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RESEARCH ARTICLE

Superficial neuromasts facilitate non-visual feeding by larval striped bass (*Morone saxatilis*)

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SUMMARY

To investigate whether mechanoreception is used in non-visual feeding in larval striped bass (*Morone saxatilis*), the ontogeny of superficial neuromasts along the lateral line was described using the vital stain FM1-43FX and fluorescent microscopy. The number of neuromasts visible along one flank increased from 11 at first feeding [5 to 7 days post-hatch (dph)] to >150 by the juvenile stage (27 dph). A neomycin dose response (0, 1, 2 and 5 mmol l^{-1}) was evaluated for neuromast ablation of bass aged 10, 13, 17 and 20 dph. Using these same age groups, the ability of bass to catch *Artemia salina* prey in both dark and light tank-based feeding trials was compared between larvae with neuromasts ablated using neomycin (5 mmol l^{-1}) and controls. Neomycin significantly reduced the incidence of feeding in the light and dark. Among larvae that fed, those in the dark treated with neomycin caught fewer *Artemia* (~5 prey h^{-1}; $P<0.05$) than controls (16 prey h^{-1} at 10 dph; 72 prey h^{-1} at 20 dph). In the light, by contrast, neomycin treatment had no significant effect on prey capture by larvae age 13 to 20 dph, but did inhibit feeding of 10 dph larvae. Verification that neomycin was specifically ablating the hair cells of superficial neuromasts and not affecting either neuromast innervation, olfactory pits, or taste cells was achieved by a combination of staining with FM1-43FX and immunocytochemistry for tubulin and the calcium binding proteins, S100 and calretinin.

Key words: mechanoreception, lateral line, foraging, aminoglycoside, olfaction.

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INTRODUCTION

Feeding success among pelagic larval fish has a major influence on growth, survival and subsequent recruitment. Marine fish larvae inhabiting clear oceanic waters are considered primarily visual feeders (Hunter, 1981; Blaxter, 1986). By comparison, non-visual feeding has received less attention, but is likely important in turbid estuaries and tropical rivers, and for feeding at night (Hoekstra and Janssen, 1985; Mookerji and Rao, 1993; Vollset et al., 2011). Striped bass [*Morone saxatilis* (Walbaum 1792)] use estuaries along the east coast of North America as nursery habitat. In Canada, the Bay of Fundy population is designated 'endangered' (COSEWIC, 2012). The sole remaining waterway supporting successful spawning is the highly turbid Shubenacadie River estuary (Rulifson and Dadswell, 1995). Non-visual feeding has been reported for the Shubenacadie population as well as for various populations in the USA, but the mechanism has not been fully elucidated (McHugh and Heidinger, 1977; Chesney, 1989; Duston and Astatkie, 2012).

Non-visual feeding among teleosts may depend on a combination of sensory modalities, including mechanoreception, olfaction and gustation. Evidence for mechanoreception comes from decreased feeding in darkness of both larvae and juvenile fish following the presumed ablation of superficial neuromasts using streptomycin, an aminoglycoside antibiotic (Jones and Janssen, 1992; Batty and Hoyt, 1995; Cobcroft and Pankhurst, 2003; Mukai, 2006). However, these studies failed to verify whether neuromasts or other sensory receptors, such as taste buds or olfactory cells, were actually damaged. The need to verify ablation was recently emphasized following the observation that many lateral line hair cells within the superficial neuromasts of fish can survive aminoglycoside exposure (Brown et al., 2011). We used vital dye stain to both describe the ontogeny of striped bass neuromasts and assess their functionality following neomycin treatment. FM1-43FX [*N*-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl) pyridinium dibromide] is taken up by intact superficial neuromasts on zebrafish larvae, causing them to fluoresce brightly, whereas ablated neuromasts show partial or no fluorescence (Owens et al., 2009). In addition, we assessed the effect of neomycin on neuromast innervation, taste buds and olfactory cells using immunocytochemical staining of two calcium binding proteins found in sensory cells, S100 and calretinin, and also tubulin (Gayoso et al., 2011; Germanà et al., 2007; Levanti et al., 2008).

First feeding by larval striped bass occurs around 7 days post-hatch (dph) and at a total length (TL) of 6 mm (MacIntosh and Duston, 2007). To determine whether superficial neuromasts are present by this stage, we described their ontogeny from 4 dph through to metamorphosis around 27 dph. Among other teleost species, superficial neuromasts are not always present at the start of feeding (Blaxter and Fuiman, 1989; Connaughton et al., 1994; Mukai and Kobayashi, 1995). Finally, to relate structure to function, feeding trials were conducted in both the light and the dark to determine the effect of superficial neuromast ablation on the ability of larvae to capture *Artemia salina* prey, using the same methods used to show non-visual feeding was dependent on prey density (Duston and Astatkie, 2012).

MATERIALS AND METHODS

Egg collection and rearing conditions

Newly fertilized striped bass eggs were collected by means of a plankton net from the Stewiacke River estuary (Nova Scotia), a tributary of the Shubenacadie River. One cohort was collected in May 2009 to describe neuromast ontogeny. The following year, four cohorts were collected between 11 and 30 June to produce larvae for neuromast ablation and feeding trials. Eggs were incubated in 80 l conical tanks at 16–17°C and salinity of 1–3 ppt. Each cohort of eggs hatched approximately 48 h after collection. Larvae from each cohort were transferred at 3 dph to one of six tanks (volume 120 l) in a recirculation system held at 20°C and 5 ppt salinity. Photoperiod was 12 h:12 h light:dark, with lights on at 07:30 h. Light intensity at the water surface was 50 lx during the day and 0 lx at night. First feeding and swim bladder inflation occurred between 5 and 7 dph. To facilitate swim bladder inflation, oil was removed from the surface using skimmers made of polystyrene. Larvae that were aged 5 to 15 dph were fed stage I *A. salina* nauplii (Aquafauna Bio-Marine, Hawthorne, CA, USA), and older larvae were fed stage II nauplii enriched with Algamac (Aquafauna Bio-Marine).

All procedures described were approved by the Dalhousie University Institutional Animal Care and Use Committee.

Ontogeny and neuromast ablation

Neuromast ontogeny was described for larvae age 4 to 27 dph (7 to 17 mm TL). In a dimly lit room (200 lx, 20°C), between four and 12 larvae were held in a cell strainer (100 μm mesh) in a six-cell tissue culture plate (Falcon, Fisher Scientific, Ottawa, ON, Canada) and immersed for 40 min in 6 ml of 3 μmol l^{-1} FM1-43FX fluorescent dye (Invitrogen, Burlington, ON, Canada) made with 1 ppt seawater (SW). Larvae were then euthanized [0.2 g l^{-1} tricaine methanesulfonate (MS-222) in 10 ppt seawater; Syndel, Nanaimo, BC, Canada], fixed in 4% paraformaldehyde (PFA; Cedarlane, Burlington, ON, Canada) in phosphate buffered saline (PBS; 100 mmol l^{-1} phosphate buffer; 147 mmol l^{-1} NaCl; pH 7.4), and kept in darkness at 4°C overnight. The next day, larvae were transferred to PBS and stored at 4°C in the dark and examined within 1 week. Larvae were examined with a Leica MZ FLIII fluorescent microscope (Leica, Wetzlar, Germany) equipped with a digital camera (coolSNAP-Pro; Media Cybernetics Manufacturing, Rockville, MD, USA). A 470 nm excitation filter was used to produce 515 nm emissions from the vital dye FM1-43FX. Each larva was viewed in the lateral plane only and the number of neuromasts was counted in the following divisions: eye (infra- and supra-orbital), preoptic (nasal), mandibular (jaw), lower head (pre-opercular and opercular neuromasts), upper head (occipital and middle neuromasts), posterior (trunk) and terminal (tail). Supratemporal neuromasts were present, but were not visible along the lateral plane. The total number of visible neuromasts along one flank varied among larvae of the same age, so the images of between four and 12 larvae were compared and a composite was hand-drawn showing the typical number (mode) and location of neuromasts visible. Olfactory pits were also stained by FM1-43FX. The drawings were scanned and traced in PaintShop Pro X2 (Corel, Ottawa, ON, Canada).

To ablate the neuromasts, we chose the antibiotic neomycin rather than streptomycin because its efficacy is better established (Harris et al., 2003; Owens et al., 2009). Four concentrations of neomycin sulphate (0, 1, 2 and 5 mmol l^{-1}; Sigma-Aldrich, Oakville, ON, Canada) were made in 1 ppt SW and tested on larvae age 10, 13, 17 and 20 dph. For each replicate of each age group, four larvae were randomly distributed between four cell strainers immersed in 1 ppt SW. One strainer was then transferred to each of the four neomycin solutions for 60 min, then rinsed twice (each 20 s in 1 ppt SW) and placed in 1 ppt SW for a 2 h chase interval to allow additional time for the neomycin to affect the neuromasts. Larvae were then transferred to FM1-43FX for 40 min, then euthanized and processed as described above. Three replicates of each dose–age combination were achieved by repeating this procedure using two additional cohorts of larvae. The intensity of fluorescent light emitted by each visible neuromast among larvae treated with neomycin was judged by eye and scored as either ‘normal’ or ‘reduced’ by comparing with controls at the same anatomical location, an approach used in similar studies (Harris et al., 2003; Murakami et al., 2003). The absence of fluorescence in some neuromasts among larvae treated with neomycin was obvious, but these could not be quantified with confidence because of subtle variations in neuromast patterns between individuals. Consequently, for statistical analysis, mean number of neuromasts exhibiting normal fluorescence was compared between neomycin treatments.

Neuromast damage due to neomycin was confirmed with immunocytochemistry staining for S100, calretinin and tubulin, and subsequent confocal microscopy. Larvae were first fixed in 4% PFA and then washed overnight at 4°C in PBS-T solution containing 2% DMSO (Sigma-Aldrich), 1% bovine serum albumin, 1% normal goat serum and 0.25% Triton X-100 in PBS. They were then incubated for 1 week with different primary antibodies diluted in PBS-T. Some were double labeled with anti-S100 (polyclonal rabbit antibody against bovine S100; 1:150 dilution; Dako, Glostrup, Denmark, catalog no. Z0311) and anti-calretinin (monoclonal mouse antibody against recombinant human calretinin; 1:150 dilution; Swant, Bellinzona, Switzerland, catalog no. 6B3). These antibodies have been shown to label neuromasts or taste receptors, respectively, in a range of other fish (Abbate et al., 2002; Germanà et al., 2007; Varatharasan et al., 2009). Other larvae were double labeled with anti-S100 and anti-tubulin (monoclonal DM1A mouse antibody against chick brain microtubules; Sigma-Aldrich, catalog no. T9026), both diluted 1:150. Larvae were then washed four times in PBS, each for 60 min, and then placed in secondary antibody diluted with PBS-T for 96 h. The secondary antibody for S100 was AlexaFluor 488 goat anti-rabbit (Invitrogen; 1:200 dilution), and for both calretinin and tubulin was AlexaFluor 555 goat anti-mouse (Invitrogen; 1:200). Larvae were then washed three times in PBS, each for 60 min, stored in PBS overnight, then mounted in glycerol and viewed with a Leica DM4000 B epifluorescent microscope. Negative controls were larvae incubated in secondary antibodies without first being labeled with primary antibodies. This resulted in little or no fluorescence in any sensory structures.

Selected preparations of neuromasts, taste buds and lateral line nerves were then examined using a Zeiss LSM 510 META confocal microscope (Carl Zeiss Canada, Toronto, ON, Canada). Stacks of 10–20 images made by sequences of focal planes at 1–2 μm intervals were overlaid to create images using Zeiss LSM 510 software. Images were then exported to PaintShop Pro for final assembly of plates.

Counts of hair cells from individual neuromasts were taken from the head, mid-trunk and tail of four larvae age 5 dph treated with 5 mmol l^{-1} neomycin and four control larvae. Hair cell numbers varied with neuromast position on the body; therefore, the means were compared between neomycin-treated and control hair cell counts within each location. Between one and three neuromasts were also examined for hair cell numbers from other larval ages (11, 13 and 20 dph). To photograph taste buds, the lower jaw was isolated following an established method (Varatharasan et al., 2009). Taste buds were examined in two control larvae age 11 and 20 dph, and up to two larvae treated with neomycin at ages 5, 14 and 19 dph.

Olfactory pits were examined in all specimens and were consistently labeled with FM1-43FX independent of neomycin treatment, but two exceptions were noted: one 14 dph larva treated with neomycin and one 17 dph control larva.

Prey capture trials

Feeding trials were repeated using larvae age 10, 13, 17 and 20 dph following established procedures (MacIntosh and Duston, 2007). The two experimental factors, each with two levels, were neomycin concentration (5 mmol l^{-1} and control) and light intensity (dark, and 22 lx at the water surface). A neomycin concentration of 5 mmol l^{-1} was chosen because it caused a significant decrease in the number of intact neuromasts (see Results) and no apparent effects on swimming behaviour of the larvae. Eight tanks were available, allowing four replicate tanks of neomycin-treated larvae and four replicate tanks of untreated controls. Light and dark trials were run on consecutive days to eliminate the risk of light illuminating the blacked-out tanks. Two cohorts of larvae that hatched 1 day apart were used, allowing the paired light and dark trials to use larvae of the same age. The response variable was the prey capture rate by individual larvae. The day before a trial, food was withheld from 17:00 h to ensure the intestinal tract of the larvae was empty for the start of the feeding trial. The following morning at 08:00 h, a random sample of larvae was quickly siphoned from the rearing tank into a white bucket and transferred to the preparation room, which was dimly lit to reduce stress on the larvae. Using a small plastic beaker, an average of 80 larvae was gently transferred in water into each of two baskets immersed in water from the rearing tank. The baskets were made of PVC pipe (5 cm high, 7.5 cm inner diameter) with a base of Nitex mesh (625 μm). The baskets of larvae were then transferred from the rearing water bath to either 150 ml of 5 mmol l^{-1} neomycin or 1 ppt SW, and left for 60 min. Both baskets of larvae were then given two rinses in 1 ppt SW (each 20 s) and allowed a chase interval of 60 min in 1 ppt SW. Then from each of the two baskets, 20 (±5) larvae were counted into each of four plastic containers. Each container of larvae was then introduced randomly to one of the eight feeding trial tanks and given 60 min to acclimate to the 30 l of 5 ppt SW at 20°C. *Artemia salina* stage I nauplii were added to each of the eight tanks in sequence with 3 min intervals between tanks to give an initial density of 200 prey l^{-1}. The mean length of the nauplii was 460±50 μm and width was 497±78 μm including appendages. Larvae were given 60 min to feed, then the tanks were quickly drained and the larvae were caught and euthanized (ice-cold solution of MS-222, 0.2 g l^{-1}). Each larva was

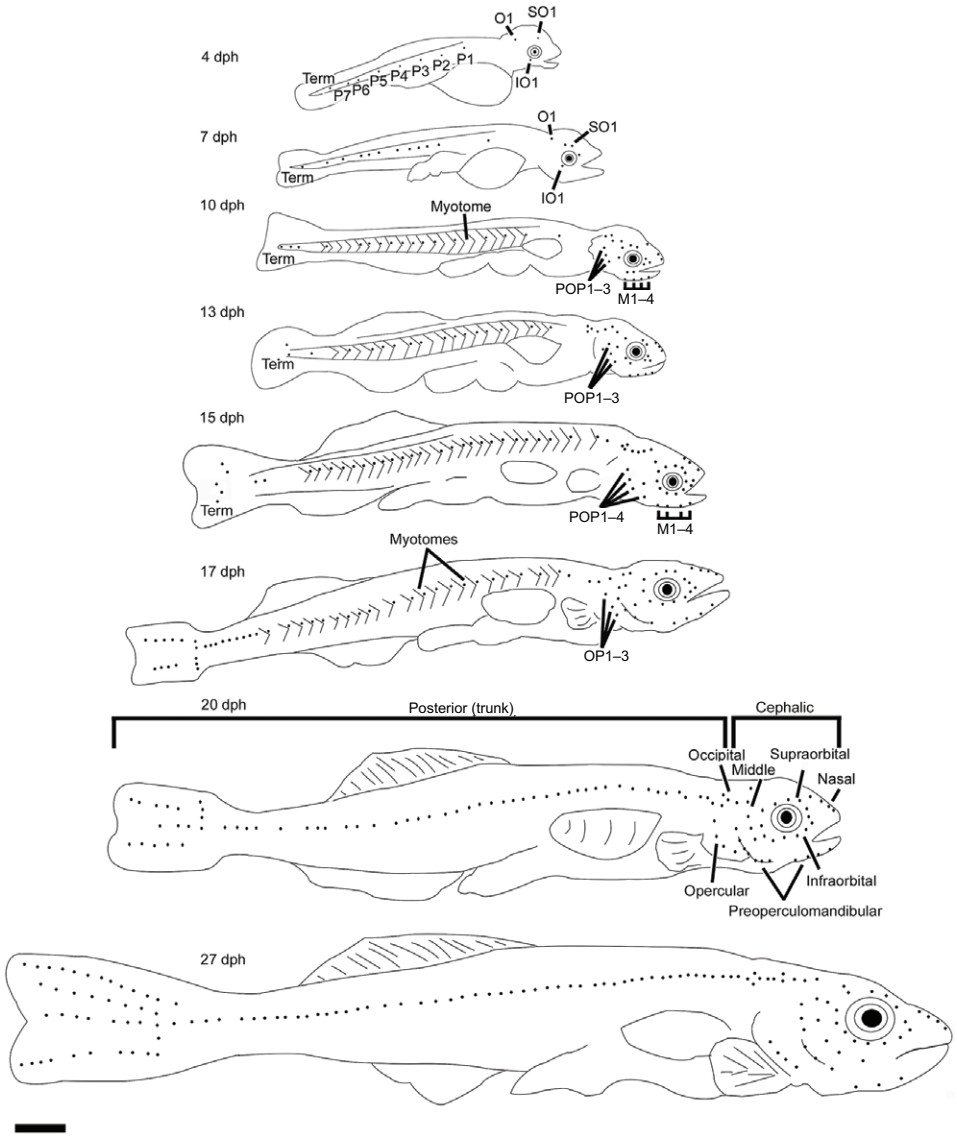

Fig. 1. Composite diagrams showing typical locations of superficial neuromasts along one flank of striped bass aged 4 to 27 days post-hatch (dph) using the vital dye FM1-43FX. Myotomes are visible at 10 dph. IO1, infraorbital neuromast; M1–4, mandibular neuromasts; O1, occipital neuromast; OP1–3, opercular neuromasts; POP1–4, pre-opercular neuromasts; SO1, supraorbital neuromast; P1–7, posterior trunk neuromasts; Term, terminal neuromasts of the tail. Scale bar, 1 mm.

examined under a dissecting microscope to determine the number of prey in the mouth, throat or stomach, and total body length (TL). Larvae ranged from an average total length of 7.4 mm (range: 6–8.2 mm) at 10 dph to 11.9 mm (range: 9–14 mm) at 20 dph.

Statistical analyses

The effect of neomycin concentration on neuromast ablation was analyzed as a split-plot ANOVA because randomization of the run order of larval age (10 to 20 dph) was not possible. The whole-plot treatment was age of the larvae (four levels: 10, 13, 17 and 20 dph) and the subplot treatment was neomycin concentration (four levels: 0, 1, 2 and 5 mmol l^{-1}). The response variable was the average number of brightly stained neuromasts visible along one flank. The experimental unit was one cell strainer containing four fish. The block consisted of three replicate strainers for each concentration and age combination. The mean number of hair cells among larvae age 5 dph (N=4) treated with neomycin compared with controls was analyzed by a one-way ANOVA.

The incidence of feeding, defined as the percentage of larvae having caught one or more prey items, was analyzed using CATMOD followed by the contrast statement for all pairwise combinations of age (10, 13, 17 and 20 dph) and treatment (light/control, light/neomycin, dark/control, dark/neomycin). Lettered groupings were generated at the 5% significance level.

Among larvae that fed, prey capture rate was analyzed as a 2×2 factorial design, repeated at ages 10, 13, 17 and 20 dph. The two independent factors were light intensity (two levels: dark, 0 lx and light, 22 lx) and drug treatment (two levels: control and 5 mmol l^{-1} neomycin). There were four replicate tanks per treatment. The response variable was the average number of *A. salina* captured per tank per hour among those larvae that captured one or more prey. To satisfy the assumptions of the repeated-measures ANOVA, a cubic root transformation was necessary. Least squares means were computed, and lettered groupings generated at the 5% level to indicate which means were significantly different. Means in the text are cited ±s.e.m. unless otherwise specified.

All statistical analyses were conducted using SAS version 9.2 (SAS Institute, Cary, NC, USA).

RESULTS

Neuromast ontogeny

The mean number of superficial neuromasts visible along one flank increased more than 10-fold during larval development from 11.5±1.5 at age 4 dph to 152±4.3 at 27 dph. Both FM1-43FX and anti-S100 stains revealed neuromasts in similar numbers and locations. The youngest larvae examined, 4 dph, had three cephalic neuromasts, and seven to nine neuromasts on the trunk and tail (Fig. 1). By age 10 dph, the number of neuromasts on the head (N=20) exceeded those on the trunk and tail (N=15), with four mandibular, three pre-opercular and approximately eight neuromasts around the eye. Myotomes were visible by age 10 dph, with trunk neuromasts located at their boundaries, slightly dorsal to the myotome points (Fig. 1). Not all myotomes had an associated neuromast, particularly on young larvae. Small intercalary neuromasts were apparent mid-way between the trunk neuromasts on a few larvae age 10 dph and older (not shown on Fig. 1). Three nasal neuromasts were visible at age 10 dph, becoming fixed at four on larvae aged 13 dph and older. Tail neuromasts were first evident at age 13 dph (Fig. 1). By 15 dph, 13 neuromasts were arranged around the eye, this number remaining fixed among older larvae. Larvae aged 15 dph had up to 32 cephalic neuromasts, and a similar number distributed between the trunk and tail (Fig. 1). Between 15 and 17 dph, the most notable changes were a doubling in number of the tail neuromasts into two linear rows and the appearance of three opercular neuromasts. From 17 dph onwards, the number of neuromasts on the trunk exceeded those on the head. By 20 dph, the cephalic neuromasts were aligning to form the infraorbital, supraorbital, middle and preoperculomandibular lines (Fig. 1). At 27 dph, the fish resembled juveniles and the neuromasts were becoming enclosed within canals. The mean numbers of head and trunk neuromasts at age 27 dph were 45±2.2 and 64±3.3, respectively. In addition, the caudal fin neuromasts exceeded 40 in number, organized in four distinct lines (Fig. 1).

Neomycin effects on neuromasts, their innervation, and taste and olfactory cells

Neomycin (5 mmol l^{-1} for 60 min) caused varying degrees of damage to individual superficial neuromasts, judging by the intensity of fluorescence following FM1-43FX staining. A few neuromasts fluoresced as brightly as untreated controls, but many other neuromasts were noticeably less intensely labeled. Still other neuromasts were completely absent from specific locations where neuromasts were likely located (Fig. 2A,B). In contrast, the olfactory pits appeared to fluoresce equally in both controls and neomycin-treated larvae, suggesting that the neomycin was not likely targeting this structure. Staining for S100 protein confirmed that the neomycin

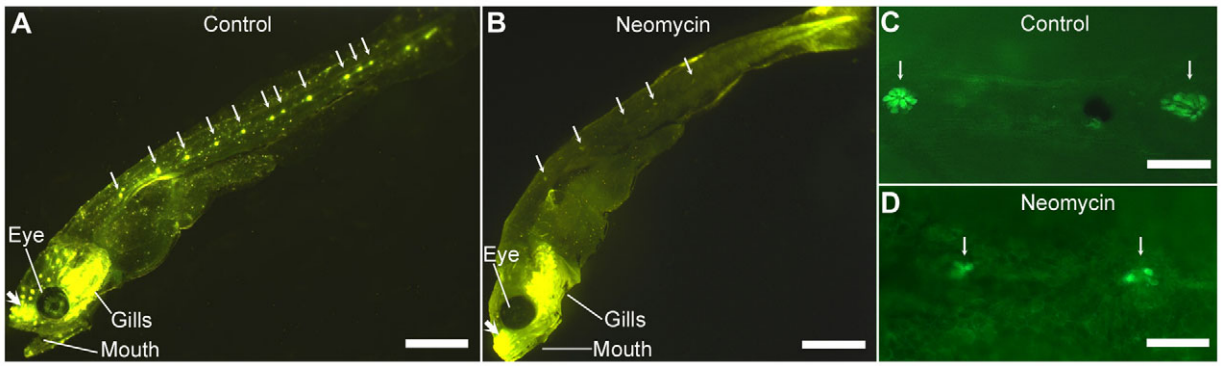

Fig. 2. (A) Control striped bass larva (10 dph) stained with the vital dye FM1-43FX. Thirty-one neuromasts are visible as bright green dots on the head (18), trunk (11; arrows) and tail (2). (B) Neomycin (5 mmol l^{-1}) treated larva (10 dph) with 14 partially ablated neuromasts on the head (8), trunk (5) and tail (1) as judged by the reduced fluorescence, and others fully ablated judging from absence of staining. Thick white arrows show olfactory pits stained in both A and B. Scale bars, 1 mm. (C) Trunk region of 13 dph control larva showing two intact neuromasts (arrows) with brightly fluorescing hair cells stained with the immunofluorescent dye anti-S100, and viewed with fluorescent microscopy. (D) Trunk region of 13 dph neomycin (5 mmol l^{-1}) treated larva showing two partially ablated trunk neuromasts (arrows) with reduced fluorescence of S100 protein and fewer visible hair cells. Scale bars, 50 µm.

treatment had varying effects on the neuromasts and the variation in fluorescence was not a histological artifact of the FM1-43FX staining procedure (Fig. 2C,D).

Each neuromast was composed of a cluster of hair cells arranged in a tight oval with a kinocilium root visible on the apical end of each cell and afferent nerve terminals contacting the base (Fig. 3A). Cephalic neuromasts of larvae age 11–13 dph contained an average of 29 hair cells (N=4) while trunk neuromasts averaged 12 hair cells (N=3). Stereocilia and cupulae were not visible. Tubulin staining revealed the lateral line nerve running underneath the neuromasts from which axons projected into the neuromast base (Fig. 3B,C).

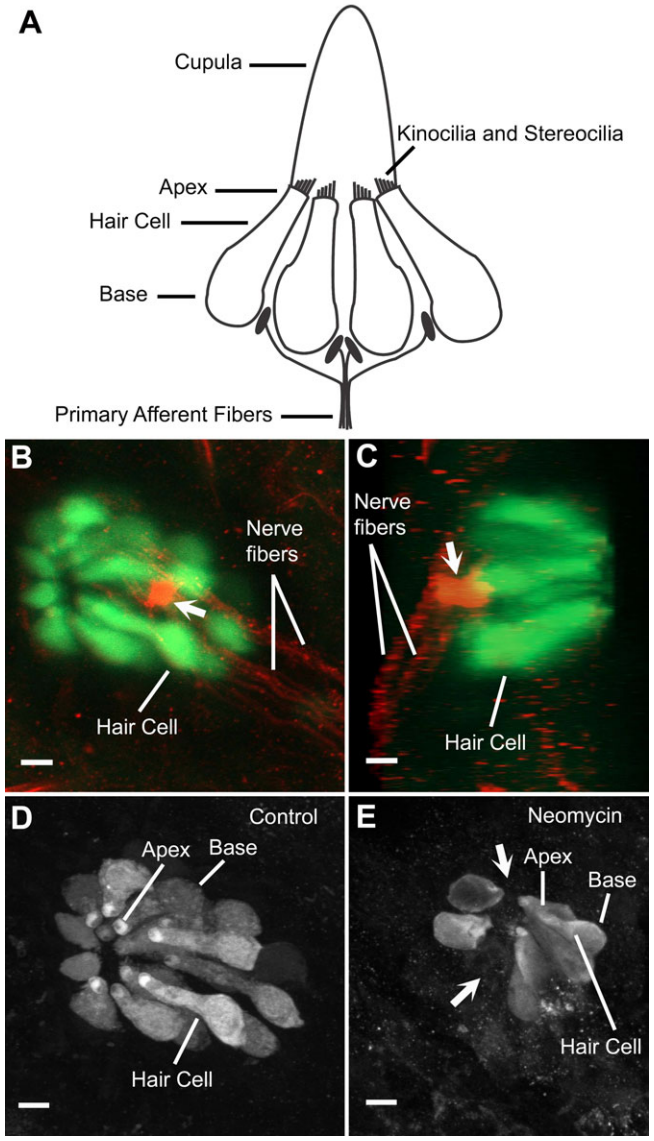

Fig. 3. (A) Schematic of a neuromast in the lateral view. (B) Trunk neuromast of a 13 dph striped bass control larva stained for S100 (green) positioned dorsally to its innervating lateral line nerve fibers stained for tubulin (red). Nerve fibers are protruding into the hair cells (arrow). (C) Same trunk neuromast rotated, showing the nerve fibers protruding into the hair cells (arrow). (D) Same trunk neuromast showing normal arrangement of 12 hair cells with the kinocilium root (dark spot) visible against the white background of the apex of each hair cell. (E) Ablated trunk neuromast of a 14 dph larva treated with neomycin (5 mmol l^{-1}) showing seven hair cells in a disorganized arrangement with apices missing kinocilium roots. Arrows indicate missing hair cells. Scale bars, 5 μm.

Among neuromasts treated with neomycin, the number of visible hair cells was reduced compared with controls, and those hair cells that remained were deformed and fluoresced poorly (Fig. 3D,E). At 5 dph, mean hair cell numbers among neomycin-treated larvae were significantly reduced (Table 1).

The toxic effects of neomycin appeared to be specific to hair cells and not to associated structures or other superficial sensory cells. For example, in a 14 dph larva, the calretinin-labeled lateral line nerve branches extending onto the tail appeared intact following neomycin treatment (Fig. 4A), while the neuromasts adjacent to the nerve branch endings appeared to show varying degrees of damage (Fig. 4B). Also, in two 19–20 dph larvae, the number of calretinin-labeled taste buds visible along the upper jaw and the olfactory pits were unaffected by neomycin and were of similar size and structure when viewed with confocal microscopy (Fig. 5).

Effect of neomycin dose on the number of intact neuromasts

The mean number of intact neuromasts visible along one lateral plane was dependent on a significant interaction between the age of the larvae and neomycin dose (P<0.001). Among untreated larvae, the mean number of intact neuromasts increased with age from 27±1.1 at 10 dph to 90±7.1 at 20 dph (Fig. 6). Among 10 and 13 dph larvae, the number of intact neuromasts was inversely proportional to neomycin concentration up to 2 mmol l^{-1}, but increasing the dose to 5 mmol l^{-1} neomycin caused no further reduction in intact neuromasts (Fig. 6). Among older larvae (17–20 dph), 1, 2 and 5 mmol l^{-1} neomycin treatments were equally effective, reducing the number of intact neuromasts by approximately 50% compared with controls (Fig. 6).

Prey capture trials

The incidence of feeding (as defined by the number of larvae that ingested at least one prey) was reduced significantly by neomycin in both 20 lx light and darkness. Younger fish were affected the most, resulting in a highly significant interaction between age and treatment on the incidence of feeding (P<0.001). Among 10 dph larvae, only 19 to 33% captured prey following exposure to neomycin compared with 77 to 87% in control larvae at that age (Table 2). By comparison, among 20 dph larvae, the reduction in the incidence of feeding caused by neomycin was smaller, but remained significant both in the light (91 *versus* 61%) and dark (60 *versus* 38%; Table 2). The effect of light *versus* dark on the incidence of feeding, independent of neomycin treatment, was significant only among larvae aged 20 dph (Table 2). Upon further analysis of only those larvae that fed, capture of *A. salina* was significantly affected by a three-way interaction between light intensity, age of larvae and neomycin dose (P<0.001). In the light, prey capture among both neomycin-treated and control larvae increased markedly with age from <30 prey $larva^{-1}$ h^{-1} at age 10 dph to >140 prey $larva^{-1}$ h^{-1} at 20 dph (Fig. 7A). Neomycin treatment had no significant effect on prey capture in three of the four age groups in the light. Only in 10 dph larvae did neomycin cause a significant decrease in prey capture in the light (Fig. 7A). In the dark, by

Table 1. Comparison of mean (±s.e.m.; N=4) neuromast hair cell numbers in three anatomical locations between control and neomycin (5 mmol l^{-1})-treated striped bass larvae age 5 dph

	Control	Neomycin
Cephalic	19.7±1.5	10.4±3.3
Middle trunk	17.8±3.6	3.3±0.7
Tail	16.0±1.8	7.7±0.4

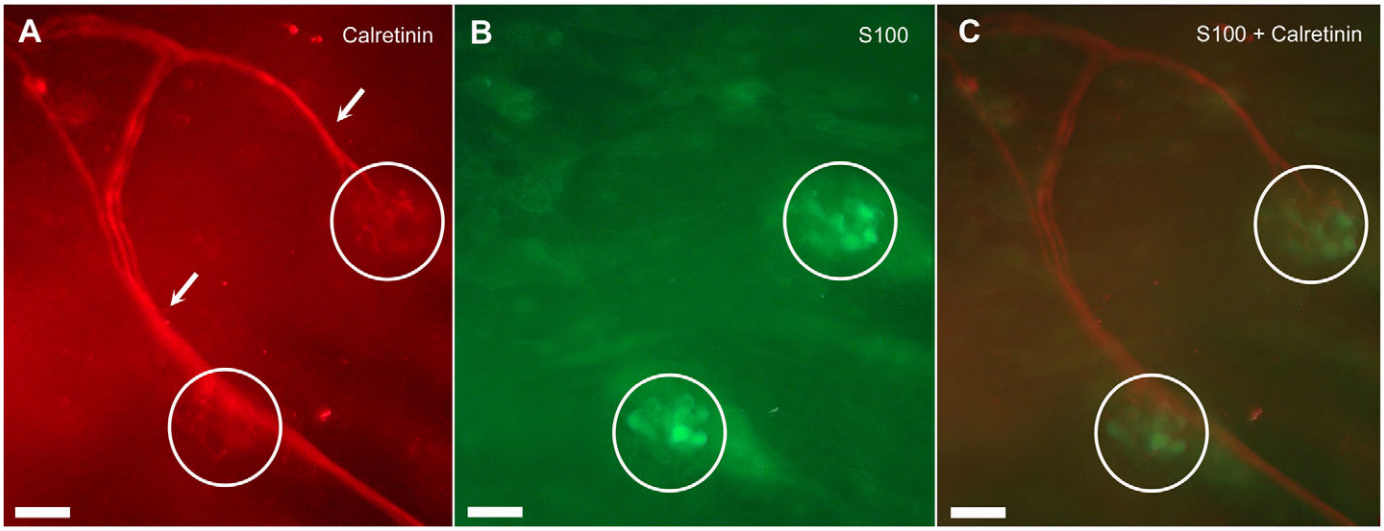

Fig. 4. (A) Lateral line nerve branches (arrows) extending to the tail of a 14 dph striped bass larva treated with neomycin (5 mmol l^{-1}) and stained for calretinin. Circles indicate nerve branch endings where neuromasts are located and innervated. (B) Neomycin treated (5 mmol l^{-1}) neuromasts (circles) correspond to the position of underlying nerve branch endings on the tail of the same larva stained with anti-S100. (C) Neuromasts stained with anti-S100 (green) are shown superimposed on the nerve branch endings stained for calretinin (red). Scale bars, 25 µm.

contrast, prey capture by neomycin-treated larvae was significantly lower than that by control larvae in all age groups (Fig. 7B). Larvae treated with neomycin caught approximately 5 prey $larva^{-1}$ h^{-1} independent of their age. By comparison, prey capture by control larvae in the dark improved progressively with age from 16 prey $larva^{-1}$ h^{-1} at 10 dph to 72 prey $larva^{-1}$ h^{-1} at 20 dph (Fig. 7B).

DISCUSSION

Non-visual feeding appears to be an important adaptation for larval striped bass in their highly turbid estuarine nursery habitat, allowing them to feed day and night at any depth. The evidence presented here complements and extends previous studies by demonstrating that non-visual feeding in fish is hindered by neomycin treatments, which appeared to specifically damage superficial neuromast hair cells, but caused no apparent damage to other sensory modalities that may aid prey detection in darkness.

The ontogeny of superficial neuromasts in striped bass is similar to that of the closely related European sea bass, *Dicentrarchus labrax* (Diaz et al., 2003). Moreover, the developmental pattern of superficial neuromasts in both species is broadly similar to that in other teleosts (Blaxter, 1987). At 4 dph, just prior to exogenous feeding, both *M. saxatilis* and *D. labrax* have three neuromasts on either side of the head and eight along each flank. Similarly, when they reach 15 mm TL, both have approximately 20 neuromasts on either side of the head, and 35 to 40 along the developing lateral line, with some aligned dorsoventrally near the caudal fin (Diaz et al., 2003). Neuromast diameter in these species is also similar, ranging from 30 to 60 µm, as are the number of hair cells per neuromast in both striped bass and sea bass, at approximately 20 to 35 (Diaz et al., 2003). Despite the similarities in neuromast development between *M. saxatilis* and *D. labrax*, there is no evidence that the latter can feed in the dark (Diaz et al., 2003;

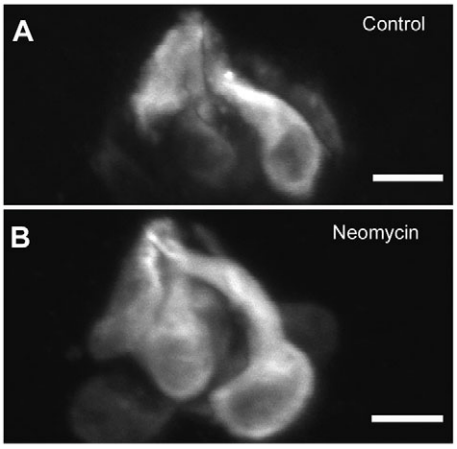

Fig. 5. (A) Taste bud from the lower jaw of a 20 dph control larva. (B) Taste bud from the lower jaw of a 19 dph larva treated with neomycin having similar structure and size as the control. Scale bars, 50 µm.

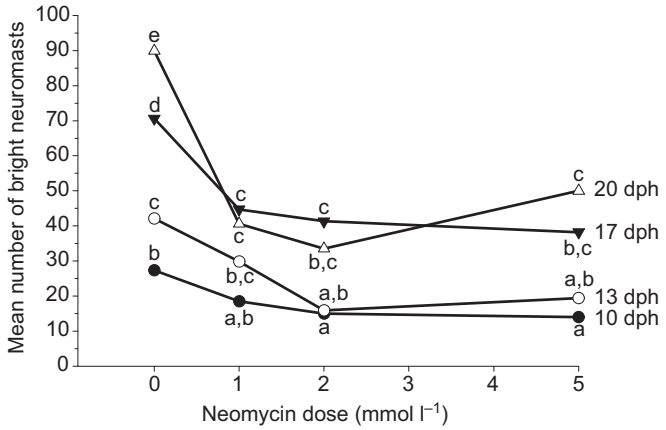

Fig. 6. Effect of neomycin dose for 1 h followed by 2 h in 1 ppt seawater on the mean number of intact superficial neuromasts along one flank of *N*=12 striped bass larvae aged 10 to 20 dph. Intact neuromasts fluoresced bright green following staining with FM1-43FX dye. Means sharing the same letter are not significantly different at the 5% level.

Table 2. Incidence (%) of feeding by striped bass larvae age 10 to 20 dph on *Artemia salina* stage I nauplii in either light (20 lx) or dark (0 lx) following treatment with neomycin (5 mmol l^{-1})

	Light		Dark	
Age (dph)	Control	Neomycin	Control	Neomycin
10	87.1[a]	33.3[d,e]	77.4[a,b]	19.1[e]
13	83.6[a,b]	48.4[c,d]	82.0[a,b]	35.0[d,e]
17	74.5[b]	37.5[d]	62.4[b,c]	38.1[d]
20	90.6[a]	61.3[c]	60.0[c]	37.9[d]

Percentages are pooled from four replicate tanks, each containing approximately 20 larvae. Percentages sharing the same letter are not significantly different at the 5% level.

Villamizar et al., 2011). However, we urge caution before concluding that a larval fish is incapable of non-visual feeding. Some fish possess adaptations that allow them to feed more effectively in the absence of visual cues. For instance, the normal length of the cupulae overlying the neuromasts is much greater for willow shiner, *Gnathopogon elongatus*, larvae than for typical visual feeders [100–250 *versus* 45–60 μm, respectively (Mukai and Kobayashi, 1991)]. Relatively long cupulae are also found in superficial neuromasts of adult blind cave fish *Astyanax hubbsi* (Teyke, 1990). Furthermore, larval weakfish, *Cynoscion regalis*, only revealed non-visual feeding capability at high prey densities (Connaughton et al., 1994), and larval cod *Gadus morhua*, once thought to be exclusively visual feeders, can forage effectively at night (Vollset et al., 2011). Indeed, in our experience with striped bass, in some trials in darkness not a single fish fed (J.D., unpublished) and other times the non-visual feeding rate varied considerably between studies for reasons unknown (MacIntosh and Duston, 2007; Duston and Astatkie, 2012). Failure to feed in the dark can be caused by damage to the cupula of neuromasts during handling of the larvae and/or conditions in the rearing tanks. Non-visual feeding by larval willow shiner on *Artemia* was eliminated by destruction of the cupulae, but subsequently resumed as the cupulae regenerated in the following hours (Mukai et al., 1994). Measures and assessments of damage to cupulae were not performed in the present study because the structures were destroyed during histological processing, but examination would be warranted in the future.

In our experiments using neomycin to affect neuromast function, we found that damage occurs with a dose dependency and a maximal effect asymptote near 5 mmol l^{-1}. This range was similar to the streptomycin dose needed to suppress the escape response of larval herring mediated by free neuromasts (Blaxter and Fuiman, 1989), but significantly greater than the aminoglycoside dose for effective ablation of hair cells in larval zebrafish (Harris et al., 2003), and suggests the need for testing effective doses for each species examined. We suggest that the relative insensitivity of bass and herring to aminoglycosides was likely due to the divalent cations in the dilute seawater incubation media, since both Ca^{2+} and Mg^{2+} protect hair cells (Coffin et al., 2009). Use of 1 ppt brackish water was necessary, however, because striped bass larvae can quickly become stressed and die in freshwater (J.D., unpublished).

Our initial assessment of the effects of neomycin relied on differences in fluorescence intensity between superficial neuromasts on individual teleost larvae after aminoglycoside treatment followed by FM1-43FX staining, similar to the approach used previously (Harris et al., 2003; Owens et al., 2009). However, we confirmed these findings using immunocytochemical staining for S100, a calcium binding protein often found in sensory neurons (Abbate et al., 2002; Germanà et al., 2007; Heizmann, 2002). Moreover, confocal imaging showed that the reduced fluorescence of striped bass neuromasts was associated with deformed and sometimes completely missing hair cells following aminoglycoside treatment, similar to zebrafish neuromasts treated with neomycin (Harris et al., 2003; Owens et al., 2007; Owens et al., 2008). Together these results indicate that numerous structural and presumably biochemical changes occur in neuromasts exposed to neomycin and that these changes result in decreased function of the hair cells as reported previously in larval fish (Blaxter and Fuiman, 1989; Mukai, 2006). It is noteworthy, however, that even at relatively high concentrations of neomycin, numerous hair cells appear to survive with no apparent damage. In fact, incomplete hair cell ablation following aminoglycoside treatment is widespread among teleost species, and this problem prompted Brown et al. (Brown et al., 2011) to urge investigators to reconsider using aminoglycosides in behavioral studies on the lateral line in fish. Based on the results presented here, we agree that it would be imprudent to assume that aminoglycoside treatment eliminates all lateral line function. However, we suggest that neomycin treatment does appear to decrease mechanosensory sensitivity and/or acuity sufficiently to impair its use in non-visual feeding. Furthermore, while our examination of other sensory systems was qualitative, it appears that 5 mmol l^{-1} neomycin caused no detectable changes in either the

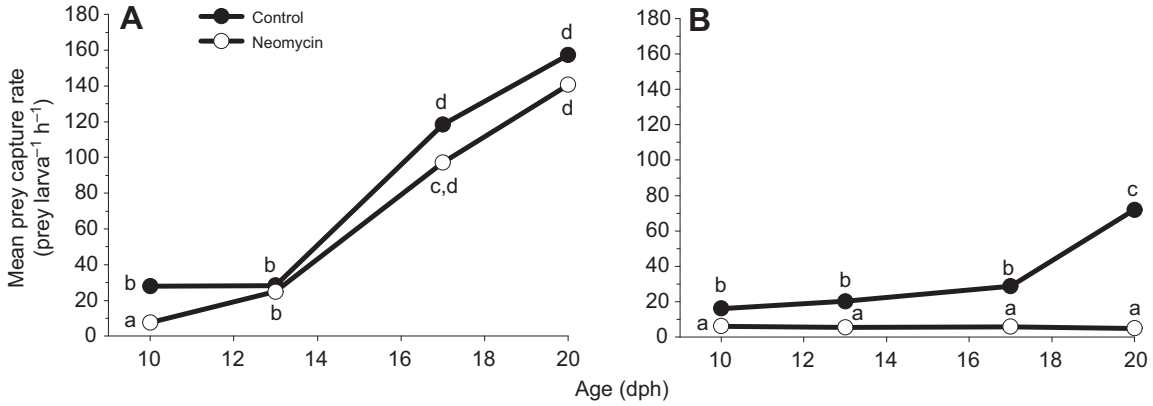

Fig. 7. Effect of neomycin (5 mmol l^{-1}) on the mean capture rates of *Artemia salina* by striped bass larvae (10 to 20 dph) either in the light (A; 20 lx) or in the dark (B; 0 lx). There were four replicate tanks per treatment, each containing approximately 20 larvae. Only those fish that caught one or more prey were included. Across both panels, means sharing the same letter are not significantly different at the 5% level.

olfactory pits or taste buds of striped bass larvae stained either with FM1-43FX or antibodies against S100 or calretinin, respectively. These findings thus bolster our confidence that the decrease in prey capture in the dark following antibiotic treatment was due specifically to impaired function of superficial neuromasts. To our knowledge, no previous study on non-visual feeding in fish has examined damage to sensory modalities other than neuromasts following aminoglycoside treatment.

In addition to showing that neuromasts are present at early larval stages and could therefore contribute to the non-visual feeding of striped bass, we also show that the ability to engage in non-visual feeding in the dark increases steadily as more neuromasts are added during the first weeks of life. Our study also provides the first experimental evidence that superficial neuromasts likely mediate non-visual feeding in larvae of this species. The three other studies linking mechanoreception to non-visual feeding in larval fish all used streptomycin and none verified its effect on either superficial neuromasts or other sensory modalities (Jones and Janssen, 1992; Cobcroft and Pankhurst, 2003; Mukai, 2006). Our findings that the incidence of feeding (number of fish that fed) decreased significantly following treatment with neomycin indicate that neuromasts provide important sensory input that guides the predatory behaviour of the larvae. However, while neuromasts may provide the only sensory input to guide feeding in the dark, we found that neomycin also decreased feeding in the light, when the larvae could presumably rely upon vision to find prey. Our results therefore suggest that even in the light, effective feeding may depend upon inputs from multiple sensory modalities with mechanosensation playing at least a facilitative role. We cannot, however, discount the possibility that neomycin may also have general toxic effects, which also cause loss of appetite in the larvae. In order to evaluate our data set more fully, we followed the approach of others (Boehlert and Morgan, 1985; Cobcroft and Pankhurst, 2003) and only analyzed the number of fish that ate at least one prey item, thereby eliminating larvae that were incapable of feeding or insufficiently motivated to feed at all. Using these latter data, we found that neomycin treatment significantly decreased feeding at night, when presumably the larvae relied exclusively on mechanoreception, but had no significant effect during the day, when vision could also be used. Larval willow shiner responded similarly after aminoglycoside treatment (Mukai, 2006). Thus, all of our analyses together consistently indicate that the neuromasts play necessary roles in non-visual feeding in the dark, but are inconclusive regarding their role in the light, when the larvae could also use vision to catch their prey. Non-visual capture rate of *Artemia* by mottled sculpin, *Cottus bairdi*, larvae exhibited a reaction distance of <0.5 mm, suggesting short-range mechanoreception (Jones and Janssen, 1992). Following streptomycin treatment, prey capture rate in the dark was barely affected, but capture of an *Artemia* occurred only if it came into direct contact with the skin of the sculpin. Hence, Jones and Janssen (Jones and Janssen, 1992) proposed that intact superficial neuromasts were needed for non-contact detection of prey.

Among older juvenile fish, a number of species appear to detect live prey in the dark by mechanoreception (Batty and Hoyt, 1995; Janssen et al., 1995; Schwalbe et al., 2012). These studies could not determine whether prey detection was by superficial neuromasts, neuromasts enclosed within canals, or both. However, physically blocking the canal organs of the blind cave fish *Anoptichthys jordani* indicated that superficial neuromasts detected prey items that produce vibratory stimuli (Abdel-Latif et al., 1990). The vibrations emitted by *Artemia* are ≤10 Hz (Barlow and Sleigh, 1980), at the low end of the detection range of superficial neuromasts, at least in goldfish, *Carassius auratus* (Goulet et al., 2012).

Describing the feeding mechanisms of larval fish is central to understanding survival, recruitment and population dynamics. Visual feeding is easily the most effective method among most species of pelagic marine fish, including striped bass, when there is sufficient light. However, the high turbidity of the estuary nursery habitat of striped bass reduces both the effective day length and water depth for visual feeding. Given this environment, the adaptation of an effective non-visual feeding mechanism in striped bass larvae is not surprising. Our study indicates that mechanoreception plays a role. The next step is to determine efficacy and effective range of this mechanism for detecting and capture of natural prey.

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AUTHOR CONTRIBUTIONS

J.A.S. led all the experimental work and conducted the data analysis. J.D. conceived the study and participated in the feeding trials and fish rearing. R.P.C. participated in the staining and microscopy. All co-authors contributed to the experimental design, results interpretation and manuscript preparation.

COMPETING INTERESTS

No competing interests declared.

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