

THE ORIENTING RESPONSE OF LAKE MICHIGAN MOTTLED SCULPIN IS MEDIATED BY CANAL NEUROMASTS

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Summary

Lake Michigan mottled sculpin, *Cottus bairdi*, exhibit a naturally occurring and unconditioned orienting response that can be triggered by both live prey and chemically inert vibrating spheres, even in blinded animals. CoCl_2 -induced reductions of the orienting response demonstrate that the lateral line is required for this behavior in the absence of non-mechanosensory cues (such as vision), but shed no light on the relative contributions of superficial and canal neuromasts to this behavior. To determine the relative roles of these two subsystems, we measured the frequency with which mottled sculpin oriented towards a small vibrating sphere before and after two treatments: (i) immersion of fish in a solution of gentamicin, an aminoglycoside antibiotic that damages hair cells in canal, but not superficial, neuromasts; and (ii) scraping the skin of the fish, which damages the superficial, but not the canal, neuromasts. To ensure that both superficial and canal neuromasts were adequately stimulated, we tested at different vibration frequencies (10 and 50 Hz) near or at the best frequency for each type of neuromast. At both test frequencies, response rates before treatment were greater

than 70% and were significantly greater than 'spontaneous' response frequencies measured in the absence of sphere vibration. Response rates fell to spontaneous levels after 1 day of gentamicin treatment and did not return to pre-treatment levels for 10–15 days. In contrast, response rates stayed approximately the same after superficial neuromasts had been damaged by skin abrasion. Scanning electron microscopy confirmed hair cell damage (loss of apical cilia) in canal, but not superficial, neuromasts of gentamicin-treated animals after as little as 24 h of treatment. The sensory epithelium of canal neuromasts gradually returned to normal, following a time course similar to behavioral loss and recovery of the orienting response, whereas that of superficial neuromasts appeared normal throughout the entire period. This study shows that the orienting response of the mottled sculpin is mediated by canal neuromasts.

Key words: mottled sculpin, *Cottus bairdi*, canal neuromast, superficial neuromast, orientation.

Introduction

The lateral line system is a hydrodynamic sensory system found in all fishes and in larval and permanently aquatic amphibians and has been implicated in many behaviors, including feeding, nuptial communication, shoaling, exploratory behaviors and orientation to abiotic flows (for a review, see Coombs and Montgomery, 1999). The end organs (neuromasts) of the lateral line system consist of small patches of mechanoreceptive hair cells that are spatially distributed on the head and body – either superficially on the skin surface or just beneath the skin surface in fluid-filled canals with pores to the surface. The apical ciliary bundles of the hair cells are embedded in a gelatinous cupula, which is viscously coupled to the surrounding water motions. The hydrodynamics and biomechanical filtering properties of the system are such that superficial and canal neuromasts have different response properties and behave as if they have been low- and high-pass-filtered, respectively (Denton and Gray, 1982; Denton and Gray, 1983; Denton and Gray, 1988; Kalmijn, 1988; Kalmijn,

1989; Montgomery et al., 1994). That is, with respect to the velocity of water motions along the skin surface, superficial neuromasts respond best to direct-current (DC) and low-frequency (less than approximately 30 Hz) alternating-current (AC) flows, whereas canal neuromasts respond best to high-frequency (approximately 30–150 Hz), AC flows (Görner, 1963; Kroese et al., 1978; Coombs and Janssen, 1990; Kroese and Schellart, 1992; Montgomery et al., 2001). Another way of looking at these response differences is that superficial neuromast responses are largely proportional to the net velocity between the fish and the surrounding water, whereas canal neuromast responses are proportional to the net acceleration.

Consistent with these response distinctions, Montgomery and colleagues have recently established a selective role for superficial, but not canal, lateral line neuromasts in the rheotactic responses of blinded fish to slow (less than 10 cm s^{-1}), DC flows (Montgomery et al., 1997; Baker and Montgomery, 1999a,b). These findings raise the question of

whether canal neuromasts might play a similarly selective role in other types of behaviors, especially where higher-frequency, AC signals are likely to be important. Unlike slow abiotic currents, which are generally continuous, many biologically relevant signals, such as those produced by moving conspecifics, prey or predators, are intermittent and have rapid, accelerating onsets or decelerating offsets. Thus, even if the long-term spectrum of a continuous signal were relatively low-frequency (e.g. the 10 Hz tail-beat of a swimming fish), the onset and offsets of swimming motions could result in higher-frequency energy with significant acceleration components. Alternatively, even if the biologically relevant signal were not intermittent, the relative movement between two continuously moving entities (e.g. predator and prey) could also create a significant acceleration component, especially at the time of initial interception. Signal onsets or transients such as these are likely to play a particularly vital role for behaviors in which increased awareness or vigilance are important, e.g. orienting behaviors in response to the initial detection of prey or alarm behaviors in response to the initial detection of predators.

Lake Michigan mottled sculpin, *Cottus bairdi*, exhibit a naturally occurring and unconditioned orienting response that can be elicited from visually deprived animals by both live (e.g. *Daphnia*) and artificial (i.e. vibrating sphere) prey (Hoekstra and Janssen, 1985; Hoekstra and Janssen, 1986; Coombs and Janssen, 1990). The orienting response is the first step in an overall sequence of prey-capture behaviors that includes subsequent step-by-step approach movements and the final strike at the prey. In the case of artificial prey, the vast majority (70–80%) of initial orienting responses to suprathreshold signals occur within 1 s of stimulus onset (Coombs and Conley, 1997a; Coombs, 1999). Thus, the orienting response is clearly a behavioral response to the initial detection of prey. In the absence of visual and chemical cues, the orienting response also clearly requires the lateral line because the frequency of orienting responses is drastically reduced, if not entirely eliminated, after the entire lateral line system has been pharmacologically inactivated (Coombs and Conley, 1997a; Abboud and Coombs, 2000).

Although these manipulations demonstrate that the lateral line system is required for the orienting response to prey, they shed no light on the relative contributions of superficial and canal neuromasts to this behavior. Given that both superficial and canal neuromasts are distributed all over the head and body of mottled sculpin in nearly equal numbers (approximately 50 per side for each type) and in similar locations (Janssen et al., 1987), there is no *a priori* reason to expect that both neuromast types could not contribute to the orienting behavior. Furthermore, because the initial orienting behavior can be elicited by vibration frequencies ranging from 3 to 100 Hz (Hoekstra and Janssen, 1985; Coombs and Janssen, 1990), the involvement of superficial neuromasts at low frequencies and canal neuromasts at high frequencies is theoretically possible. Electrophysiological evidence, however, suggests that acceleration-sensitive canal neuromasts mediate the orienting response at both low and high frequencies (Coombs

and Janssen, 1990). When threshold signal levels are expressed in units of acceleration, behavioral threshold curves for the orienting response are nearly identical in sensitivity and bandwidth to the tuning curves of acceleration-sensitive canal neuromast fibers, but not to those of velocity-sensitive superficial neuromast fibers. Thus, a comparison of behavioral and neurophysiological threshold tuning curves suggests that acceleration-sensitive canal neuromasts, rather than velocity-sensitive superficial neuromasts, are responsible for the initial orienting response.

In this study, we tested this hypothesis directly by measuring the frequency with which mottled sculpin oriented towards a vibrating sphere before and after two different treatments: one (immersion in a weak solution of gentamicin) to cause selective damage to canal neuromasts, and the other (skin abrasion) to cause selective damage to superficial neuromasts. Gentamicin is an ototoxic antibiotic that has been shown to damage hair cells in canal, but not superficial, neuromasts of the lateral line system in several teleost species (Song et al., 1995; Montgomery et al., 1997). To ensure that both superficial and canal neuromasts were adequately stimulated, we tested at both low and high frequencies (10 and 50 Hz) near or at the best frequency for each type of neuromast. Finally, we used scanning electron microscopy to verify that each treatment had caused selective damage to either canal or superficial neuromasts and to document the time course of recovery following gentamicin treatment.

Materials and methods

Experimental animals

Mottled sculpin (*Cottus bairdi* Girard), standard length 7–10 cm, were captured in baited minnow traps at depths of 2–4 m in inshore waters of Lake Michigan. The fish were housed in 38 or 761 aquaria and maintained at the same temperature as the water used in the experimental tanks (12–17 °C). Water-filled, plastic-lined nets were used to transport animals from their home tanks to the experimental tank to prevent damage to superficial neuromasts. Fish were fed small pieces of squid several (3–5) days a week in the experimental tank as a part of the test procedure. Protocols for animal handling are on file and were approved by Loyola's Institutional Animal Care and Use Committee (Protocol no. 101).

Stimulus generation

Signal vibrations were created by a small plastic sphere (6 mm in diameter) attached to a minishaker (Bruel & Kjaer 4810) by a stainless-steel rod (a 16-gauge, blunt-tipped syringe needle 12 cm long). The axis of vibration was in the vertical plane. A modular hardware system for signal generation (Tucker Davis) under computer control regulated the timing of stimulus presentation and the frequency and amplitude of the sphere oscillations. Signals were generated with a D/A converter (10 kHz sampling rate), low-pass-filtered (5 kHz cut-off), attenuated and amplified before being sent to the minishaker. Source vibrations were pulsed on (500 ms) and off

(500 ms) (10 ms rise/fall time) for a total of 5 s for each signal trial. Peak-to-peak vibration amplitudes for both 10 and 50 Hz signals were set to levels that produced reliable, daily orienting frequencies in the range 80–100 % before treatment. These levels were approximately 30 dB (10 Hz) and 50 dB (50 Hz) above the minimum signal levels required to evoke the orienting response as measured in previous studies (Coombs and Janssen, 1990). As such, the peak-to-peak velocity levels of the two signals at the source were nearly equivalent (approximately $0.11\text{--}0.18\text{ m s}^{-1}$), but the peak-to-peak acceleration level of the 50 Hz signal (approximately 60 m s^{-2}) was approximately eight times greater than that of the 10 Hz signal (approximately 7 m s^{-2}). Root mean square (rms) voltage levels to the minishaker were monitored daily to ensure that vibration amplitudes did not change during the course of the experiment.

Behavioral procedures

Behavioral methods were similar to those used in previous studies and consisted of videotaping the orienting responses of fish to a small dipole source (Coombs, 1999). Fish were first placed in a vibration-isolated (TMC) tank, measuring $50\text{ cm}\times 50\text{ cm}\times 36\text{ cm}$ and filled to depth of 8 cm with aquarium water. A rectangular Plexiglas enclosure ($21\text{ cm}\times 20\text{ cm}$) was used to corral fish and position them so that they were within 3–6 cm of the source and so that the long axis of their body was tangential to one of several concentric circles drawn on the bottom of the experimental tank, each with a fixed radial distance (3, 6 or 9 cm) from the source center. Test trials were either ‘signal’ (source vibrating) or ‘blank’ (source present but not vibrating); trial types were randomly generated by computer so that, on average, 50 % of the trials would be blank and the other 50 % would be signal. After positioning the fish, the experimenter initiated the trial by hitting a computer key and observed the fish’s behavior, rewarding it with a small piece of squid if, and only if, it moved towards the source during a signal trial. Trials were aborted if the fish moved before trial onset. A video camera placed below the experimental tank was used to record stimulus onset (time-locked to a light-emitting diode), the angular relationship and distance between the fish and source at signal onset and the subsequent response of the fish to the signal.

Food reward and adequate motivation (i.e. hunger) are necessary for maintaining, but not for establishing, the unconditioned orienting response (Coombs and Janssen, 1990; Coombs, 1995). Thus, a food reward serves two purposes. In addition to maintaining the behavior, it provides a way to monitor the fish’s motivational state from day to day and within a test session from trial to trial. To reduce the likelihood that low response rates were due to motivational rather than to sensory factors, we made sure that fish would accept a small piece of squid before beginning each test session and terminated the test session when the fish no longer accepted the food reward for correct responses to signal trials. Thus, the number of trials during any given test day and the number of

test days per week were contingent upon the animal’s motivational state (see Results for actual values).

Selective inactivation of various sensory organs

A possible chemosensory contribution to the orienting response was ruled out by using a chemically inert, plastic sphere as the stimulus source. To eliminate visual cues, all fish were blinded, either by removing the entire eyeball or by removing the lens and then aspirating the retina under anesthesia (0.01 % MS-222) (Coombs and Conley, 1997a). Blinded animals were allowed a minimum of 2 weeks of recovery time after eye surgery before being used in behavioral experiments. To selectively inactivate canal neuromasts, fish were immersed in a 0.002 % solution of gentamicin in buffered aquarium water for 4 days (following the procedure of Song et al., 1995). To prevent the degradation of gentamicin efficacy, fresh solutions of gentamicin were made up daily. Superficial neuromasts were selectively damaged by gently scraping the skin on both sides of anesthetized fish with a scalpel blade (following the example of Montgomery et al., 1997).

Behavioral data analysis

To eliminate observer bias and to provide more precise criteria for defining the orienting response than was practical during the real-time execution of the experiment, we used *post-hoc* measurements of the fish’s position before and after trial onset to compute orienting frequencies. The fish-to-source angles and distances before and after the fish’s first movement following the onset of either a signal or blank trial were determined from digitized video frames (Snappy, Inc) using SigmaScan Pro imaging software (SPSS). Fish-to-source distance was defined as the horizontal distance between the fish’s snout and the source center. Fish-to-source angle was defined as the horizontal angle formed between the long axis of the fish’s head and a line drawn from the center of the source to the midline of the fish’s head at the rostral-caudal level of pectoral fin insertion. Differences between pre- and post-response angles and distances were expressed as a percentage of the pre-response angle or distance to provide a measure of how much the original angular or distance gap had closed after the orienting response. Time-stamped (17 ms resolution) video frames were also used to compute reaction times (the time elapsed between signal onset and the initiation of the orienting response). Response movements during signal trials were scored as orienting responses if (i) the fish closed the angular or distance gap by a minimum of 10 % and (ii) the reaction time of the response was less than the total duration of the trial (5 s). The same criteria were used for blank trials to determine the ‘false alarm’ rate or the frequency of orienting responses in the absence of source vibration.

For the purpose of statistical analysis, daily orienting and false alarm rates for each individual were averaged over two test periods of five consecutive days (Monday–Friday), the week before and the week after the initial treatment day (Sunday). Although testing continued for up to an additional 25 days to determine the time course of recovery from drug

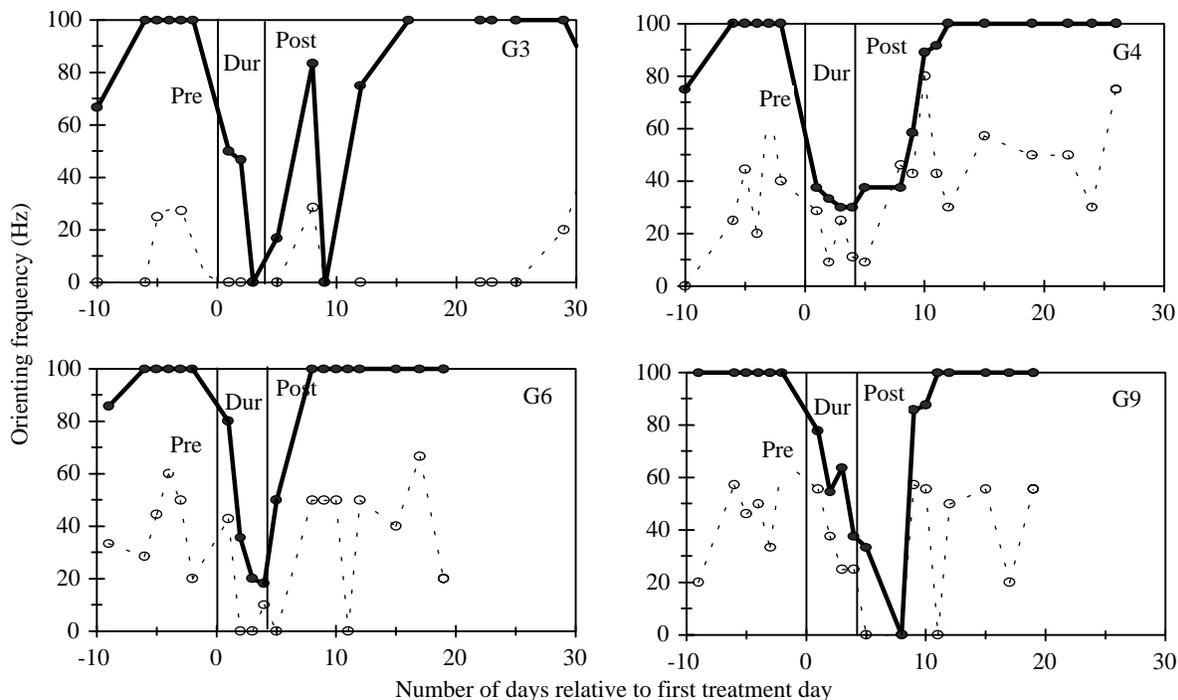


Fig. 1. Time course of daily orienting frequencies of four Lake Michigan mottled sculpin *Cottus bairdi* (G3, G4, G6 and G9) in the presence (solid lines, filled circles) and absence (dashed lines, open circles) of 50Hz sphere vibration before (Pre), during (Dur) and after (Post) gentamicin treatment.

treatment, data from these days were not included in the statistical analyses so as not to confound treatment effects with recovery effects. The arcsine-transformed means from each 5-day pre- and post-initial treatment day periods were used in three separate, two-way repeated measures (RM) analyses of variance (ANOVAs; one for each experiment: 50 Hz + gentamicin, 10 Hz + gentamicin and 10 Hz + skin abrasion), with trial (signal *versus* blank) and treatment (pre *versus* post) conditions as the two factors. Various *post-hoc*, pair-wise comparisons of the means were also run (see Results for details).

Scanning electron microscope procedures

Scanning electron microscopy was used to assess the effects of gentamicin treatment on the apical surfaces of both canal and superficial neuromasts. Samples were selected from each of four treatment groups: (i) 24 h in gentamicin (two animals), (ii) 12 days after the start of gentamicin treatment (two animals), (iii) after full behavioral recovery (26 and 30 days following treatment, one animal each) and (iv) 24 h after skin scrape (one animal; this animal was only scraped on the left side, allowing the right side to be used as an internal control). Animals were anesthetized and fixed by immersion in 2-4% glutaraldehyde in phosphate buffer. After 5-10 days of fixation, animals were subsequently dehydrated in a graded series of ethanol. Whole heads, mid-sagittally-transected heads and trunk and tail segments were transferred to liquid CO₂ and critical-point-dried. These specimens were mounted on aluminum stubs and sputter-coated with gold and palladium.

The morphology of superficial neuromasts was examined first, and each specimen was then dissected to expose the canal neuromasts. The specimens were then sputter-coated again. As many neuromasts as possible were examined from all superficial and canal groups. Each neuromast was evaluated for the presence or absence of apical damage on the basis of the number, size and density of intact ciliary bundles. A subjective score (see Table 1) was given to each neuromast, with a score of 1 indicating normal apical morphology and a score of 5 indicating complete destruction of all ciliary bundles.

Results

Gentamicin treatment nearly eliminated orienting responses to vibratory dipole sources, yet scraping the surface of the skin had no apparent effect on behavioral responses. Using scanning electron microscopy, we confirmed that gentamicin treatment damages canal neuromasts but leaves superficial neuromasts unaffected. Scraping the skin surface resulted in extensive epidermal damage, including the destruction of many superficial neuromasts, without affecting canal neuromasts. We will first describe the time course of the behavioral effects of both treatments, and then give a detailed description of the morphological effects over the same period.

Behavioral results

The time course of daily signal- and blank-trial response rates before, during and after gentamicin treatment are shown in Figs 1 and 2 for the 50 and 10 Hz signal conditions,

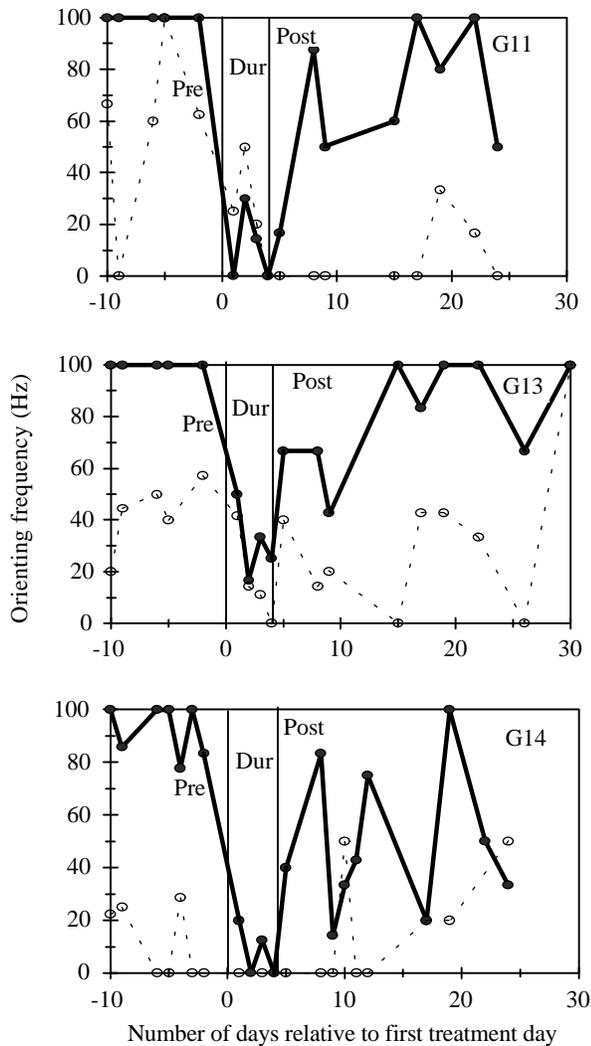


Fig. 2. Time course of daily orienting frequencies of three Lake Michigan mottled sculpin *Cottus bairdi* (G11, G13 and G14) in the presence (solid lines, filled circles) and absence (dashed lines, open circles) of 10 Hz sphere vibration before (Pre), during (Dur) and after (Post) gentamicin treatment.

respectively. The total number of trials used in computing daily signal- or blank-trial response frequencies ranged from two to 16 across all individuals and days, but the average number of trials per day for different individuals ranged from 5.1 to 7.9 (blank-trial condition) and from 4.7 to 8.3 (signal-trial condition). Note that, because response rates are based on limited numbers of trials per day, daily response rates tend to fluctuate in rather large step sizes. Daily response frequencies to signals before treatment varied between 67 and 100% for all fish and were almost always greater than daily response frequencies to blank trials. Daily signal-trial response rates fell to the level of blank-trial response rates after 1 day of gentamicin treatment and did not return to pre-treatment levels until another 10–15 days had passed (Figs 1, 2). In contrast, daily signal- and blank-trial response rates after skin scrape treatments remained in the normal, pre-treatment ranges for up

to 12 days post-treatment (results not shown). Although the orienting response after gentamicin treatment appears to be more variable and to recover more slowly for the 10 Hz signal (Fig. 2) than for the 50 Hz (Fig. 1) signal, this is probably because peak-to-peak acceleration amplitudes were lower for the 10 Hz signal than for the 50 Hz signal (see Discussion for the importance of acceleration to the orienting response).

Group mean response rates (mean of individual means) for 5-day test periods before and after the first day of treatment are summarized in Fig. 3 for each experimental condition. Gentamicin, but not skin-scrape, treatment had significant effects on response frequencies ($P < 0.005$, 10 Hz; $P < 0.0001$, 50 Hz; two-way RM ANOVA). Signal-trial response rates to both test frequencies were significantly reduced after gentamicin treatment ($P < 0.01$, 10 Hz; $P < 0.001$, 50 Hz, Bonferroni *post-hoc* comparisons). Although mean blank-trial response rates were also somewhat lower after all treatments (Fig. 3), they were only significantly lower after gentamicin treatment in the 50 Hz case ($P < 0.01$) (Fig. 3A). Trial condition (signal *versus* blank) also had a significant effect in all three experiments ($P < 0.001$, two-way RM ANOVA). In all three pre-treatment cases, signal-trial response rates were significantly greater ($P < 0.01$, Bonferroni *post-hoc* comparisons) than blank-trial response rates. For post-treatment cases, however, only the skin scrape treatment resulted in signal-trial response rates that were significantly greater than blank-trial response rates ($P < 0.01$, Bonferroni *post-hoc* comparison).

To determine whether there may have been more subtle differences in the accuracy or latency of the orienting response before and after the skin scrape treatment or between the two test frequencies before gentamicin treatment, we also analyzed reaction times and fish-to-source angles and distances before and after the orienting response. Given the low numbers of positive responses after gentamicin treatment, we analyzed pre-treatment values only, comparing orienting accuracy and latency between 10 and 50 Hz signal conditions.

Pre-treatment mean reaction times for different individuals ranged from 260 to 490 ms for the 50 Hz signal and from 630 to 800 ms for the 10 Hz signal. Reaction times in the 10 Hz condition were significantly longer than 50 Hz reaction times ($P < 0.005$, Mann–Whitney two-tailed test); these differences are probably because the peak-to-peak acceleration level of our 50 Hz signal was approximately eight times greater than that of our 10 Hz signal (see Discussion). Pre- and post-treatment reaction times for the skin scrape experiment were not significantly different (Mann–Whitney two-tailed test).

Mean changes in the angular position of fish in the skin scrape experiment were tightly clustered around 70% for all individuals, both before and after treatment, with no significant treatment differences (paired *t*-test, two-tailed, on arcsine-transformed percentages). Because the mean starting angle of the fish was approximately 90° relative to the source, a 70% change in the angle means that the fish's snout was 63° closer to the source after the orienting response. There were also no significant differences in the angular accuracy of the orienting

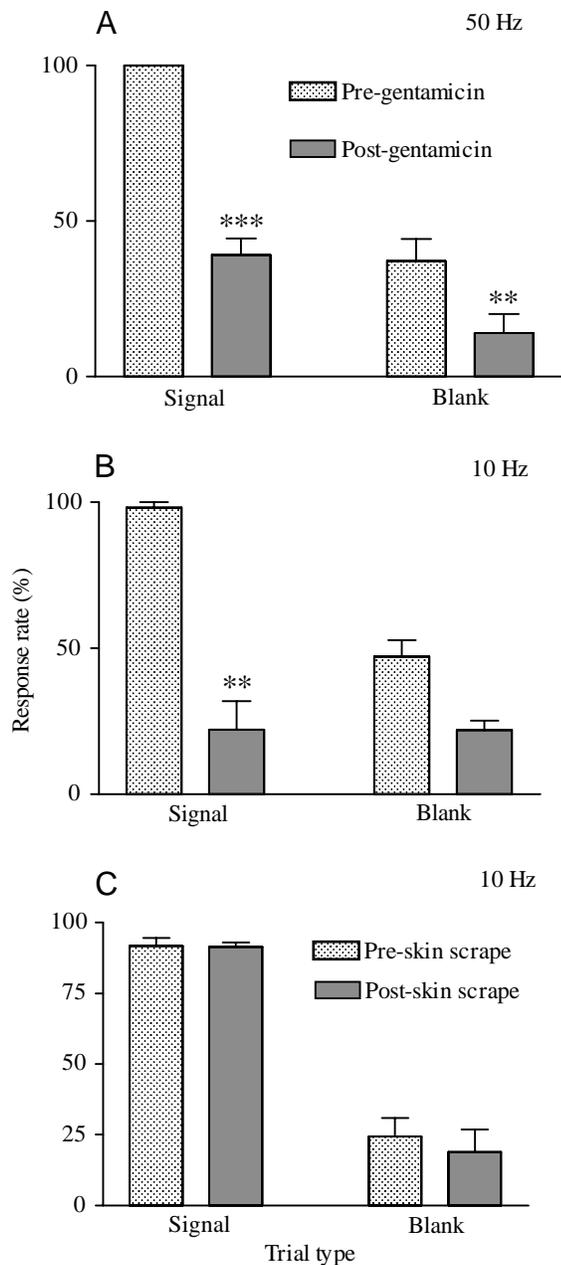


Fig. 3. Group mean signal- and blank-trial response rates for 5-day test periods before and after gentamicin-induced damage to canal neuromasts (50 Hz signal, A; 10 Hz signal, B) or skin-scrape-induced damage to superficial neuromasts (10 Hz signal, C). Asterisks indicate statistically significant pre/post-treatment differences in response rates to signal and blank trials (*** $P < 0.001$; ** $P < 0.01$). See Results section for statistical significance of signal- and blank-trial differences in response rates before and after treatment. Values are means + S.E.M., $N = 4$ (A) and 3 (B,C).

responses to 10 and 50 Hz signal trials prior to gentamicin treatment.

Mean changes in fish-to-source distances before and after the skin scrape treatment varied from 54 to 64% among individuals, but were not significantly different. However, changes in distance before treatment of any kind were

significantly less for the 10 Hz signal than for the 50 Hz signal (Mann-Whitney two-tailed test, $P = 0.04$), an effect that is consistent with the lower peak-to-peak acceleration amplitudes of the 10 Hz signal (see Discussion). For the average starting distance of 6 cm, this means that the fish moved, on average, approximately 4.2 cm closer to the source in the 50 Hz case (a 70% change), but only 3.5 cm closer in the 10 Hz case (a 59% change).

Morphological effects of gentamicin and skin scrape treatments

To assess the effects of gentamicin treatment, two individuals from each of three time periods (1, 12 and >20 days after the initial treatment day) were examined using scanning electron microscopy. These sample periods were chosen on the basis of behavioral results, which showed reductions in the orienting frequency to as low as 0% after 1 day, a period of recovery in which the orienting frequency varied between 0 and 100% near the twelfth day and a return to normal orienting frequencies (>70%) by 20 days (Figs 1, 2). Animals showing full behavioral recovery (G4, Fig. 1; G13, Fig. 2) were used for the last category. Neuromasts were sampled from several different locations on the head, trunk and tail of the fish according to their known distributions (Janssen et al., 1987). Out of a total of 287 canal neuromasts and 307 superficial neuromasts examined, a little less than half in each category were obscured by cupular remains and could not be evaluated with respect to hair cell damage. Thus, the following results are based on a total of 188 canal and 171 superficial neuromasts (approximately 30 of each type per individual specimen). Although some specimens allowed for more complete observations (more neuromasts or neuromasts from more locations) than others, the pattern of results was similar in each specimen.

The effects of gentamicin on canal neuromasts varied greatly (Table 1), ranging from complete or nearly complete loss of apical cilia after the first day of treatment (Fig. 4A,B) to complete recovery from loss 20 days post-treatment (Fig. 4C,D). The percentage of neuromasts (average of two individuals) showing these different effects are summarized as a function of days post-treatment for both canal (Fig. 5A) and superficial (Fig. 5B) neuromasts.

The most severe effects were seen in canal neuromasts after 24 h of treatment (Figs 4A,B, 5A). More than 90% of the canal neuromasts in these specimens were damaged to some extent, with most showing severe damage, ranging from nearly bare

Table 1. *Subjective scale used to score the effects of gentamicin treatment on neuromasts*

Score	Corresponding morphology
1	Intact, normal density
2	Mostly normal, short cilia or reduced density
3	Reduced density, some bare patches
4	Greatly reduced density, mostly bare
5	Totally bare surface, no cilia present

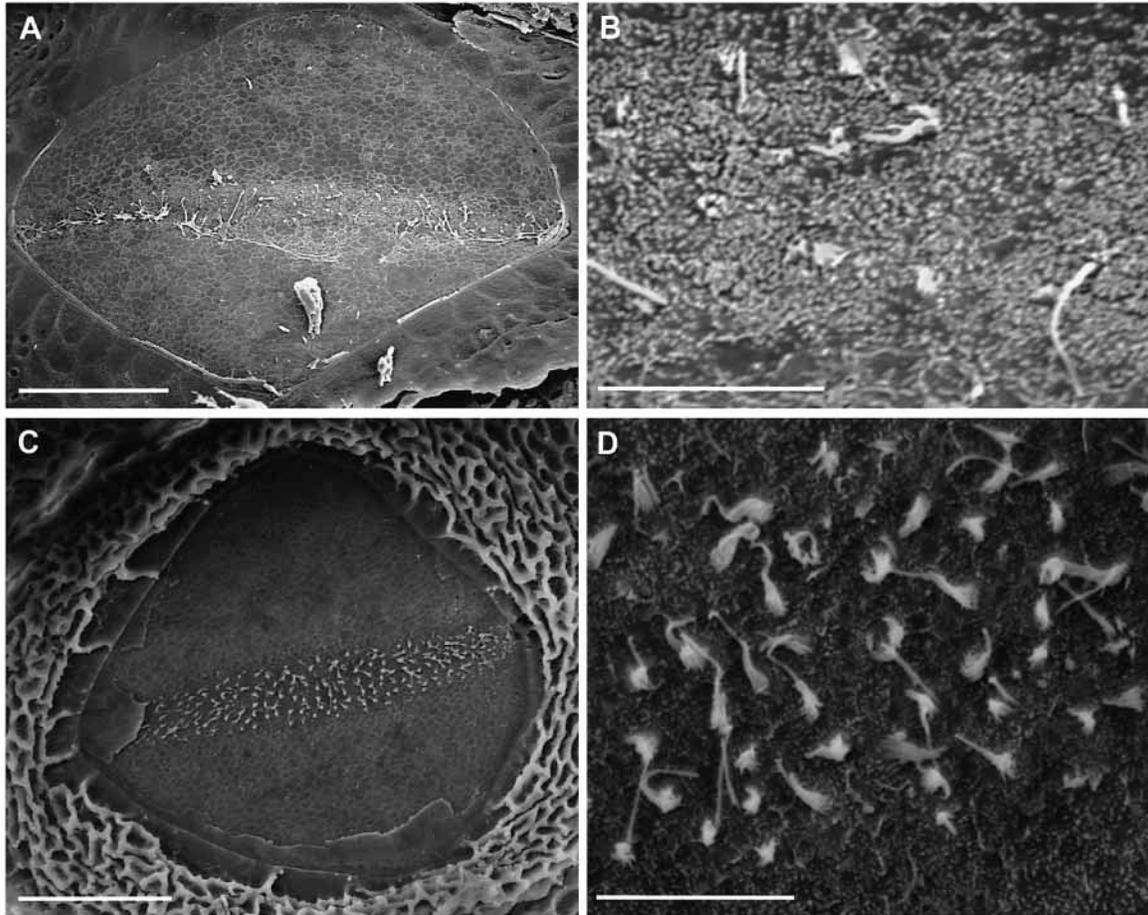


Fig. 4. Scanning electron micrographs of the hair cell sensory epithelium of canal neuromasts under low (A,C) and high (B,D) magnification. (A,B) The effects of gentamicin on a canal neuromast after 24 h of exposure. (C,D) A neuromast that has recovered from gentamicin-induced damage 26 days post-treatment (taken from animal G4, see Fig. 1). Scale bars, 10 μ m in A and C, 50 μ m in B and D.

apical surfaces to a very sparse distribution of abnormally short ciliary bundles. Half-way through the typical time course of behavioral recovery, nearly half the canal neuromasts had recovered a high density of normal ciliary bundles. By the end of behavioral testing, the canal neuromasts had nearly all recovered, with less than 20% showing any abnormalities (Fig. 5A), most commonly a slightly reduced density or small bare patches within the sensory epithelium (Fig. 4C,D).

In contrast, gentamicin had no apparent effect on superficial neuromasts (Fig. 5B). At all the time points examined, however, approximately 15–20% of superficial neuromasts showed some damage, generally bare patches within the sensory epithelium. Note that this also describes the canal neuromasts 26–30 days after gentamicin treatment. This small proportion of damaged neuromasts may be the normal condition in life, with loss of hair cells caused by senescence, by mechanical damage from handling or by friction between the animal and rocks in the aquaria. However, some proportion of these damaged neuromasts may also be due to breakage after fixation. Cilia may become strongly attached to the cupula after fixation (Barber and Emerson, 1979), causing breakage if the cilia is pushed off during critical-point drying or dissection (to open the canals).

The effect of skin scrapes was also examined under the scanning electron microscope. The skin on both the scraped and unscraped sides of the fish was visibly traumatized, perhaps caused by increased handling or by desiccation during the procedure. Although the scraped side showed substantially more damage, both sides showed the following symptoms. The skin surface was covered with an excess of slime, completely obscuring the apical morphology of whole regions of the epidermis. Those areas that were not obscured by slime showed evidence of severe osmotic stress – either swollen cell profiles and/or sunken or shriveled apical surfaces.

A quantitative comparison between sides is confounded because we were unable to locate entire groups of neuromasts on the treated side. Many, if not most, of these ‘missing’ neuromast groups were probably destroyed, but it was often difficult to confirm this because of the degree of osmotic swelling and excess slime. Nonetheless, the scraped side was clearly more damaged than the unscraped side. For instance, of the group of four neuromasts normally surrounding the nostril, three were seen on the unscraped side but none could be identified on the scraped side. In fact, only eight out of 50 total possible superficial neuromasts (not including those that

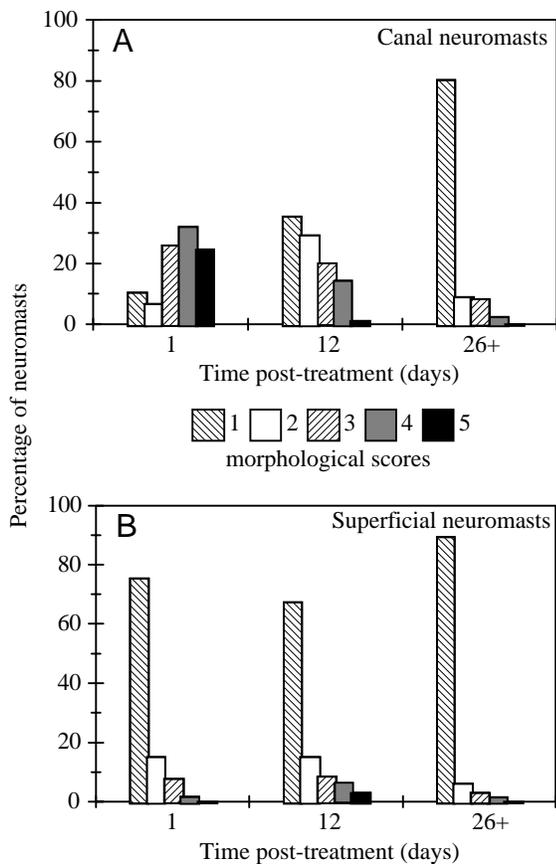


Fig. 5. Histograms of the rating of neuromast morphology (1–5) during the course of gentamicin treatment. (A) Canal neuromasts; (B) superficial neuromasts. For a description of morphological categories, see Table 1.

continue down the trunk canal line) could be identified on the scraped side, seven of which appeared to have normal sensory surfaces. In contrast, 47 superficial neuromasts were identified on the unscraped side, with 96% of these having either an intact cupula ($N=7$) or an exposed sensory epithelium that appeared normal ($N=38$). Because the skin scrape treatment had no effect on the orienting response, we did not use scanning electron microscopy to examine the time course of recovery from this treatment.

Discussion

This study demonstrates that canal neuromasts play a vital, if not exclusive, role in the orienting response of mottled sculpin to prey-like sources. Several lines of evidence support this thesis. First, gentamicin-induced damage to canal neuromasts eliminates the orienting response to vibratory sources, regardless of whether the vibration frequency is near the best frequency for canal (50 Hz, Fig. 1) or superficial (10 Hz, Fig. 2) neuromasts. Second, physical ablation of superficial neuromasts has no effect on the likelihood, accuracy or latency of the orienting response, even when the signal frequency (10 Hz) is near the best frequency for superficial

neuromasts (Fig. 3C). Finally, the time course of gentamicin-induced changes in the orienting behavior (Figs 1, 2) parallels the time course of hair cell damage and recovery from damage in canal neuromasts (Figs 4, 5).

These findings are supported by other, more indirect, lines of evidence for the importance of canal neuromasts to the orienting response. For example, it has been reported (Coombs and Janssen, 1990) that threshold levels of the orienting response (50% response rate) could be elicited by vibration frequencies ranging from 10 to 100 Hz as long as the vibration amplitude was maintained at the same minimum level of acceleration, as would be expected if the behavior were mediated by acceleration-sensitive canal neuromasts. Likewise, it has been reported that signals with significant acceleration components (10 and 20 Hz alternating-current water jets) were more effective than those without acceleration components (direct-current water jet) in eliciting the orienting behavior (Janssen et al., 1990). Finally, the present study showed that the signal frequency with the higher peak-to-peak acceleration amplitude (50 Hz) resulted in better performance (i.e. the fish moved closer to the source) and faster reaction times than the signal frequency with the lower acceleration amplitude (10 Hz).

Both the otolithic endorgans of the auditory system and the canal neuromasts of the lateral line system are acceleration-sensitive endorgans, so it is conceivable that both may play a role in the acceleration-dependent orienting response. Indeed, conditioned suppression of respiration has been used to show that mottled sculpin can detect 50 Hz dipole sources with their inner ear when the entire lateral line system is inactivated by CoCl_2 (Braun and Coombs, 2000). The exact contribution, if any, of the inner ear to the orienting response is unknown, however, and will probably remain so until effective methods are developed for selectively blocking the ear without affecting the animal's sense of balance and overall behavior. Nevertheless, the findings of this and previous studies (Hoekstra and Janssen, 1986; Coombs and Conley, 1997a; Coombs and Conley, 1997b) make it clear that acceleration inputs to the ear alone are insufficient for producing the orienting response in blinded animals because the orienting response disappears when the lateral line, but not the inner ear, is pharmacologically blocked. Thus, these results taken together with those showing an excellent match between tuning curves from acceleration-sensitive lateral line fibers and behavioral threshold curves for the orienting response (Coombs and Janssen, 1990) make a strong, if not conclusive, case for the response being driven by the acceleration-sensitive canal neuromasts of the lateral line system, rather than the acceleration-sensitive endorgans of the inner ear.

The case for distinct behavioral roles for canal and superficial neuromasts

The present results, in conjunction with those showing that superficial neuromasts appear to play an equally exclusive role in rheotaxis (Montgomery et al., 1997; Baker and Montgomery, 1999a,b), suggest that there may be differences

in the behavioral roles subserved by canal and superficial neuromasts. Behavioral tasks requiring rapid responses and/or fast integration times (e.g. alerting behaviors in response to signal onsets) are more likely to rely on acceleration-sensitive, high-frequency canal neuromasts than velocity-sensitive, low-frequency superficial neuromasts, which may be better suited for behaviors requiring the integration of information over longer periods. Similarly, behavioral tasks requiring that information be integrated across spatially distributed endorgans (e.g. to determine the general direction of bulk water flow) may utilize superficial neuromasts, whereas behavioral tasks requiring the spatial segregation of information (e.g. for discriminating fine spatial details or for determining precise locations) may utilize canal neuromasts. The impaired ability of characid blind cavefish (*Astyanax fasciatus*) to perform spatial discrimination tasks after the canal system has been selectively blocked (Abdel-Latif et al., 1990; Montgomery et al., 2001) is yet another piece of behavioral evidence pointing to the importance of canal neuromasts in spatial tasks. This finding is especially surprising in the light of the large numbers (thousands) of superficial neuromasts over the entire head and body of this fish and challenges the natural presumption that the proliferation of superficial neuromasts is an adaptation serving the exquisite ability of these fish to form hydrodynamic images of their environment (Montgomery et al., 2001).

It would appear that the orienting response of the mottled sculpin involves elements of both speed and spatial precision. That is, for an animal to orient towards and eventually capture its prey, it needs to be aware of the prey's presence as soon as possible and it also needs to determine its location. It is certainly clear from this study that orienting responses are rapid and closely linked to stimulus onset. Mean reaction times of untreated fish were all less than 1 s, meaning that, on average, fish were responding shortly after the first but before the second of a series of five short vibration bursts during the signal trial. Reaction times were even shorter (<500 ms) for the 50 Hz signal trials, occurring before the end of the first signal burst. Given that the acceleration level of our 50 Hz signal was approximately eight times greater than that of our 10 Hz signal, the faster reaction times to the 50 Hz signal indicates a dependence of response latency on acceleration level, as would be expected if speed of response were linked to acceleration-sensitive canal neuromasts.

Information about prey location is also an obvious requirement for the orienting response, which for normal untreated fish in this study resulted in a narrowing of the angular (azimuth) and distance gaps between the fish and the 50 Hz source by an average of 76% and 71%, respectively. Theoretical (Denton and Gray, 1983) and experimental (Coombs et al., 1996; Coombs and Conley, 1997b; Conley and Coombs, 1998) considerations suggest that prey location, including both the azimuth and distance to the prey, can be encoded by the pattern of excitation along the spatially distributed endorgans of the lateral line and that bilateral computations are not needed. For the excitation patterns to be useful, the excitation levels of neuromasts at different locations

should be preserved and transmitted to the central nervous system *via* separate input channels.

Although the data are limited, it appears that the circuitries of canal and superficial neuromasts are somewhat different in this regard. In studies on the cichlid *Sarotherodon niloticus*, it was found (Münz, 1979; Münz, 1985) that individual trunk canal neuromasts are innervated by as many as 20 different afferent fibers, but that fewer than 4% of these afferents appear to innervate more than one canal neuromast. In contrast, peripheral fibers innervating superficial neuromasts in the same species typically branch to innervate several, if not all (approximately 5–10), of the superficial neuromasts found in a single row on any given trunk scale. Although fibers innervating superficial neuromasts on one scale do not usually branch to innervate those on adjacent scales, physiological recordings from single afferent fibers indicate that, in some cases, the group of mutually innervated superficial neuromasts extends over a much larger area, e.g. along the entire length of a caudal fin ray. Thus, the two systems differ in their degree of spatial integration; there is minimal, if any, peripheral integration of information across different canal neuromasts but moderate degrees of integration among groups of superficial neuromasts. In *Sarotherodon niloticus*, then, the functional units of the canal system are individual neuromasts, but within the superficial system, multiple neuromasts contribute to each functional unit (Münz, 1985).

Most importantly, there is no evidence to indicate that a single fiber contacts both superficial and canal neuromasts. Thus, it would appear that information from the two subsystems is preserved separately at the level of peripheral nerve fibers. The demonstration of distinct behavioral roles for each subsystem (the present study; Montgomery et al., 1997; Baker and Montgomery, 1999a,b) further suggests that these two systems may remain distinct at higher levels of the central nervous systems. The degree and type of integration that take place between and even within these subsystems at central levels is almost entirely unknown, however, and deserves further study.

Superficial neuromast diversity and the purported role of superficial neuromasts in prey orientation

These assertions about the critical role of canal neuromasts for orienting and other tasks requiring spatial analysis appear to be in conflict with several reported examples in the literature for the involvement of superficial neuromasts in prey detection and orientation by amblyopsid (Poulson, 1963) and characid (Abdel-Latif et al., 1990) blind cave fish, by surface-feeding fish such as the topminnow *Aplocheilichthys lineatus* (Bleckmann et al., 1989), by various species of larval fish, including the mottled sculpin (Jones and Janssen, 1992; Mukai et al., 1994), and by aquatic amphibians such as the axolotl (*Ambystoma mexicanum*) (Takeuchi et al., 1990). This apparent contradiction may be reconciled, however, by a re-evaluation of superficial neuromast diversity. In fact, some superficial neuromasts are probably homologous to individual canal neuromasts, having similar or identical topology,

peripheral innervation patterns and connections in the central nervous system. Superficial neuromasts in larval fish that will later invaginate into the dermis and become enclosed in canals as the fish matures represent the clearest case. Heterochronic processes that result in the adult retention of this embryonic, epidermal phase should not be expected, *a priori*, to affect the cellular phenotype, circuitry or intrinsic function of any given neuromast, regardless of its final position within the integument.

An extensive nomenclature has been developed to parse the variation seen in superficial neuromast morphology (Lekander, 1949; Srivastava and Srivastava, 1968; Coombs et al., 1988). Much of this complexity can be reduced to four categories of superficial neuromast: (i) larval neuromasts, (ii) replacement neuromasts, (iii) accessory neuromasts and (iv) pit organs. As described above, larval neuromasts are canal neuromasts that have yet to be enclosed in canals but will be enclosed at a later developmental stage. Replacement neuromasts are similar to larval neuromasts in that they correspond, in their topography, innervation and presumed developmental origin to canal neuromasts of adult fish in closely related taxa (Coombs et al., 1988). They differ from larval neuromasts only in that they fail to become enclosed in canals – probably as a result of a paedomorphic or heterochronic process in which the normal progression to the canal formation stage is cut short. Accessory neuromasts are superficial organs that accompany canals or are found in various locations on the head and body, including the supernumerary neuromasts of some characids (Schemmel, 1967) and cyprinids (Puzdrowski, 1989; Lekander, 1949) and the rows of mutually innervated superficial neuromasts on the trunk scales of *Sarotherodon niloticus* (Münz, 1985). These superficial neuromasts never become enclosed in canals. Finally, the pit organs make up a special class of superficial neuromasts that generally occur in depressions on the skin in as many as six stereotypical locations on the dorsal surface of the head, cheek and lower jaw (Northcutt, 1989; Webb and Northcutt, 1997). Ancestral gnathostome vertebrates possessed both canal neuromasts and lines of pit organs, but only teleost fishes have added additional superficial neuromasts, the accessory system (Northcutt, 1989).

Of these various categories, at least two (larval and replacement) have a clear relationship with canal neuromasts. While larval and replacement neuromasts may not necessarily have all the properties of canal neuromasts (e.g. the biomechanical filtering properties of the canal), they may nevertheless share many features, such as relative size, intrinsic properties of the hair cells, axis of best sensitivity and patterns of peripheral innervation and connections within the central nervous system. Thus, prey orientation by the various groups of fish purported to use superficially located neuromasts may be subserved by a class of superficial neuromasts that are homologous to canal neuromasts. Indeed, this idea is supported by reports of larval mottled sculpin undergoing a transitory loss in sensitivity to live prey (*Artemia*) during the process of canal formation (Jones and Janssen, 1992). These results suggest that the developmental precursors of canal neuromasts, rather than

other classes of superficial neuromast, are responsible for prey detection by larval sculpin, as demonstrated by the transitory loss in sensitivity during canal formation.

The proposition that particular superficially located neuromasts are individually homologous to canal neuromasts and share common circuitry and functional roles clearly requires further testing. In the absence of any evidence to the contrary, however, current examples of prey orientation by superficially located neuromasts cannot rule out this hypothesis as a viable explanation. In addition to the larval sculpin, dorsally situated superficial neuromasts thought to be specialized for surface-feeding by topminnow, are almost certainly ‘replacement lines’ of the supra- and infra-orbital canals (Bleckmann et al., 1989). Likewise, many superficial lines in aquatic amphibians such as *Ambystoma mexicanum* are regarded as homologous to similarly placed canal lines in bony fishes (Northcutt, 1992).

There is only one example that we are aware of that implicates accessory superficial neuromasts in an orientation task. It has been reported (Abdel-Latif et al., 1990) that blind cave fish continue to approach dipole sources even after the lateral line canals have been occluded. But these data are questionable in the light of the fact that the behavior was evoked by vibration frequencies (50–90 Hz) and source distances (20 cm or 4–5 body lengths) beyond the normal frequency range of superficial neuromasts (DC, 30 Hz) (Münz, 1989) and the normal operating range of the lateral line system (1–2 body lengths) (Kalmijn, 1988; Kalmijn, 1989; Denton and Gray, 1983; Coombs, 1999; Braun and Coombs, 2000). Furthermore, the approach behavior in this case was operantly conditioned and, thus, somewhat different from the naturally occurring, unconditioned orienting behaviors reported for most other studies. Thus, it is at least possible, if not likely, that the conditioned behavior reported for this fish relied on the sensitivity of the inner ear rather than the superficial neuromasts of the lateral line. Indeed, goldfish, which are very similar to blind cavefish in having a specialized connection (Weberian ossicles) between the swimbladder and inner ear, have been classically conditioned to respond to dipole sources in the 50–800 Hz range, even in the absence of lateral line input (Coombs, 1994).

Evidence for differential susceptibility of superficial neuromasts to gentamicin treatment

The suggestion that some superficially located neuromasts may be homologous to canal neuromasts raises the question of whether these same classes of superficial neuromast might also be susceptible to gentamicin-induced damage. In this regard, it has been reported (Song et al., 1995) that replacement superficial neuromasts positioned in the ‘gap’ of an interrupted trunk canal show the same degree of gentamicin-induced damage as canal neuromasts. Similarly, superficial neuromasts forming a caudal continuation of the trunk canal on the mottled sculpin were also found to be damaged by gentamicin in the present study. Paradoxically, neuromasts tentatively classified as replacement lines of the supraorbital and infraorbital canals

in the mottled sculpin (Janssen et al., 1987) were not affected by gentamicin. If these neuromasts are truly replacement neuromasts (i.e. directly homologous to canal neuromasts in related taxa), it is possible that their biochemistry has changed to eliminate their gentamicin-sensitivity. However, it is also possible that these neuromasts have been incorrectly classified. Given that the current classification is based on topographic location only and not on any other well-defined operational criteria (e.g. innervation pattern), it can only be provisional at best.

Mottled sculpin have few clear examples of accessory neuromasts, but these probably include the neuromasts that accompany the trunk canal and those along the caudal fin proper. These neuromasts were all unaffected by gentamicin treatment. The superficial neuromasts examined previously (Song et al., 1995) were also accessory neuromasts, and these were gentamicin-resistant as well. It is not clear which superficial neuromasts were examined by Baker and Montgomery (Baker and Montgomery, 1999), but the abundance of supernumerary neuromasts in *Astyanax fasciatus* makes accessory neuromasts the most likely candidates. These authors also report that these neuromasts were not sensitive to gentamicin.

There are very few data on the gentamicin susceptibility of pit organs. Song et al. do not report any results on pit neuromasts in the oscar (Song et al., 1995). Similarly, it is unclear which superficial neuromasts of sculpin should be considered as pit organs but, based on location and hair cell orientation, the dorsal line of the supraorbital canal and the dorsal trunk line (following the nomenclature of Janssen et al., 1987) are likely candidates. These were unaffected by gentamicin treatment.

In general, these results indicate that at least some replacement neuromasts show the same susceptibility to gentamicin as canal neuromasts, whereas accessory neuromasts and pit organs do not. Future studies on more obvious replacement neuromasts, such as those found in closely related fish taxa showing phylogenetic trends in canal reduction (e.g. Esocidae) (Nelson, 1972), should examine the gentamicin-susceptibility of neuromasts in this category. Likewise, the effects of gentamicin should be explored in non-teleost fishes (e.g. gars, sturgeons and bowfin), in which accessory neuromasts are not present and pit organs are more clearly defined and easily recognized.

Concluding remarks

All the available evidence seems to suggest that the lateral line may be considered as two subsystems: a system of velocity-sensitive superficial neuromasts that responds slowly and that integrates spatial information at the periphery, and a system of acceleration-sensitive canal neuromasts that responds more rapidly and that segregates spatial information. While the superficial system appears to serve behaviors dependent on large-scale stimuli, such as abiotic currents, the canal-based system subserves behaviors requiring information

about fine spatial details, such as the ability to locate spatially punctate sources. Further, the distinction between these systems may extend beyond peripheral filtering properties to circuitry and processing within the central nervous system. Indeed, the examples from amphibians and topminnows suggest that, even without an actual canal, there may still be a behaviorally distinct subset of superficial (replacement) neuromasts that behave like canal neuromasts. Further studies on the inherent filtering properties, central representation, divergence and integration of the two subsystems are clearly warranted, but it appears that we should seek a classification of neuromasts that is dependent on better-defined operational criteria than the mere presence or absence of a canal.

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