Report

Evolution of a Behavioral Shift Mediated by Superficial Neuromasts Helps Cavefish Find Food in Darkness

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Summary

How cave animals adapt to life in darkness is a poorly understood aspect of evolutionary biology [1]. Here we identify a behavioral shift and its morphological basis in *Astyanax mexicanus*, a teleost with a sighted surface-dwelling form (surface fish) and various blind cave-dwelling forms (cavefish) [2–4]. Vibration attraction behavior (VAB) is the ability of fish to swim toward the source of a water disturbance in darkness. VAB was typically seen in cavefish, rarely in surface fish, and was advantageous for feeding success in the dark. The potential for showing VAB has a genetic component and is linked to the mechanosensory function of the lateral line. VAB was evoked by vibration stimuli peaking at 35 Hz, blocked by lateral line inhibitors, first detected after developmental increases in superficial neuromast (SN) number and size [5–7], and significantly reduced by bilateral ablation of SN. We conclude that VAB and SN enhancement coevolved to compensate for loss of vision and to help blind cavefish find food in darkness.

Results and Discussion

Vibration Attraction Behavior

Cavefish swim toward water disturbances created by small clay particles dropped into cave pools [8]. In contrast, surface fish scatter and flee when faced with the same type of stimulus in a natural lighted habitat. In the laboratory, cavefish are attracted to an oscillating object in water [9], prompting us to quantify this behavior by using a vibrating rod as a stimulus (Figure 1; see also Movie S1 available online). In the absence of the rod, cavefish and surface fish usually swam along the edges of the assay chamber. When the rod was inserted into the water but was not vibrated, both forms of *Astyanax* responded weakly by occasionally exploring the center of the chamber. However, when the rod was vibrated at 50 Hz, a frequency often used for analyzing fish orientation behaviors [10, 11], cavefish, but not surface fish, were strongly attracted, as determined by an increased mean number of approaches (NOA) to the rod, a higher mean duration of swimming in the vicinity of the rod, and a shorter mean latency before beginning to swim toward the rod (Figures 1D–1F; Table S1). We defined vibration attraction behavior (VAB) as the increase in attraction to a vibrating rod above a background level of 4 NOA—observed with the nonvibrating rod—during a 3 min assay period (Figure 1D; Figure 2A). VAB is stable in laboratory-raised cavefish, as determined by replicated values in assays on the same individuals conducted a month apart (mean

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NOA ± standard error of the mean [SEM] of first and second assays: 12.7 ± 3.0 and 12.0 ± 2.4, respectively; see Table S1).

Laboratory- and/or field-conducted behavioral assays showed that Pachón, Los Sabinos, and Piedras cavefish exhibit VAB, whereas Molino cavefish, which evolved independently [12, 13], as well as most surface fish, did not show VAB (Table S2). VAB was more robust in field-collected Pachón cavefish compared to those raised in the laboratory, suggesting that VAB may decline over generations in the laboratory. The complete absence of light and fluctuating food resources are hallmarks of the cave environment [1] that are not experienced by cavefish raised in the laboratory. To determine the roles of these environmental factors, we conducted VAB assays with surface fish raised in the dark or after starvation. Neither manipulation resulted in the induction of VAB (Figure S1A; Table S1), prompting us to investigate the possibility of a genetic basis for VAB. Accordingly, VAB was examined in the F1 progeny of a surface fish and Pachón cavefish cross. The mean distribution of VAB in the offspring was intermediate between those of the parents (Figure S1B; Table S1), suggesting that VAB is an inherited trait. Thus, VAB has a genetic basis, although the contribution of unknown environmental factors cannot be excluded. This conclusion is consistent with previous results showing that both environmental and genetic factors contribute to animal behavior [14, 15].

Significance of VAB in Finding Food

Laboratory-raised Pachón cavefish showed a wide range of VAB values, and some individuals did not display this behavior (Figure 2A). Additionally, most laboratory-raised surface fish lacked VAB, although some individuals were identified with relatively low VAB (Figure 2A). These differences allowed for testing the significance of VAB in feeding by competitive prey-capture experiments using pairs of fish with and without VAB (Figure 2B). Cavefish are better competitors than surface fish for limited quantities of food in the dark [16]. Consistent with this observation, cavefish dominated over surface fish in frequency of strikes at prey in the dark, but this advantage disappeared in the light (Figure 2Ba). Key findings of the competition experiments were (1) that surface fish with VAB showed significantly more strikes in the dark than surface fish without VAB, a difference that disappeared in the light (Figure 2Bb), and (2) that cavefish with VAB predominated over those without VAB in light or darkness (Figure 2Bc). The results suggest that VAB has a role in feeding and may be beneficial for survival of *Astyanax* in caves, although additional factors, such as enhanced olfaction, may also be involved in prey capture by cavefish. Small invertebrates such as copepods disturb the water at 30–40 Hz when swimming [17], which is in the detection range of the lateral line system (see below and [18]). Cavefish themselves create 30–90 Hz turbulence when in motion [19], suggesting that moving cavefish produce water disturbances that other cavefish might be able to follow [16], either to a source of food or perhaps to find mates.

In the absence of macroscopic predators, cavefish could be free to express VAB in caves, which could be a risky endeavor for surface fish outside of caves. Surface fish expressing VAB in their natural surface environment could be under negative
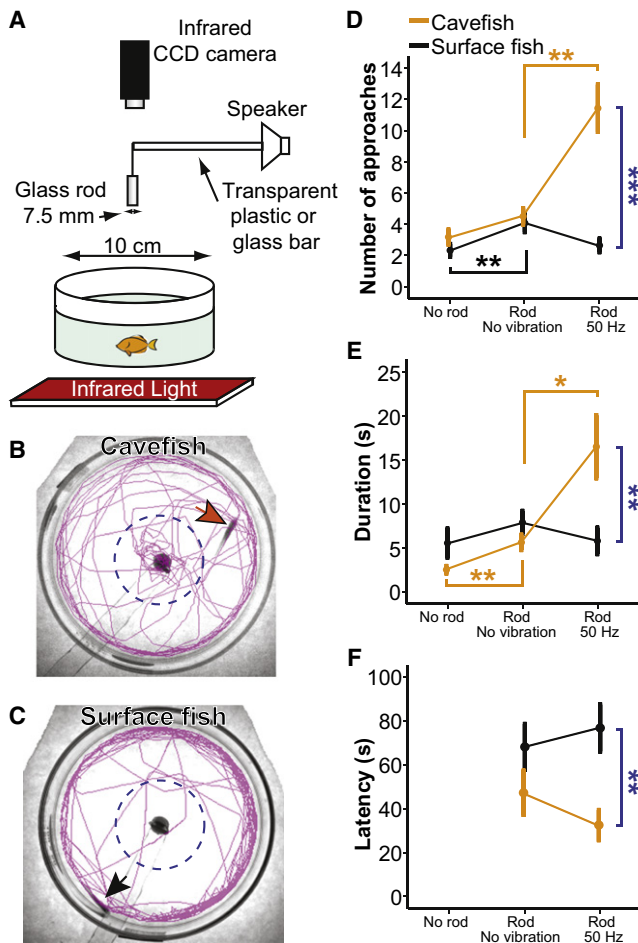
Figure 1. Quantification of Vibration Attraction Behavior

(A) Diagram showing the assay system used to record vibration attraction behavior (VAB) in darkness. An infrared light illuminates the cylinder-shaped assay chamber from below. Swimming is recorded with a charge-coupled device infrared camera positioned above the assay chamber. A glass rod is inserted into the assay chamber to produce vibration stimuli.

(B and C) Path (purple lines) of swimming in Pachón cavefish (B) and surface fish (C) during the 3 min assay period. Dotted lines indicate 2 cm diameter quantification area surrounding the grass rod (dark spot in the center of the chamber). Arrows indicate the starting positions of the fish.

(D–F) VAB quantified in cavefish (orange lines and points) and surface fish (black lines and points) as number of approaches (NOA, D), duration (E), and latency (F) in the absence of a rod (left), the presence of a stationary rod (middle), and the presence of a vibrating rod (50 Hz, right). Values are means ± standard error of the mean (SEM). Cavefish: n = 40; surface fish: n = 44. Orange, black, and blue asterisks indicate significant differences among cavefish, among surface fish, and between cavefish and surface fish, respectively (*p < 0.05, **p < 0.01, ***p < 0.001). See also Movie S1, Figure S1, and Tables S1 and S2.

selective pressure because of the risk of predation. Nevertheless, a small proportion of laboratory-raised surface fish showed low levels of VAB that were abolished by lateral line inhibitors (see below and Figure S2B), suggesting that the VAB phenotype is present at low frequencies in natural populations. When introduced into a dark cave, surface fish with VAB would have an advantage over those lacking this phenotype and thus could serve as the founders of cavefish populations. Because VAB levels gradually increased through selection in subsequent generations, the ability of cavefish to successfully find food in the darkness would be enhanced.
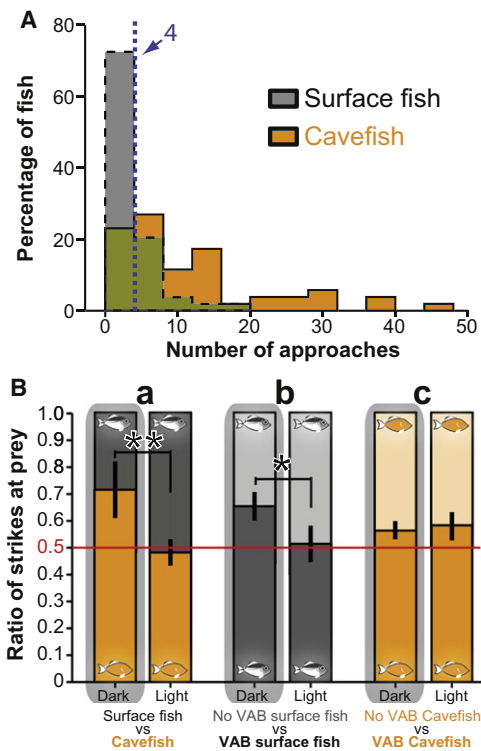

Figure 2. Significance of VAB in Feeding Determined by Competitive Prey-Capture Experiments

(A) VAB levels in surface fish and Pachón cavefish measured as NOA. Surface fish: n = 54, gray area; cavefish: n = 52, orange area. Vertical dashed line represents NOA cutoff value for classifying fish with (above 4) and without (below 4) VAB with a stimulus of 50 Hz.

(Ba–Bc) Prey-capture competition assays. Bars show the proportion of strikes at prey between pairs of surface fish (gray bars) and cavefish (orange bars) with or without VAB during a 1 min assay period in darkness (Ba–Bc, left bars) and in light (Ba–Bc, right bars). A total of eight pairs of cavefish versus surface fish (Ba), five pairs of surface fish with and without VAB (Bb), and ten pairs of cavefish with and without VAB (Bc) in the dark and light are shown. Values are mean ratio of strikes ± 95% confidence intervals of the mean. *p < 0.05, **p < 0.01. See also Table S1.

Therefore, natural selection for VAB may be one of the ways in which surface-fish-like ancestors became adapted to caves and evolved into cavefish, emphasizing the importance of behavioral diversity in adapting animals to new environmental challenges.

Superficial Neuromasts Underlie VAB

We determined the tuning of VAB by measuring responses to stimulus frequencies ranging from 5 to 500 Hz. This allowed us to discriminate between possible roles of the lateral line and inner ear sensory systems. The results showed that VAB has a relatively low frequency range (10 to 50 Hz) with a peak at 35 Hz (Figure 3A), considerably lower than the best sensing range of the *Astyanax* inner ear (200–6000 Hz) [20] but within the frequencies detectable by the lateral line (20–80 Hz) [18]. To confirm the role of the lateral line, we measured VAB after treatment with cobalt or gentamicin, which inhibit the function of neuromasts without detectable effects on the inner ear [21–24]. The results showed that these inhibitors abolished VAB (Figure S2), indicating that VAB is mediated by the lateral line. This conclusion is supported by the absence of changes
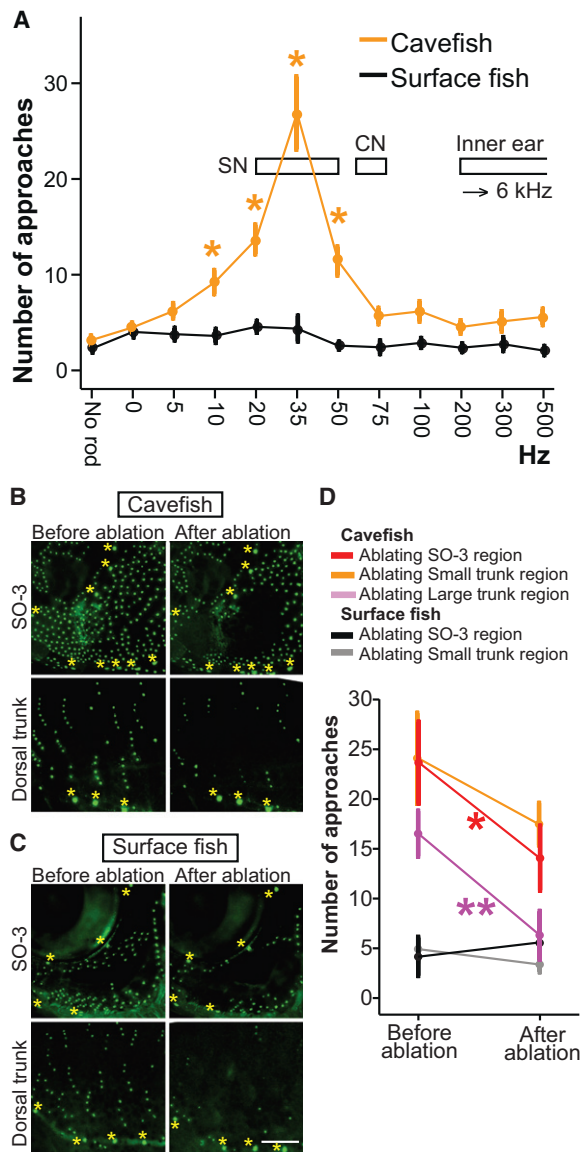
Figure 3. VAB Is Controlled by Superficial Neuromasts

(A) The relationship between VAB and vibration frequencies (Hz) in Pachón cavefish and surface fish. Values are mean NOA ± SEM. Cavefish: n = 40 at no rod and 0 Hz; n = 35 at 5 Hz, 10 Hz, 20 Hz, 100 Hz, and 200 Hz; n = 19 at 35 Hz and 75 Hz; n = 51 at 50 Hz; n = 8 at 300 Hz; n = 27 at 500 Hz. Asterisks denote frequencies at which cavefish VAB is significantly above the 0 Hz condition at $p < 0.05$ (see Table S1). Surface fish: n = 44 at no rod and 0 Hz; n = 38 at 5 Hz, 10 Hz, 20 Hz, and 100 Hz; n = 19 at 35 Hz and 75 Hz; n = 54 at 50 Hz; n = 35 at 200 Hz; n = 8 at 300 Hz; n = 27 at 500 Hz. Open horizontal boxes indicate the best sensing range of superficial neuromasts (SN), canal neuromasts (CN), and inner ear (right) in *Astyanax* [18, 20]. See also Figures S2 and S3.

(B and C) DASPEI-stained Pachón cavefish (B) and surface fish (C) before and after SN ablation. Scale bar is 1.0 mm; magnification is the same in (B) and (C).

(D) VAB in cavefish and surface fish before SN ablation and 2 days after bilateral SN ablation. Values are means ± SEM. *$p < 0.05$, **$p < 0.01$. Cavefish: n = 16 for SO-3, n = 10 for small region of the trunk, and n = 11 for large region of the trunk. Surface fish: n = 13 for SO-3 and n = 6 for small region of the trunk. A vibrating rod at 35 Hz was used. See also Table S1 and Figure S4.

in auditory structure and capacity between the two forms of *Astyanax* [20, 25].

The lateral line system consists of canal neuromasts (CN) and superficial neuromasts (SN) [26]. Adult cavefish and surface fish have about the same number of CN, but cavefish have several-fold more SN than surface fish [5–7]. Cavefish CN and SN are also larger and contain more sensory hair cells than those of surface fish [5, 7]. SN are the best candidates for a role in VAB because maximal VAB was evoked at 35 Hz, which coincides with the peak frequency of SN ([18]; see Figure 3A), and previous studies showed that CN ablation did not affect the attraction of cavefish to a vibrating sphere [9]. The following experiments were conducted to determine the type of neuromasts involved in VAB. First, we explored the timing between VAB onset and neuromast development in surface fish and cavefish (Figure S3). The number of CN did not change during development, but CN size increased about 2-fold, from 1 to 4 months postfertilization (mpf) (Figures S3C and S3D). In contrast, the number and size of SN increased starting at 2 mpf, and this increase was greater in cavefish than in surface fish (Figures S3C and S3D). VAB was first detected at 3 mpf in cavefish (Figure S3B), which is consistent with a role for SN. Second, we determined the effects on VAB of ablating SN bilaterally in the cranial third suborbital bone (SO-3 [27]) and the trunk (Figure S4), body areas with different neuromast densities. Cavefish lacking SN in the SO-3 region (ablated SN: 115 ± 8, mean ± SEM) or in a large trunk region containing about the same SN number as the SO-3 region (ablated SN: 119 ± 6) showed significant reduction in VAB (Figures 3B and 3D; Figures S4A and S4C). Decreased VAB was also observed when SN were ablated in a smaller dorsal trunk region (ablated SN: 50 ± 3), but this change was not significant (Figure 3D; Figure S4B). In contrast, bilateral ablation of SN in similar regions of surface fish controls had no behavioral effects (Figures 3C and 3D). CN were usually not affected in these experiments (Figures 3B and 3C). Therefore, the ablation experiments provide strong evidence that SN enchantment is responsible for cavefish VAB and demonstrate the primary and cooperative effects of SN located in the head and trunk on this behavior.

To determine whether increased SN number, size, or both are responsible for VAB, we compared VAB and SN number or diameter in the SO-3 regions of individual cavefish, surface fish, and their F1 progeny (Figure 4). The results showed that VAB was positively correlated with both elevated SN number and size (Figures 4A and 4B). Importantly, F1 hybrids showed intermediate VAB levels and exhibited SN numbers between those of their surface fish and cavefish parents (Figures 4A and 4C–4E), but the size of F1 hybrid SN was similar to large cavefish SN (Figures 4B–4D). The results suggest that both SN number and size are involved in cavefish VAB, although number may be the more important factor in determining the magnitude of VAB. Nevertheless, surface fish with low levels of VAB did not show significant increases in SN number or size (Figures 4A and 4B), implying that SN enhancement may not be necessary for modest levels of VAB.

We have found that enhancement of SN may be responsible for the evolution of relatively high levels of VAB in cavefish. The cavefish SN cupulae (hair cell stereocilia covered by a gelatinous case) are about 300 μm in length compared to about 42 μm in surface fish [7]. The long cavefish cupulae extend beyond the motionless boundary layer, which attenuates water movements along the surface of the body [28, 29]. McHenry et al. [29] determined the thickness of the boundary
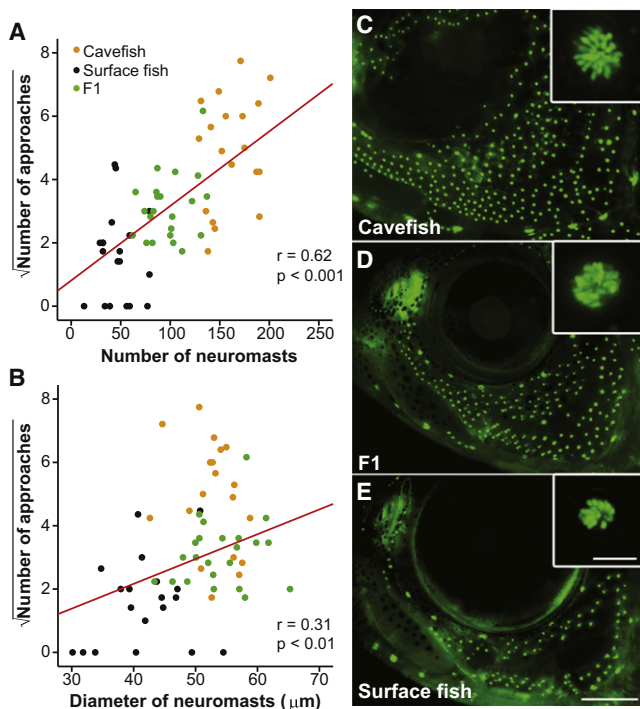
Figure 4. The Relationship between VAB and SN Number and Size in Pachón Cavefish, Surface Fish, and the F1 Progeny of a Surface Fish × Cavefish Cross

(A and B) Scatter plots showing the relationship between the SN number (A) or diameter (B) and the square root of NOA. Both SN number and diameter are significantly correlated with VAB (see Table S1). Cavefish: n = 20; surface fish: n = 19; F1 hybrids: n = 23. Linear regression line is in red. A vibrating rod at 35 Hz was used.

(C–E) DASPEI staining of neuromasts in the SO-3 region of a cavefish (C), an F1 hybrid (D), and a surface fish (E). Scale bar in (E) is 1.0 mm; magnification is the same in (C)–(E). Insets in (C)–(E) show the size of a typical SN from the SO-3 region of fish in (C)–(E). Scale bar in inset (E) is 50 μm; magnification is the same in each inset.

layer to be 40 μm in low water currents produced by vibration stimuli below 100 Hz. Large cavefish SN could therefore detect low vibration-frequency stimuli (below 50 Hz) in an otherwise calm cave pool. A dense cluster of neuromasts oriented in the same direction likely share common innervation, forming a small sensing unit [7], and this neural network could produce low-noise, high-quality information by averaging activity patterns within a group of neuromasts [30]. Taking advantage of a dense cluster of large SN, cavefish with sophisticated sensors may be able to evaluate and react to vibrating stimuli better than surface fish.

Astyanax cavefish have evolved a suite of constructive traits, including larger jaws [31], more taste buds [31, 32], modified brain structures [33], changes in behavior [34–39], and larger and more numerous SN [5–7] compared to their surface fish counterparts. Prior to this study, the relationship between the constructive morphological traits and the evolution of behavioral changes had not been examined. The current results have linked one of the constructive traits—SN enhancement—to a behavior that is advantageous for feeding in a dark environment. An increase of neuromasts has also been reported in other cavefish species [40], and thus lateral line enhancement could be a general phenomenon that mediates the adaptation of teleosts to life in caves.

Experimental Procedures

Biological Materials
The experiments were performed on laboratory-raised [5, 27, 31, 41] and wild-caught *Astyanax mexicanus* cavefish and surface fish. Fishes used in the assays ranged from 1 month to 2 years old and were fed living *Artemia salina* larvae. Wild-caught surface fish were collected from Nacimiento del Río Choy and Río Tampaón, both in San Luis Potosí, Mexico. Wild-caught Pachón and Piedras cavefish were collected at Cueva de El Pachón and El Sótano de Las Piedras, respectively, in San Luis Potosí, Mexico. All methods described here were approved by the University of Maryland Animal Care and Use Committee and conformed to National Institutes of Health (NIH) guidelines.

Vibration Attraction Behavior Assays
VAB was assayed in the dark using cylindrical chambers (Pyrex 325 ml glass dish, 10 cm diameter × 5 cm high, Corning) filled to 30–35 mm with water in the laboratory. Fish were placed in the assay chamber 4 or 5 days before the experiment to acclimate and were transferred to a dark room 1 day before the assay. Before the assay was initiated, the fish were subjected to another 3 min acclimation period in the recording chamber. Stimulus was created by using a 7.5 mm diameter glass rod inserted 15–18 mm into the water, supported by a 1 mm thick and 95 mm long transparent plastic plate (up to 100 Hz stimuli) or a 3 mm thick and 115 mm long glass bar (200–500 Hz stimuli). Manual stimuli were produced at 3–4 Hz (2.9 mm amplitude based on Fourier analysis of captured videos performed with MatLab Software R2007a, MathWorks) or mechanically at 5–500 Hz (Table S3). The axis of the vibration was in the horizontal plane. An EISCO function generator (Ambala) or a Leader LG1301 function generator with an audio speaker (Pro Speakers, Apple) were used to produce the stimulus, which was calibrated by Fourier analysis of recorded videos taken with a high-speed charge-coupled device (CCD) camera (DRS Lightning RDT). Individual fish were subjected to either a single assay in the absence of a rod, in the presence of a nonvibrating rod, or in the presence of a vibrating rod or to three successive assays using a random sequence of these three conditions. Swimming movements were video recorded for a 3 min period under the illumination of an infrared light (880 nm wavelength, BL1960-880 black light, Advanced Illumination). An infrared CCD camera (Qicam IR, Qimaging) with a zoom lens (Zoom 7000, Navitar) was used to capture images at 10 frames/s using StreamPix 3.36.0 video recording software (NorPix). ImageJ 1.35 software (NIH) was used for video analysis.

Wild-caught fish were maintained for 1 or 2 days in 2 l plastic bottles containing either water from their original location or purified tap water, and VAB was assayed at a field location. Fish were transferred to the assay chamber at least 1 hr before the assay, were given time to acclimate in the dark, and were subjected to a 3 min acclimation period on the recording stage. Manual stimulation was provided by a glass rod for 3 min as described above, and behavior was recorded with an infrared-light-equipped infrared camcorder (DCR-SR200C, Sony). NOA was counted with reference to a transparent plastic disc (2 cm diameter) attached to the vibrating glass probe.

Genetic and Environmental Effects on VAB
A female surface fish was crossed with a male Pachón cavefish to obtain F1 hybrids. To test for the effects of darkness on VAB, we raised surface fish from hatched larvae for 3–4 months in constant darkness prior to the behavioral assay. Controls were reared under a normal 14 hr light:10 hr dark diurnal cycle. For starvation experiments, surface fish (4 months to 1 year old) were transferred to an assay chamber and starved for 5 days prior to the assay.

Prey Capture Competition Assay
Surface fish and cavefish with or without VAB were marked by an Alcian blue tattoo near their dorsal fins. Five days prior to the assay, a pair of tattooed fish was transferred to a test aquarium (12 × 20 × 10 cm), subjected to either continuous darkness or a 14 hr light:10 hr dark diurnal cycle depending on whether subsequent assays were to be conducted in dark or light, respectively, and fed *Artemia* larvae once per 2 days. On the day of the assay, three drops of living *Artemia* larvae were added to the aquarium, and strikes at prey were recorded for 1 min with the infrared camcorder. Strikes were counted for every fish, and the total number of strikes for pairs was calculated. Then the ratio of strike numbers of a dominating fish was calculated. Each competition assay was repeated twice, and the ratios of strike numbers were averaged across the two trials.

Vibration Frequency Analysis

Vibration frequency analysis was conducted in three ways: (1) in the absence of a vibrating glass rod, (2) in the presence of a stationary glass rod (0 Hz), and (3) in the presence of a vibrating rod at 5–500 Hz (Figure 3A). The sequence of vibration frequencies was randomly chosen for each fish, and a resting period of at least 25 min was applied between the assays. VAB was recorded as described above.

Cobalt and Gentamicin Treatment

Before treatment, fish were assayed for VAB as described above, followed by a 4- or 5-day resting period. The fish were then immersed in a 0.08 mM solution of $CoCl_2$ (Sigma) in Ca^{2+}-free standardized water (25 mM KCl, 50 mM KNO_3, 50 mM NaH_2PO_4, 200 mM NaCl, 100 mM $MgSO_4$, adjusted to pH 7.2 with NaOH; [21, 22]) or in a 0.002% solution of gentamicin sulfate salt (Sigma) in conditioned water (conductivity approximately 600 μS) for 14–19 hr [23] prior to VAB assays. Control fish were immersed in Ca^{2+}-free standardized water containing 3.75 mM Ca^{2+} for the cobalt experiment or in conditioned water for the gentamicin experiment.

Neuromast Vital Staining

For vital staining with 2-(4-(dimethylamino)styryl)-N-ethylpyridinium iodide (DASPEI, Invitrogen) [42], fish were immersed in 25 μg/ml DASPEI dissolved in conditioned water (conductivity approximately 600 μS) for 1 hr, followed by immersion in ice cold 66.7 μg/ml Ethyl 3-aminobenzoate methanesulfonate salt (MS222, Sigma) in conditioned water, viewed with a fluorescence microscope (Axioskop 2 equipped with 2.5× Plan-Neofluar lens and a fluorescein isothiocyanate filter set; Zeiss) and photographed with a Zeiss Axiocam CCD camera. Neuromasts were quantified on images of DAS-PEI-stained fish using ImageJ software. CN were counted in the infraorbital lateral line, and SN were counted in the SO-3 region [27]. The longer diameter of CN and SN was measured in the 10 largest stained neuromasts in the same area.

Superficial Neuromast Ablation

Before ablation, VAB was measured and neuromasts were stained with DASPEI and photographed. SN were ablated by applying 0.5 μl of Vetbond nontoxic tissue adhesive (3M) along either the SO-3 or a dorsal trunk region using the plastic tip of a 2.5 μl Eppendorf pipetman. Application of Vetbond to CN was avoided. After application of tissue adhesive to one side, the fish was exposed to air for 10 s, and tissue adhesive was then applied to the same area on the opposite side, followed by a second cycle of air drying. Treated fish were placed in a 10 cm diameter cylindrical chamber containing conditioned water at room temperature. Within a day of active swimming, the tissue adhesive usually "peeled off" the body, resulting in a void in the underlying field of SN.

Statistics

The specifics and results of the statistical tests are provided in Table S1. Statistical tests were conducted with PASW 17.0 software (SPSS).

Supplemental Information

Supplemental Information includes four figures, three tables, and one movie and can be found with this article online at doi:10.1016/j.cub.2010.07.017.

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