acquired per minute) offered a general metric of feeding success; and (3) the capture probability (the ratio of capture rate to strike rate) indicated the overall effectiveness of feeding strikes.

Experimental manipulation of the LLS

We tested the role of the LLS by experimentally compromising its ability to sense flow. This was achieved by placing fish in a 250 µmol l^{-1} solution of neomycin sulfate (Fisher BioReagents, Fair Lawn, NJ, USA) for 45 min prior to experiments. This treatment kills most hair cells in the neuromasts (Harris et al., 2003; Van Trump et al., 2010), but leaves the hair cells of the inner ear intact and thereby has no adverse effect on sensing sound, body orientation, or acceleration. Experiments were performed within 2 h of the treatment because of the rapid ability of these hair cells to regenerate (McHenry et al., 2009). Fish were only used if they exhibited routine swimming behavior and motivation to feed. These experiments were performed on zebrafish of varying age (7, 10, 12, 13, 15, 20, 25, and 30 dpf) to consider the effects of age on foraging performance.

Experiments were conducted to test the role of the LLS with and without the aid of vision. Untreated (i.e. with LLS) and treated (i.e. without the LLS) fish were filmed in either the light or dark at all ages (N=480). The mean values and 95% confidence intervals of our measurements of capture rate, strike rate, and capture probability were compared between untreated and treated groups. All statistical procedures for these experiments, and all others described in this study, were completed with custom scripts in MATLAB (v.2013a), available upon request from the corresponding author.

We tested the degree to which feeding depends on flow sensing by cranial neuromasts. These experiments were prompted by preliminary recordings and observations in the literature (e.g. Westphal and O'Malley, 2013), which showed that fish tended to pursue *Artemia* near the head when foraging in darkness. We developed a method to compromise flow sensing in the head while leaving the rest of the body unaltered. This was achieved by embedding the body of an anesthetized (0.0017 g l^{-1} of buffered MS-222; Finquel, Argent Chemical Laboratories, Redmond, WA, USA) juvenile (25 dpf) in a block of 5% agarose (low-melting point; Fisher Scientific, Fair Lawn, NJ, USA; heated to 60°C and cooled to 35°C). Once set, the agarose surrounding the head was removed with a scalpel to expose the head while leaving the body embedded. This served to protect the body, but not the head, from exposure to the neomycin solution for 45 min. The Petri dish was filled with a solution of neomycin sulfate (250 µmol l^{-1}) to act on only the exposed cranial neuromasts. After a recovery period (10 min), foraging experiments were performed in the manner described above. We were unable to devise a comparable approach to compromise just the trunk lateral line because embedding the head in agarose prohibited the operculum from circulating the gills. We additionally performed a sham treatment where fish were anesthetized and embedded in agarose, but the head was exposed to water lacking neomycin.

Comparisons of capture and strike rates between treatment, control, and sham groups were performed by one-way ANOVA. This test was appropriate because the measurements were normally distributed (Kolmogorov–Smirnov, P>0.05) and showed homogeneity of variance (Levene's test, P>0.05). A Tukey's HSD test was performed to determine significant differences between groups. Capture probabilities were compared between groups with a

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Kruskal–Wallis test, because these data did not meet the assumption of a normal distribution. Pairwise comparisons, using a Nemenyi test (using a Tukey distribution), determined significant differences between groups.

Lateral-line morphometrics

We tested whether the improvements in foraging with age were reflected in changes in lateral-line morphology. Our morphometric analysis focused on the cranial LLS because our preliminary results suggested a major role for this region in foraging. We measured the number of neuromasts and, for each neuromast, the number of hair cells and the diameter (at the base) and height of the cupula. The cupula is an extracellular component of a superficial neuromast that extends from the surface of the body. The dimensions of the cupula and number of hair cells are the major structural features that determine the sensitivity of a superficial neuromast (McHenry et al., 2008; Van Trump and McHenry, 2008; van Netten and McHenry, 2013). These measurements were performed for the supraorbital (SO1–SO4), infraorbital (IO1–IO3), and mandibular (MN1–MN3) neuromasts in fish of varying age (15, 25, and 30 dpf). Hair cells were visualized in anesthetized larvae embedded in agarose (0.5%) with a live fluorescent stain [2% DASPEI, 2-(4-(dumethylamino) styryl)-N-ethylpyridinium iodide; Invitrogen, Eugene, OR, USA], using a stereomicroscope (Zeiss Discovery V.20, Carl Zeiss, Thornwood, NY, USA) with a GPF filter set (450–490 nm) and fluorescence illuminator (120 W Mercury Vapor Short Arc, X-Cite series 120q, Lumen Dynamics, Mississauga, ON, CA).

Cupulae were visualized in a separate group of anesthetized fish that were perfused with polystyrene microspheres (0.1 µm diameter, Polysciences, Warrington, PA, USA). The microspheres adhered to the surface and provided sufficient contrast to see the peripheral shape of the cupula (Van Trump and McHenry, 2008) under Nomarski optics using a compound microscope (Zeiss Axioskop 2 FS plus, Carl Zeiss). The cupula diameter was measured from photographs (AxioCam HRc, Carl Zeiss) taken with the microscope focused on the base of the cupula. The diameter is a sufficient descriptor of the base dimensions because our preliminary observations found the base to be circular in shape. The cupula height was measured by noting the z-position of the microscope as we focused between the base and distal edge of the cupula. We performed a one-way ANOVA with *post hoc* comparisons (Tukey's HSD test) for all of these measurements to test the effect of age. These measurements met the assumptions of a normal distribution (Kolmogorov–Smirnov, $P>0.05$) and homogeneity of variance (Levene's test, $P>0.05$).

Learning experiments

We conducted experiments that tested whether zebrafish learn to use their LLS during the first month of age. This was achieved by raising two groups of fish that were not permitted exposure to the flow generated by live *Artemia* prey. One group was fed dead *Artemia* (group 3, described in Results) and the LLS of the other group (group 4) was compromised throughout the rearing period. This was achieved by daily exposure to a neomycin solution (250 µmol l^{-1} neomycin sulfate for 45 min) prior to feeding with live *Artemia*. Both groups were consequently naïve to the flow created by live *Artemia* at the time that we performed feeding experiments at the juvenile age (30 dpf). Neomycin treatments ceased 2 days prior to these experiments to afford the time for regeneration of the LLS. This timing was selected because previous experiments showed that hair cells and responses to flow stimuli recover in zebrafish within 25 h of neomycin treatment (McHenry et al., 2009). Fish were only included in experiments if they exhibited routine swimming behavior and motivation to feed. In addition, fish treated with daily neomycin treatments were used only if they exhibited an escape in response to the flow of a pipette. We stained these fish with DASPEI to visually confirm the presence of neuromasts following our experiments.

Feeding experiments were recorded for 15 juveniles from each rearing regime and compared with the control (Group 1, described in Results) and lateral-line-compromised group (Group 2) of the same age. We tested for differences in capture and strike rates between treatment groups with a one-way ANOVA with *post hoc* comparisons (Tukey's HSD test). These measurements met the assumptions of a normal distribution (Kolmogorov–Smirnov, $P>0.05$) and homogeneity of variance (Levene's test, $P>0.05$). Capture probabilities were compared between treatment groups with a Kruskal–Wallis test, because these data did not meet the assumption of normality. A pairwise comparison, the Nemenyi test (with a Tukey distribution), was used to determine significant differences between groups.

RESULTS

Changes in foraging with age

Zebrafish improved in their ability to feed on *Artemia* with monotonic increases in metrics of forage performance over the first month of age. Juveniles struck at prey with greater frequency and with a higher capture rate than larvae (Fig. 1). For example, the capture rate of juveniles (1.38±0.17 min^{-1}, mean±95% CI, N=15 at 30 dpf) was approximately an order of magnitude greater than that of 1-week-old larvae (0.11±0.03 min^{-1}, N=15 at 7 dpf; Fig. 1C). This was because the older fish would strike at greater frequency, with a strike rate (1.98±0.19 min^{-1}, 30 dpf) that was twice that of larvae (0.73±0.12 min^{-1}, 7 dpf; Fig. 1D), and each strike was approximately four times more likely to capture prey (strike probability: 0.18±0.09 at 7 dpf and 0.70±0.09 at 30 dpf; Fig. 1E).

We tested the role of flow sensing on foraging by compromising the LLS with a treatment of neomycin (Fig. 1A,B). This experimental manipulation had little effect when fish could see, as indicated by the lack of significant differences in the capture rate, strike rate, and capture probability when foraging under illumination (indicated by 95% confidence intervals, Fig. 1C–E). However, we did find substantial differences when fish foraged in the dark, particularly in individuals older than 15 dpf. For example, the control group at the juvenile age (30 dpf) exhibited a mean capture rate (0.82±0.25 min^{-1}, N=15) that was four times greater than that of fish with a compromised LLS (0.18±0.07 min^{-1}, N=15; Fig. 1F). In addition, both the strike rate (2.26±0.48 min^{-1}, N=15, Fig. 1G) and capture probability (0.37±0.09, N=15; Fig. 1H) of untreated juveniles were twice the values of the group with a compromised LLS (1.04±0.23 min^{-1}, 0.18±0.11, N=15; Fig. 1G). Therefore, flow sensing by the LLS appears to enhance feeding in the dark by eliciting fish to strike both more frequently and with greater effectiveness.

The role of the cranial lateral line in foraging

We tested the role of the cranial LLS on foraging in juvenile fish by chemically treating just the cranial neuromasts and leaving the posterior lateral line intact. We found that the foraging performance of fish with a compromised cranial system was indistinguishable from those with an entirely compromised LLS. In particular, the mean (±95% CI) capture rates of juveniles (25 dpf) without cranial neuromasts (0.07±0.05 min^{-1}, N=15) did not significantly differ from that of fish with an entirely compromised LLS (0.09±

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0.06 min^{-1}, N=15), but was significantly lower than that of both sham (0.47±0.11 min^{-1}) and untreated fish (i.e. 'with LLS', 0.53±0.18 min^{-1}) (N=15, one-way ANOVA, $F_{3,59}$=16.2, P<0.001, Tukey's HSD, P<0.05; Fig. 2A). This result emerged despite the fact that the strike rate in fish without functional cranial neuromasts (1.13±0.34 min^{-1}) did not differ significantly from that of the sham group (1.63±0.29 min^{-1}; Fig. 2B). The strike rate of cranial-treated fish was also indistinguishable from that of fish lacking the entire LLS (0.58±0.18 min^{-1}), but significantly lower than that of control fish (1.95±0.36 min^{-1}, N=15 in each group, $F_{3,59}$=16.7, P<0.001, P<0.05; Fig. 2B). It is therefore unclear whether larvae strike less often when they lack only cranial neuromasts. However, our results do suggest that the cranial system contributes to the effectiveness of

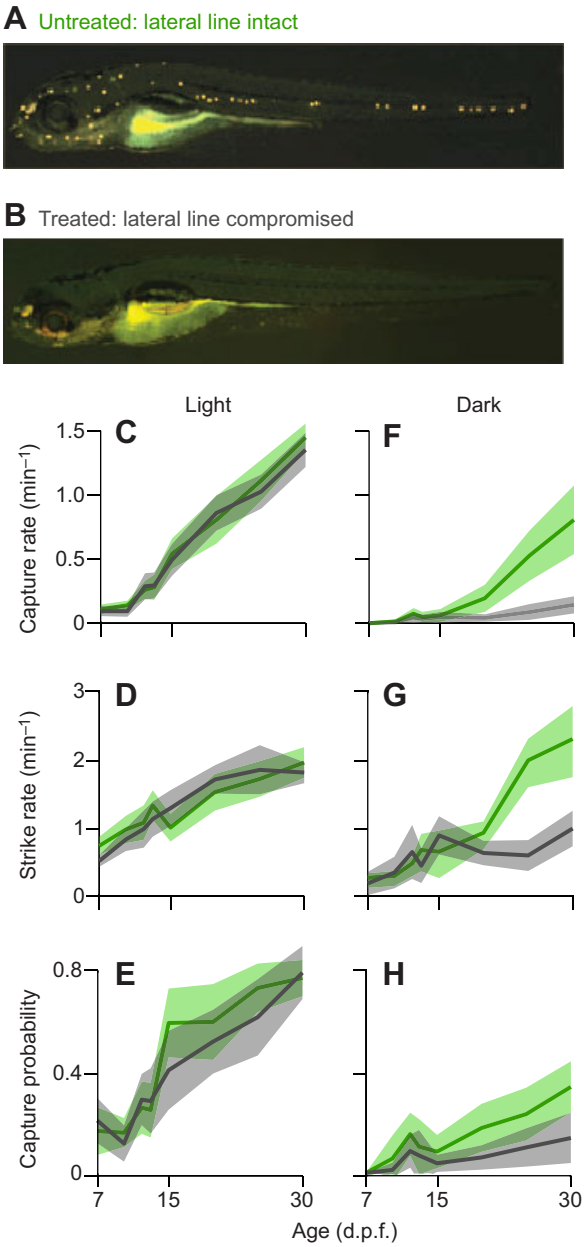

Fig. 1. Effects of the lateral line system in *Danio rerio* on foraging with age. (A) The hair cells of the superficial neuromasts of the lateral-line system (LLS) are stained with DASPEI under fluorescent illumination. This causes each receptor to appear as a yellow point along the body of an untreated larva (5 dpf) with a functional LLS. (B) DASPEI staining fails to reveal any lateral line hair cells in a larva (5 dpf) exposed to neomycin sulfate. (C–H) The results of experiments of fish of varying age that foraged on *Artemia* under (C–E) illuminated and (F–H) darkened conditions are presented through three performance metrics. The measurements (mean±95% CI) of capture rate (C,F), strike rate (D,G) and capture probability (E,H) are shown (N=15 fish at each age).

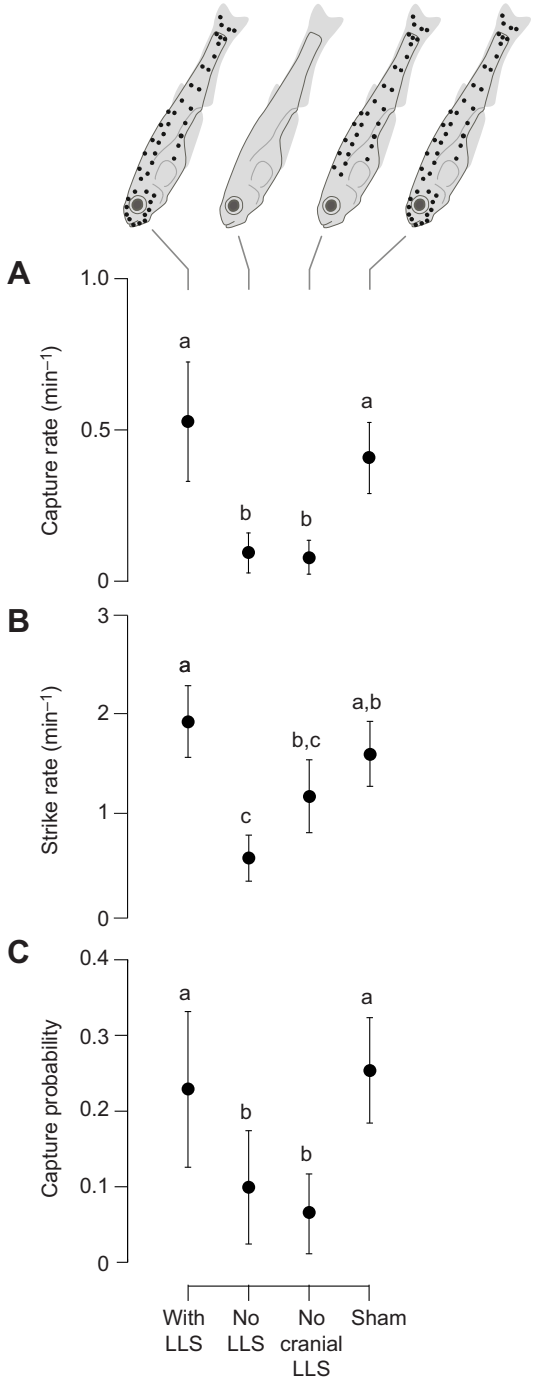

Fig. 2. Effects of the cranial lateral line on foraging. The results of foraging experiments for four treatment groups are reported (mean±95% CI) in terms of the (A) capture rate, (B) strike rate, and (C) capture probability of juvenile zebrafish (25 dpf) foraging on *Artemia*. The groups consisted of a control with an untreated LLS ('with LLS'), a group where each fish had its entire LLS compromised by exposure to neomycin sulfate ('no LLS'), another where just the cranial lateral line was compromised ('no cranial LLS'), and a sham group that was handled in the same manner as the 'no cranial LLS' group. Different letters indicate significant differences between groups (N=15 fish in each group).

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a strike. This was indicated by the capture probability of fish without cranial neuromasts (0.05±0.04), which was significantly lower than that of both the sham (0.25±0.05) and control groups (0.26±0.09, N=15), and did not differ from that of fish lacking the entire LLS (0.01±0.08, N=15, Kruskal–Wallis, H=22.8, P<0.01, pairwise comparisons, P<0.05; Fig. 2C).

We examined whether ontogenetic changes in the morphology of cranial neuromasts might account for the improvement in the ability of fish to forage in the dark. The mean (±95% CI) number of neuromasts in 15-dpf fish (41.8±3.3) was not significantly different from that of juveniles of 25 dpf (45.8±4.2) and 30 dpf (47.3±2.8, one-way ANOVA, $F_{2,44}$=2.51, P=0.093, N=15 in each group; Fig. 3). Measurements of the cupula of neuromasts in the cranial region revealed that neither its height nor diameter differed significantly with age (one-way ANOVA, d.f.=2, 26, P>0.05, N=9; Fig. 4B–D). We similarly found that the number of hair cells did not vary significantly with age for all cranial neuromasts (one-way ANOVA, d.f.=2, 44, P>0.05; Fig. 3B), with the exception of the fourth supraorbital (one-way ANOVA, $F_{2,44}$=4.30, P=0.02). At that site, larvae of 25 dpf (11.1±2.06) had significantly fewer hair cells than fish of 15 dpf (13.0±1.9, N=15) or 30 dpf (14.9±1.3, N=15, Tukey's HSD, P<0.05). This is not a pattern consistent with ontogenetic improvement in sensitivity of neuromasts. These results suggest that the morphology of the cranial LLS does not vary in a manner that could enhance the mechanical sensitivity between 15 and 30 dpf. Therefore, morphological changes cannot account for the improvement in foraging in the dark with age (Fig. 1G).

The effects of learning on foraging

We tested whether the improvement in foraging with age was a consequence of associative learning. Two groups of fish were raised under different feeding regimes to produce 30-dpf fish that were naïve to the flow generated by live *Artemia*. One group was fed dead *Artemia* (Group 3 in Fig. 5) and the other was fed live food, but was raised with a compromised LLS through daily neomycin treatments (Group 4 in Fig. 5). Fish in the compromised group were permitted to regenerate their LLS prior to our measurements of foraging performance. We performed foraging experiments in the dark on these naïve groups with the same experimental design as the fish that were exposed to the flow of prey.

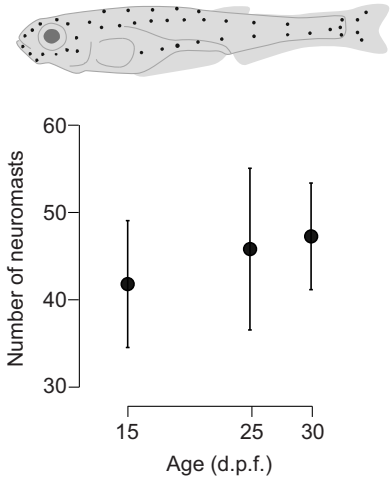

Fig. 3. Total number of neuromasts with age. The number of neuromasts (mean±95% CI) on the head and body in lateral view of zebrafish (15 to 30 dpf), which did not differ significantly among ages (one-way ANOVA, P=0.09, N=15 fish at each age).

We found that fish that were naïve to the flow of *Artemia* were less effective at foraging than fish raised with exposure to flow. For example, fish raised on dead *Artemia* (Group 3: 0.41±0.13 min^{-1}; Fig. 5A) and those with a compromised LLS (Group 4: 0.27±0.13 min^{-1}) exhibited less than half the capture rate of the control fish (Group 1: 0.82±0.25 min^{-1}), which was a significant difference (one-way ANOVA, $F_{3,59}$=12.5, P<0.001, Tukey's HSD, P<0.05). Both of the naïve groups (Groups 3 and 4) were statistically indistinguishable from the fish that were unable to sense flow while foraging in the dark (Group 2 in Fig. 5) in terms of capture rate (one-way ANOVA, $F_{3,59}$=9.65, P<0.001, Tukey's HSD, P<0.05; Fig. 5B) and capture probability (Kruskal–Wallis, H=15.7, P<0.01, pairwise comparisons, P<0.05; Fig. 5C). However, fish raised on dead *Artemia* (Group 3) showed intermediate values in both strike rate and capture probability (Fig. 5B,C). Therefore, larvae may gain some advantage to being raised with a functional LLS, even when naïve to the flow generated by *Artemia*. These results are consistent with the hypothesis that larval fish improve in their ability to forage in the dark (Fig. 1F–H) because they learn to associate *Artemia* with the flow generated by this prey for propulsion.

DISCUSSION

Our results offer new insight into the role of the LLS in the foraging of larval zebrafish. We found that: (1) foraging in the dark improves with age, (2) the cranial neuromasts play a major role in this foraging, and (3) the improvement in foraging with age is an effect of larvae learning to sense the flow of prey. These results have emerged from the results of observations and manipulative experiments that used a variety of techniques.

Foraging in the dark improves with age

Our experiments allowed us to consider the role of the LLS when prey are visible. We observed an order-of-magnitude increase in capture rate over one month of age in foraging under illumination (Fig. 1C). This increase was generated by fish striking with greater frequency (Fig. 1D) and higher capture probability (Fig. 1E). None of these trends were adversely affected when we removed the ability of larvae to sense flow. When performing similar experiments, Westphal and O'Malley (2013) measured a subtle (~25%) decrease in feeding rate in older larvae (15 and 30 dpf). Nonetheless, it can be concluded that the LLS is not necessary for foraging when guided by vision.

In contrast, we found that flow sensing plays a major role in foraging in the dark. This was indicated by the substantial reductions in performance that we observed when we compromised the LLS (Fig. 1F–H), which is consistent with previous results (Westphal and O'Malley, 2013). The effect of this experimental treatment was most acute on fish older than 15 dpf (Fig. 1F), which struck at prey at a lower frequency (Fig. 1G) and less accurately (Fig. 1H) without the aid of the LLS. Therefore, the assistance offered by the LLS in foraging increases with the age of zebrafish.

Cranial neuromasts are important for foraging

We found that the ability to sense the flow of prey is largely facilitated by cranial neuromasts. Fish retaining a functional posterior lateral line, but compromised cranial system, exhibited a capture rate that was indistinguishable from that of fish lacking all functional neuromasts (Fig. 2A). Without the aid of flow sensing at the head, fish would strike less often (Fig. 2B) and with lower accuracy (Fig. 2C) than control and sham groups. These results are

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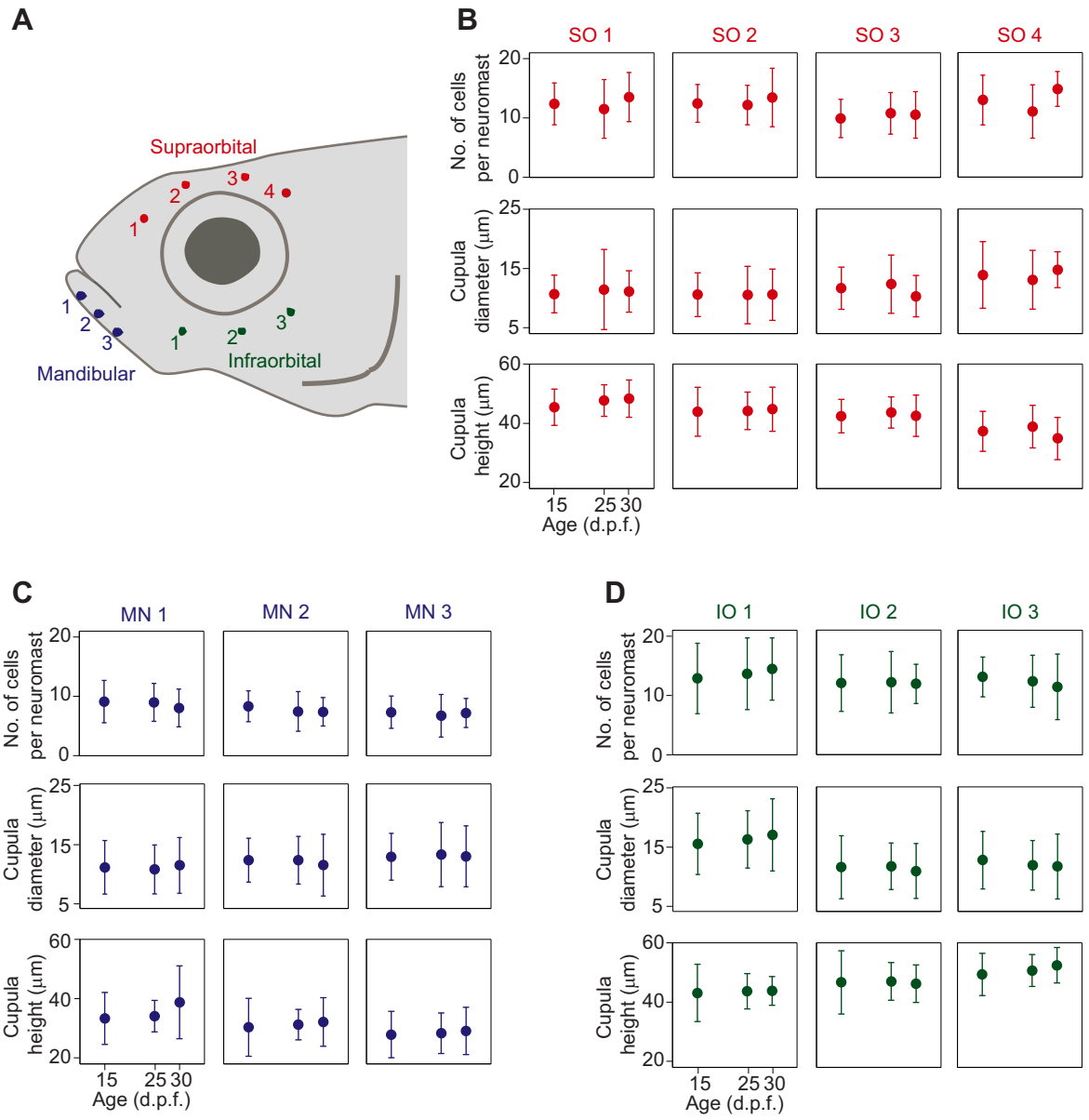

Fig. 4. Morphology of cranial neuromasts with age. (A) Illustration of the locations of cranial neuromasts measured in zebrafish. (B–D) Morphometric features measured for the (B) supraorbital, (C) mandibular, and (D) infraorbital neuromasts among zebrafish at 15, 25, and 30 dpf (mean±95% CI, *N*=9, at each age). For each neuromast, we measured the number of hair cells and the diameter and height of the cupula.

consistent with descriptions of how juveniles change their orientation and strike at *Artemia* that come in close proximity to the head, without contacting the surface of the body (Westphal and O'Malley, 2013). Therefore, the flow detected by cranial neuromasts appears to prompt fish to both initiate feeding and align a strike toward the prey. These results do not preclude a role for the posterior lateral line, which is considered to play a role in predator evasion (Stewart et al., 2013, 2014). We found that fish foraging without the entire LLS would strike less often than those lacking just the cranial system (Fig. 2B), but the capture probability was indistinguishable between these groups (Fig. 2C). Therefore, the posterior lateral line may serve a minor role in stimulating foraging, but it does not enhance the effectiveness of a strike.

We found no evidence that change in the morphology of neuromasts enhances flow sensing with age. Our analysis focused on the cranial neuromasts, because of their above-mentioned prominent role in foraging. These neuromasts did not increase in number between 15 and 30 dpf (Fig. 3), which is the period where we found the largest increase in foraging performance (Fig. 1). In addition, the size and shape of the neuromasts did not change in any way that would serve to enhance sensitivity (Fig. 4). This finding was based on our measurements of the number of hair cells and the dimensions of the cupula, which are the major features that determine the mechanical sensitivity of a superficial neuromast (McHenry et al., 2008; van Netten and McHenry, 2013). It is surprising that these features did not vary with age, because previous studies suggested that this period coincides with a proliferation in the number of cranial neuromasts (Nuñez et al., 2009) and the advent of canal neuromast development at 22 dpf (Webb and Shirey, 2003). These changes are likely to impact the role of the cranial LLS, but they correspond to developmental transformations that apparently occur after the ages that we considered. The discrepancy between

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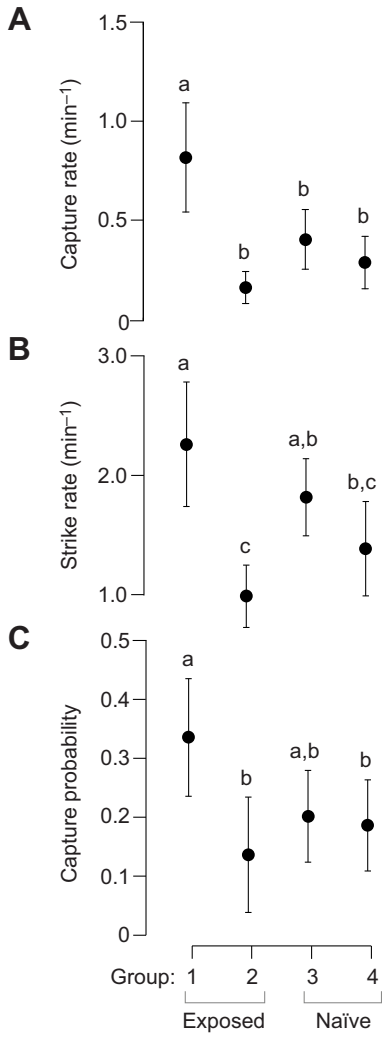

Fig. 5. Effects of learning on foraging performance in the dark. The effects of learning were addressed through measurements of foraging performance in the dark for four groups of juvenile zebrafish (30 dpf, *N*=15 for each group) that were raised under distinct conditions. Groups 1 and 2 were raised on live *Artemia* and were thereby exposed to the flow generated by prey. Group 1 serves as a control because the LLS was not manipulated, whereas foraging in Group 2 was performed on fish with a LLS compromised by exposure to neomycin. The other two groups were naïve to the flows created by *Artemia* by the time of the foraging experiment. This was achieved by raising fish that either were fed dead *Artemia* (Group 3) or were exposed to neomycin daily to compromise the LLS, (Group 4). Values (mean±95% CI) of (A) capture rate, (B) strike rate, and (C) capture probability are shown with different letters indicating significant differences between groups (Tukey's HSD).

present and past studies is likely due to differences in the rearing conditions (Schilling, 2002). In support of this interpretation, our juveniles (30 dpf) were substantially shorter in body length (~9.0 mm) than juveniles in at least one of these prior studies (~10.4 mm) (Webb and Shirey, 2003).

Larvae learn to sense flow

We tested the hypothesis that larvae improve in their ability to forage in the dark (Fig. 1F) as a consequence of learning. The larvae reared for our experiments were fed live *Artemia* daily under illuminated conditions. According to our hypothesis, larvae acquired an association between the flow generated by the swimming of *Artemia* and the reward of ingested prey. Therefore, larvae were capable of recognizing the flow of *Artemia* without vision when we performed a foraging experiment in the dark. This hypothesis predicts that larvae raised under conditions that prohibit the opportunity to associate a flow stimulus with feeding will be as ineffective at foraging in the dark as larvae lacking the LLS. This was indeed what we found. Larvae that were either raised with a daily treatment of neomycin or were fed dead *Artemia* exhibited a capture rate that was indistinguishable from those with a compromised LLS (Fig. 5A). We therefore interpret the ability of larvae to improve in their feeding in the dark with age (Fig. 1F) to be a consequence of associative learning.

Our results are consistent with previous studies on learning in zebrafish. Under classical conditioning, adult zebrafish learn to direct their swimming in response to visual stimuli (e.g. Darland and Dowling, 2001; Sison and Gerlai, 2010; Bianco et al., 2011) and to forage in response to synthetic chemicals (Braubach et al., 2009). Young larvae (6–8 dpf) can similarly be trained to exhibit tail-flicking behavior in response to light by pairing it with touching by a probe (Aizenberg and Schuman, 2011). The ability to perform associative learning increases with age. In experiments that used swimming in response to visual cues, Valente et al., (2012) found larvae younger than 4 weeks old to be unresponsive to classical conditioning, but to respond to operant conditioning by electric shocks as young as 3 weeks old. Our results suggest that larvae may begin to learn by 15 dpf when offered the reward of prey.

Our results are significant to the advancement of the understanding of the larval ecology of fishes and the neurophysiology of learning. Fish capable of learning to identify flow can forage when vision is compromised, such as in dark or turbid waters. This suggests that the sensory systems of fishes are sufficiently plastic that they may generally be recruited for essential functions. Such plasticity is likely to permit foraging under situations that challenge competitors and predators. This ability to learn has been demonstrated in zebrafish, which are an exciting model system for neurophysiology because of the techniques of functional imaging and optogenetics (e.g. Fetcho and Higashijima, 2004; Portugues et al., 2013). Through these approaches, it is increasingly possible to examine the physiological basis of learning.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

The authors collaborated equally on the design of the study, writing of the manuscript and data analysis. A.C. performed all experiments and statistical tests.

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