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Evolutionary shift towards lateral line dependent prey capture behavior in the blind Mexican cavefish

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ABSTRACT

Feeding strategies are dependent on multi-modal sensory processing, that integrates visual, chemosensory, and mechanoreceptive cues. In many fish species, local environments and food availability dramatically influence the evolution of sensory and morphological traits that underlie feeding. The Mexican cavefish, *Astyanax mexicanus*, have developed robust changes in sensory-dependent behaviors, but the impact on prey detection and feeding behavior is not known. In the absence of eyes, cavefish have evolved enhanced sensitivity of the lateral line, comprised of mechanosensory organs that sense water flow and detect prey. Here, we identify evolved differences in prey capture behavior of larval cavefish that are dependent on lateral line sensitivity. Under lighted conditions, cavefish strike *Artemia* prey at a wider angle than surface fish; however, this difference is diminished under dark conditions. In addition, the strike distance is greater in cavefish than surface fish, revealing an ability to capture, and likely detect, prey at greater distances. Experimental ablation of the lateral line disrupts prey capture in cavefish under both light and dark conditions, while it only impacts surface fish under dark conditions. Together, these findings identify an evolutionary shift towards a dependence on the lateral line for prey capture in cavefish, providing a model for investigating how loss of visual cues impacts multi-modal sensory behaviors.

1. Introduction

The ability to locate and acquire food is central to survival and successful reproduction (Illius et al., 2002). Animals have evolved diverse foraging and prey capture mechanisms, with different organisms utilizing a variety of sensory systems to localize food sources (Catania, 2012; Daghfous et al., 2012; Moss and Shettleworth, 1996). Many species, from humans to fish, heavily utilize visual systems to find food (Bianco and Engert, 2015; Troscianko et al., 2011). In the absence of visual cues, organisms must rely on alternative sensory input to locate food sources. For instance, nocturnal species seek food in dimly lit areas, relying almost exclusively on auditory cues (Payne, 1971; Wagner et al., 2013), and many rodents with poor visual acuity are largely reliant on olfactory sensory cues (Doty, 1986; Rattazzi et al., 2015; Yao et al., 2016). The diversity of sensory processes used in foraging and prey detection suggests that the sensory basis for foraging is under stringent evolutionary pressure. While diverse strategies utilizing sensory systems in foraging behavior are well described, much

less is known about how these systems evolve in response to environmental perturbation.

The blind Mexican cavefish, *Astyanax mexicanus,* provide a unique opportunity to study how foraging strategies evolve in response to strong environmental pressures. *A. mexicanus* exist in two distinct morphological forms: an eyed surface-dwelling form found in aboveground rivers and streams of northeast Mexico and parts of Southern Texas, and 29 populations of cave-dwelling forms, mostly found within the Sierra del Abra region of northeast Mexico (Gross, 2012; Jeffery, 2001; Mitchell et al., 1977). Many of these populations of cavefish are geographically and hydrologically restricted, suggesting they evolved independent of one another (Bradic et al., 2013; Mitchell et al., 1977; Ornelas-García et al., 2008). Moreover, cave-dwelling fish have converged on a suite of morphological and behavioral traits, such as eye loss, which evolved via different genetic mechanisms (Borowsky, 2008a; Duboué et al., 2011; Wilkens and Strecker, 2003; Yoshizawa et al., 2012), making this emerging model organism a powerful system to study the principles of convergent evolution.

Contrasting dramatically with the ecology of the surface rivers and streams, the perpetually dark caves have relatively few primary producers; therefore, the cavefish diet is primarily restricted to small organic matter, bat guano, and insects (Espinasa et al., 2017; Mitchell et al., 1977). To compensate, cavefish have developed a number of behavioral adaptations amenable to the subterranean environment, including increased vibration-associated behavior, hyperphagia, and changes in feeding angle (Aspiras et al., 2015; Kowalko et al., 2013; Wilkins, 1988; Yoshizawa et al., 2010). In addition, cavefish have evolved enhanced mechanosensory, olfactory, and taste sensitivity, presumably to compensate for reduced reliance on visual cues (Bibliowicz et al., 2013; Schemmel, 1967; Varatharasan et al., 2009; Yoshizawa et al., 2010). Despite these enhanced sensory changes, the contributions to evolved differences in feeding behavior remain poorly understood.

Here, we describe a prey capture assay that we developed for studying feeding strategies in larval *A. mexicanus,* to investigate how different sensory modalities contribute to feeding behavior. Prey capture has been extensively studied in zebrafish, and functional imaging in transgenic zebrafish has been used to map retinal, motor, and central brain circuits required for prey detection and capture (Bianco and Engert, 2015; Muto et al., 2017). In zebrafish larvae, this behavior is almost exclusively driven by visual inputs, yet other fish species are reliant on mechanosensation and olfaction (Bianco and Engert, 2015; Braubach et al., 2009; Schwalbe et al., 2012). We demonstrate that both surface and cave forms of *A. mexicanus* have the potential to utilize the lateral line in sensing and finding food. In surface fish, the visual system is dominant over the lateral line, yet in cave forms, the lateral line is the primary sensory system by which fish can detect food. Our results demonstrate that several foraging strategies exist within single organisms for finding food, and adaptation to the cave environment resulted in dependence on mechanosensation for prey capture.

2. Results

To determine whether prey-seeking behavior differs between surface fish and cavefish, we recorded 29–33 day post-fertilization (dpf) surface fish and Pachón cavefish larvae (Fig. 1A) consuming *Artemia* brine shrimp using a high-speed camera set at 100 frames per second. To quantify prey capture behavior, we measured the strike angle by quantifying the angle between the brine shrimp target and the fish's center point along the midline (Fig. 1B). In addition, we calculated the strike distance by determining the distance between the closest location on the fish head to the *Artemia* at the start of the strike motion (Fig. 1B). Serial time-lapse images of single prey capture events revealed that surface fish approach prey directly, bending the most caudal region of their tail (Fig. 1C). In zebrafish, this movement has been classified as a J turn (McElligott and O'Malley, 2005). Conversely, Pachón cavefish approach prey using a C-bend turn that involves turning the head toward adjacent prey (Fig. 1D). Quantification under lighted conditions began at zeitgeber time 0 (ZT0), the onset of lights on, when the overall activity is most similar between surface fish and Pachón cavefish (Duboué et al., 2011). Analysis revealed strike angle and strike distance were significantly greater in Pachón cavefish than surface fish, indicating that cavefish both bend their heads towards prey and attack from a greater distance (Fig. 1E, F). Differences in strike angle (Fig. S1A), but not distance (Fig. S1B), emerged as early as 8 dpf, indicating a developmental component to lateral line mediated prey capture.

It is possible that the reduced attack angle and strike distance during prey capture in surface fish is the result of reliance on visual cues that are absent in cavefish. To investigate this in animals lacking visual cues, we measured prey capture in complete darkness, while maintaining the time of testing at the onset of lights on (ZT0) used for measurements under lighted conditions. We compared images from the high-speed camera in light and dark conditions and found that under dark conditions, the prey capture behavior between surface and cavefish appeared similar (Fig. 2A, B). Unlike lighted conditions, the strike angle of surface fish was significantly greater in dark conditions, while darkness had no effect on Pachón cavefish (Fig. 2C). Further, under dark conditions, the strike angle did not differ between surface fish and cavefish (Fig. 2C). These findings suggest that differences between surface and Pachón cavefish observed under lighted conditions are primarily due to use of visual cues in surface fish. Strike distance was significantly reduced in the dark in surface fish but not cavefish (Fig. 2D), suggesting optimal foraging in surface fish occurs during the light, and cavefish may have improved ability to detect prey compared to surface fish in the dark. Quantification of successful capture rate revealed no differences between light and dark conditions in either surface or cavefish (Fig. 2E). However, the total number of attempts were significantly greater under both conditions in Pachón cavefish (Fig. 2F). Further, in surface fish, the strike rate was reduced in the dark, suggesting they are less successful at capturing prey during dark conditions (Fig. 2F). These findings reveal different foraging strategies where attempts are increased in cavefish with reduced success rate.

To determine whether the lateral line modulates prey capture, we ablated the lateral line and measured the effects on prey capture. Bathing fish in the antibiotic gentamicin efficiently ablates all lateral line neuromasts by disrupting hair cells (Van Trump et al., 2010). Neuromasts were ablated 24hrs prior to behavioral testing, and the effectiveness of ablation was confirmed by staining fish with the mitochondrial marker DASPEI following prey capture tests (Fig. 3A). Gentamicin treatment fully ablated the lateral line in surface fish and Pachón cavefish (Fig. 3B-E). Ablating the lateral line did not impact strike angle or distance in surface fish under light conditions, however, all prey capture was abolished under dark conditions, indicating that surface fish are dependent on the lateral line for prey capture in dark conditions (Fig. 3F, G). In cavefish, ablation of the lateral line significantly reduced both strike angle and distance under both light and dark conditions, revealing a critical role for the lateral line under both conditions (Fig. 3F, G).

The antennae strokes of a moving *Artemia* generate oscillations of approximately 7 Hz, and based on previous literature, we reasoned that the lateral line may detect water movement induced by these oscillations (Kirchner et al., 2014). To test this hypothesis, we presented surface and Pachón larvae with dead *Artemia* that had been flash-frozen, then thawed immediately prior to the experiment, in accordance with published protocols (Schwalbe et al., 2012). Under lighted conditions, cavefish and surface fish displayed a reduced attack angle, and all prey capture was abolished in surface fish under dark conditions (Fig. 4A). While cavefish were able to capture prey under light and dark conditions, the strike angle was dramatically reduced compared to previous experiments using live *Artemia,* and was similar to findings in gentamicin treated fish (Fig. 4A). A significant reduction in strike angle was observed in surface fish under lighted conditions, presumably because the use of dead *Artemia* eliminates the effect of prey movement on strike angle. Strike distance in surface fish was unaffected by the absence of moving *Artemia* under lighted conditions, while cavefish showed reduced strike distance under both lighted and dark conditions (Fig. 4B). Taken together, these findings suggest prey capture of dead *Artemia* largely phenocopies lateral line ablation, supporting the notion that prey capture is induced by detection of *Artemia* movements by the lateral line.

3. Discussion

We used high-speed imaging to examine state-dependent and population-specific differences in prey capture behavior in *A. mexicanus*. This assay provides quantifiable metrics of strike angle, which likely reflects the sensory modality used to detect prey, lateral line
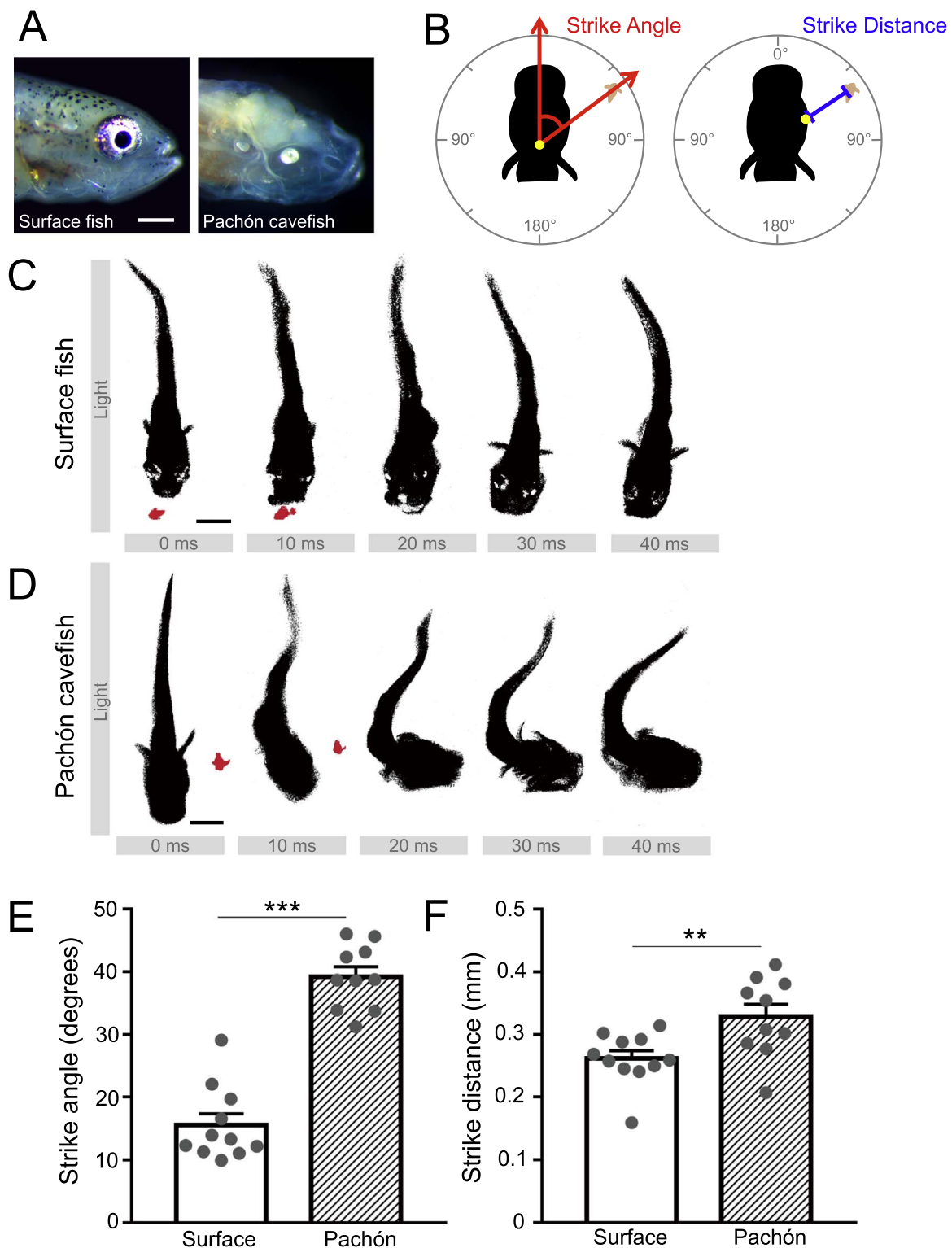
Fig. 1. *Evolved differences in prey capture between surface and Pachón cavefish.* (A) Comparison of morphological differences between larval surface fish and Pachón cavefish morphs. Cavefish (right) exhibit altered cranial structure compared to surface fish (left), and lack pigmentation and functional eyes. Scale bar = 0.5 mm. (B) Quantification of strike angle (left) and strike distance (right) used for behavior analysis. (C) Example time-lapse of a stereotypical surface fish strike, in lighted conditions. Surface fish move towards their prey head-on and propel themselves forward to capture. Scale bar = 1 mm. (D) Example time-lapse of a stereotypical cavefish strike, in lighted conditions. Cavefish remain immobile prior to striking nearby prey, using a lateral C-shaped motion. Scale bar = 1 mm (E) Strike angle in larval surface (N = 11) and cavefish (N = 10; Unpaired *t*-test, t = 9.772, df=19, *P* < 0.0001). (F) Strike distance in larval surface (N = 11) and cavefish (N = 10; Unpaired *t*-test, t = 2.884, df=19, *P* = 0.0095). Error bars represent + /- standard error of the mean. ** denotes *P* < 0.01, *** denotes *P* < 0.001.

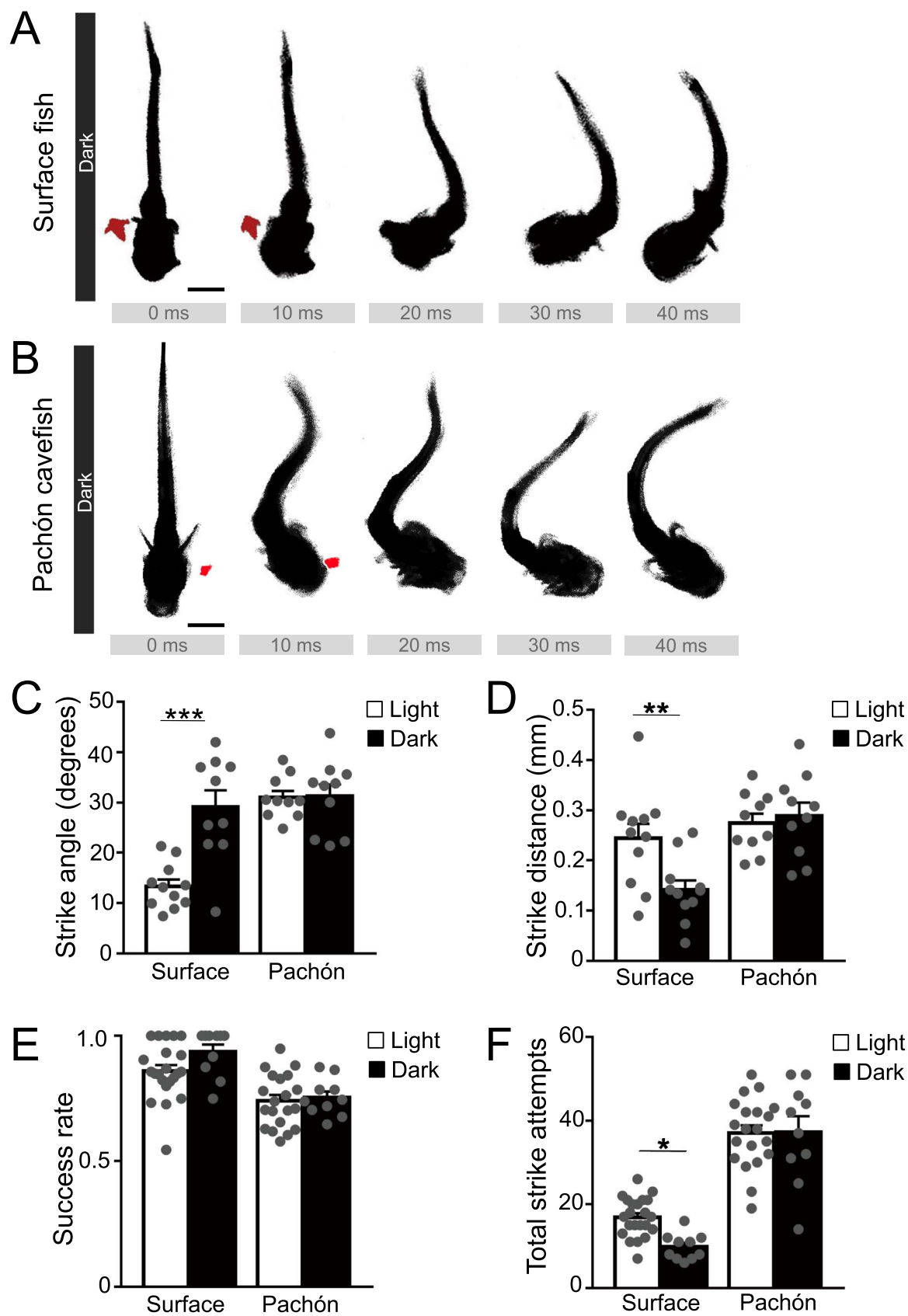sensitivity in prey detection, or a stereotyped motor response induced by prey detection. In addition, we quantify strike distance, which likely reflects the sensitivity of prey detection. Further, we identify state-dependent modulation of prey capture behavior in *A. mexicanus* surface fish. These findings reveal that *A. mexicanus* surface fish have the ability to modulate between prey capture behaviors in accordance with light availability, uniquely suiting them for adaptation to the dark cave environment.

3.1. Evolutionary shift in prey capture behavior

Comparative analysis of prey capture behavior in surface fish and Pachón cavefish revealed an evolutionary shift towards dependence on non-visual cues in cavefish, indicative of evolution in a perpetually dark environment. Our findings reveal a greater strike distance in cavefish fry under dark conditions, suggesting that cavefish are more attuned to foraging in the dark. A number of factors may account for this increase in strike distance, including enhanced detection of prey, or increased motivation to feed, that have previously been documented in cavefish (Aspiras et al., 2015; Yoshizawa et al., 2010). Suction also plays a critical role in prey capture, and it is possible that increased suction in cavefish contributes to the greater strike distance we observed in Pachón cavefish (Camp et al., 2015). Supporting the notion that feeding is enhanced in cavefish, a competition assay in 17 dpf fry revealed cavefish consumed more *Artemia* than surface fish under dark conditions (Espinasa et al., 2014). Further, this is not related to the presence of eyes because there was no effect of eye-ablation on food consumption in surface fish (Espinasa et al., 2014). In addition, multiple lines of evidence in adult fish reveal enhanced foraging capabilities in adult cavefish. In a competition assay, Pachón cavefish were able to out-compete surface fish under dark, but not lighted conditions, and this was later related to the degree of vibration attraction behavior in individual fish (Hüppop, 1987; Yoshizawa et al., 2010). Further, the expansion of lateral line superficial neuromasts surrounding the eye orbit were found to increase vibration attraction behavior in cavefish, revealing an adaptive function of lateral line expansion (Yoshizawa et al., 2010). While vibration attraction behavior was not detected in 30 dpf fry (Yoshizawa et al., 2010), it is possible that alternative changes in the lateral line allow for increased strike distance and angle in cavefish.

3.2. Relation to stereotyped turning behavior in zebrafish

High resolution imaging of prey capture in *A. mexicanus* reveals robust differences in stereotyped turning behavior, which are dependent on light availability and differ between surface and cavefish. The stereotypic capture behavior of zebrafish larvae in response to prey has been studied extensively (Budick and O'Malley, 2000; Gahtan et al., 2005). This pathway has primarily been studied under lighted conditions, since zebrafish demonstrate a significant reduction in prey capture behavior both in darkness and when vision is impaired (Gahtan et al., 2005). This visually driven behavior in zebrafish results in a stereotypic J-turn movement, initiated by the tail (Patterson et al., 2013). In *A. mexicanus*, surface fish exhibit a J-turn under lighted conditions, while the stereotyped movement shifts to C-shaped movements in dark conditions. Further, cavefish use C-shaped movements

to attack prey under both light and dark conditions. In zebrafish, C and J turns are dependent on distinct neural circuits and are activated by distinct sensory inputs (Fajardo et al., 2013; Liu and Fetcho, 1999), supporting the notion that independent neural circuits regulate context and population-specific foraging in *A. mexicanus*.

The findings that *A. mexicanus* surface and cavefish readily detect prey in the dark are notable because conflicting accounts exist on the ability of zebrafish to detect prey in the absence of visual cues. While it was previously reported that prey capture in zebrafish is entirely dependent on visual processing (McElligott and O'Malley, 2005), other studies suggest there is consumption under dark conditions (Gahtan et al., 2005; Westphal and O'Malley, 2013). Interestingly, little is known about the mechanism of prey detection under dark conditions, and one study suggests it is dependent on tactile cues through direct contact between paramecium prey and larvae (Patterson et al., 2013). Further, this study suggests zebrafish larvae use the same J-turn behavior under light and dark conditions (Patterson et al., 2013). In contrast, findings in *A. mexicanus* indicates surface fish larvae have two different foraging strategies under light and dark conditions, and are fully capable of capturing prey in the dark. These data reveal the presence of light-dependent plasticity of prey capture behavior that is not present, or has yet to be identified, in zebrafish.

3.3. A role for the lateral line in prey capture behavior

Our results reveal an essential role for the lateral line in cavefish prey capture behavior, as well as in surface fish when visual cues are not present. While to our knowledge, these results are the first to document a role for the lateral line in *A. mexicanus* larvae, the adult lateral line regulates vibration attraction behavior (VAB) that promotes foraging, particularly under dark conditions (Yoshizawa et al., 2010). In adult cavefish, ablation of the lateral line with gentamicin abolishes vibration attraction behavior, and this effect was localized to a population of superficial neuromasts near the eye-orbit that are present in greater number in cavefish (Yoshizawa et al., 2010). Examination of VAB over the course of development revealed VAB is not present in fish under five months old, suggesting the prey capture observed in this study and VAB are regulated by distinct processes (Yoshizawa et al., 2010). These discrepancies are likely explained by the assays measuring different aspects of foraging behavior. While the VAB assay focuses on the attraction to a vibrating source, our prey capture analysis focuses on the mechanics of striking at prey. Early in development, fish likely consume smaller animals, and therefore the lateral line may be involved in the initiation and execution of strikes at nearby prey, but dispensable for responding to vibrations at longer distances. Alternatively, it is possible that differences in frequency account for discrepancies in lateral line dependent behavior. Significant VAB is detected at 10–50 Hz in adult fish while *Artemia* are estimated to generate 7 Hz frequency (Kirchner et al., 2014; Yoshizawa et al., 2010). Identification of lateral line neuromasts contributing to prey detection would allow for further investigation of how prey-sensation is represented within the brain, and how it integrates with other sensory modalities.

While our findings reveal a critical role for the lateral line in prey capture, a small number of prey capture events remained in cavefish
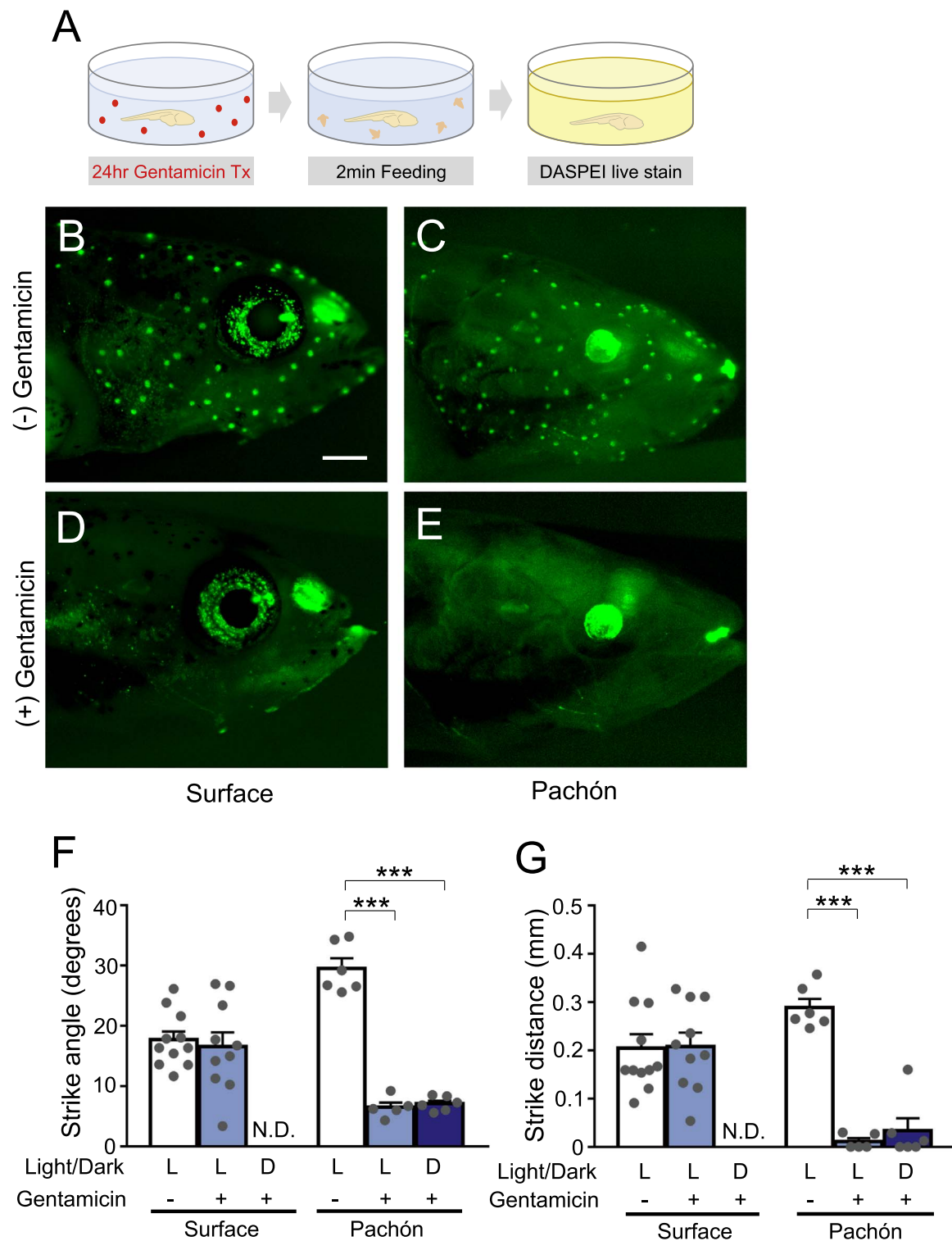
Fig. 3. *Ablation of lateral line in Pachón cavefish reduces strike angle and distance.* (A) Diagram of experimental paradigm for gentamicin treatment. Fish are treated for 24 h in dissolved gentamicin sulfate, followed by a 2-min feeding/behavioral recording, and 1 h in a DASPEI solution to confirm complete ablation of neuromasts. (B-E) Visualization of lateral line neuromasts in surface fish (left) and Pachón cavefish (right) before (top) and after (bottom) gentamicin treatment. Scale bar = 0.5 mm. (F) Strike angle of surface fish and Pachón cavefish, with or without prior gentamicin treatment, in lighted or dark conditions. Surface fish control(N = 11) vs. light/gentamicin(N = 10; Unpaired *t*-test, t = 0.4196, df=19, *P* = 0.6795). Pachón cavefish control (N = 6) vs. light/gentamicin(N = 5; *P* < 0.0001). Pachón cavefish control vs dark/gentamicin(N = 6; *P* < 0.0001; One-Way ANOVA, F(2, 14) = 136.9, *P* < 0.0001. (G) Strike distance of surface fish and Pachón cave fish, with or without gentamicin treatment, in lighted or dark conditions. Surface fish control(N = 11) vs. light/gentamicin(N = 10; Unpaired *t*-test, t = 0.09121, df=19, *P* = 0.9283). Pachón cavefish control(N = 6) vs. light/gentamicin(N = 5; *P* < 0.0001). Pachón cavefish control vs. dark/gentamicin(N = 6; *P* < 0.0001; One-Way ANOVA, F(2, 14) = 63.46, *P* < 0.0001. N.D. = No Data/Strikes. Error bars represent + /- standard error of the mean. *** denotes *P* < 0.001.
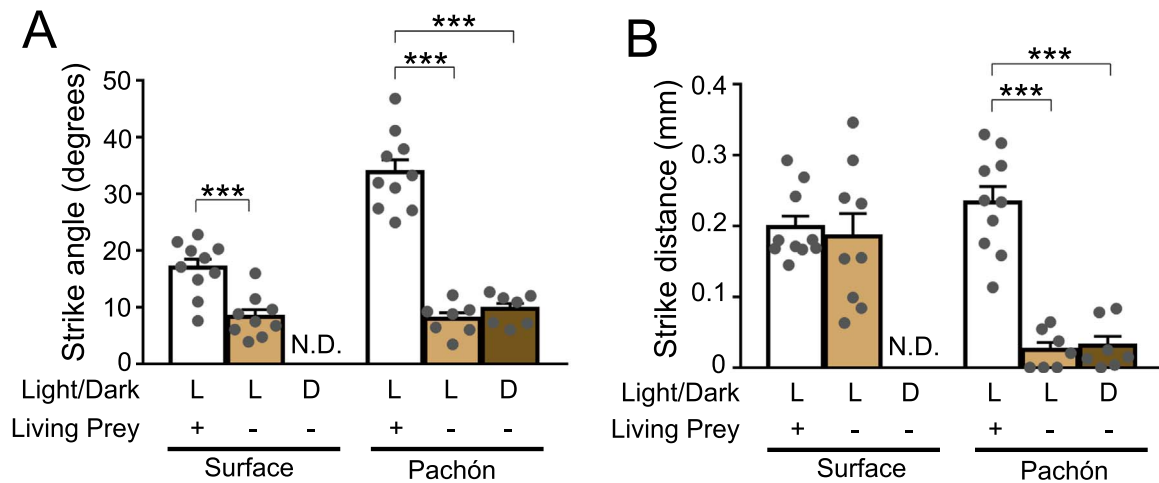
Fig. 4. *Effect of immobile prey on strike dynamics in light and dark conditions.* Fish were fed either live brine shrimp or non-living (flash-frozen) shrimp. (A) Strike angle in surface and Pachón cavefish, between lighted and dark conditions, on mobile (living) or immobile (dead) prey. Surface fish control(N = 10) vs. light/immobile prey(N = 9; Unpaired *t*-test, t = 4.372, df=17, $P = 0.0004$). Pachón cavefish control(N = 10) vs. light/immobile prey(N = 7; $P < 0.0001$). Pachón cavefish control vs dark/immobile prey(N = 7; $P < 0.0001$; One-Way ANOVA, $F_{(2, 21)} = 74.38$, $P < 0.0001$. (B) Strike distance in surface and Pachón cave fish, between lighted and dark conditions, on mobile (living) or immobile (dead) prey. Surface fish control(N = 10) vs. light/immobile prey(N = 9; Unpaired *t*-test, t = 0.3753, df=17, $P = 0.7120$). Pachón cavefish control(N = 10) vs. light/immobile prey(N = 7; $P < 0.0001$). Pachón cavefish control vs dark/immobile prey(N = 7; $P < 0.0001$; One-Way ANOVA, $F_{(2, 21)} = 45.56$, $P < 0.0001$. N.D. = No Data/Strikes. Error bars represent +/- standard error of the mean. *** denotes $P < 0.001$.

lacking functional lateral lines; these tended to occur when food was immediately in front of the fish, perhaps indicating the presence of an alternative enhanced sensory modality, such as taste or olfaction, that allowed larvae to detect prey. Supporting this, cave morphs of *A. mexicanus* have previously been reported to have both increased numbers of taste buds (Varatharasan et al., 2009; Yamamoto et al., 2009) and enhanced chemosensory response (Bibliowicz et al., 2013) compared to their surface counterparts.

A number of other species use the lateral line to detect prey (Boord and Montgomery, 1989; Coombs et al., 2012). In the peacock cichlid, *Aulonocara stuartgranti*, the lateral line is required for detecting movements generated by *Artemia* (Schwalbe et al., 2012). *A. stuartgranti* consume live and dead *Artemia* equally in light conditions, yet nearly all consumption was abolished in both light and dark conditions following lateral line ablation (Schwalbe et al., 2012). Similarly, we find that consumption of dead *Artemia* is dramatically reduced in both surface fish and cavefish under dark conditions, accompanied by reduced strike angle and distance. These findings largely phenocopy the effect of gentamicin treatment, suggesting that the lateral line is detecting *Artemia* movement.

This assessment can be tested by examining response to non-nutritive foods. For example, zebrafish will approach and consume air bubbles (Muto and Kawakami, 2013). Testing consumption of air bubbles in lateral line ablated fish would test the hypothesis that residual feeding in cavefish is due to gustatory or olfactory processing. An alternative possibility is that increased locomotion previously observed in cavefish at 30 dpf (Duboué et al., 2011, 2012) may result in an increased number of random contacts with dead *Artemia,* resulting in consumption. Therefore, these findings lay the groundwork for future studies examining the requirements of altered sensory modalities in prey capture behavior.

3.4. Multi-modal processing of prey-seeking behavior

Prey capture serves as a model for examining innate multimodal behavior and decision making. This process is described as having multiple steps that include object recognition, approach, a decision to attempt a strike, object capture, and assessment, where the animal decides to consume or expel the object (Muto and Kawakami, 2013). The response to objects is dependent on their size and angle, resulting in approach or avoidance (Bianco et al., 2011). Functional imaging in

partially immobilized zebrafish has identified a visual pathway in the pre-tectal region that is activated by prey-stimulus and required for the initiation of prey capture behavior (Semmelhack et al., 2014), but it is unclear how this pathway interfaces with decision making processes or other sensory modalities. Studies examining the neural basis of prey capture primarily focus on visual circuitry in zebrafish (Muto and Kawakami, 2013), and our findings that surface morphs of *A. mexicanus* integrate visual and mechanosensory stimuli provide the unique opportunity to examine how different sensory processes are integrated to generate a stereotyped J-turn behavior under lighted conditions, and C-shaped movements under dark conditions. Whereas J-bends are driven through activation of midbrain neurons receiving input from tectal and pretectal areas (Fajardo et al., 2013), C-bends are largely thought to arise through activation of Mauthner cells (Burgess and Granato, 2007; Lee and Eaton, 1991). Since Mauthner cells receive input from cells of the inner ear and the lateral line (Eaton et al., 1988), and ablation of the lateral line reduces prey capture in cavefish, this suggests a possible lateral-line to Mauthner mechanism for prey capture in cavefish, and a mostly tectal or pre-tectal one for surface fish. Further analysis in *A. mexicanus*, such as ablation of Mauthner cells may provide insight as to how the integration of these processes evolved in cavefish.

The ability to detect prey in darkness, and the differences in strike angle and distance between surface fish and cavefish, may have important consequences for foraging ability in natural conditions. The lack of reported prey capture in zebrafish larvae under dark conditions suggests these fish exclusively feed during the day. By contrast, both surface and cave populations of *A. mexicanus* larvae appear capable of detecting and capturing prey in dark conditions. While *Artemia* do not represent a natural food source for cavefish, these findings are likely to be ecologically relevant, as stomach content analysis of fry from the Pachón cave revealed consumption of diverse crustaceans including water fleas, copepods, and isopods (Espinasa et al., 2017), though a different study on adult cavefish from the Micos caves suggested fish consume bat guano (Mitchell et al., 1977). A better understanding of the diets in surface fish and cavefish would provide insight into the evolutionary basis for differences in prey-seeking behavior.

While little is known of *A. mexicanus* foraging behavior in the wild, these findings raise the possibility that *A. mexicanus* surface and cavefish feed during the day and night. Adult cavefish lack behavioral

and molecular locomotor rhythms in their natural cave environment, but behavioral rhythms are present under light-dark conditions (Beale et al., 2013; Yoshizawa et al., 2015). Further, bat colonies in caves may provide circadian and nocturnal rhymes, resulting in the necessity of nighttime foraging (Mitchell et al., 1977). In the Somalian cavefish, *Preatichtys andruzzi*, food-entrainable oscillators are present that can both synchronize the clock and behavioral rhythms with feeding (Cavallari et al., 2011). Taken together, these findings raise the possibility that nighttime foraging in cavefish may impact circadian function in *A. mexicanus*.

The presence of dark feeding in surface morphs may provide insight into the unique ability of *A. mexicanus* to inhabit subterranean environments and adapt to cave life. Numerous fish species populate a wide range of freshwater rivers surrounding the caves and are occasionally swept in to the subterranean habitat. Species such as cichlids have been observed residing in these caves; yet *A. mexicanus* remains nearly exclusive among species that repeatedly colonize the cave environment in NE Mexico (Mitchell et al., 1977). Cave-dwelling *A. mexicanus* have enhanced lateral line sensitivity relative to surface conspecifics, however some surface fish demonstrate a moderate level of vibration attraction behavior (VAB), suggesting natural populations of surface fish harbor individuals which are suited for finding food in darkness. Further, surface x Pachón cavefish F_1 progeny show a higher number of neuromasts and VAB compared to surface fish, suggesting this sensory enhancement is genetic and can dramatically enhance food finding abilities in the dark within a single generation (Teyke, 1990; Yoshizawa et al., 2010). These reports, combined with our findings, suggest *A. mexicanus* prey capture under dark conditions, through a lateral line-dependent mechanism, allowed for early colonization of the caves.

3.5. Applications for investigating the evolution of prey capture

Our analysis is limited to Pachón cavefish, yet lateral-line or feeding dependent changes have been identified in multiple populations of independently evolved cavefish, including those from the Tinaja and Molino caves (Yoshizawa et al., 2015). It will be of interest to determine whether evolved differences in strike angle and distance identified in cavefish occur in multiple populations of cavefish. In addition, the identification of multimodal regulation of prey capture in *A. mexicanus,* in combination with well-defined visually-mediated prey capture circuits in zebrafish, provide a unique opportunity for examining how sensory processes are integrated. A recently sequenced genome and the application of gene editing and transgenesis techniques in *A. mexicanus* (Elipot et al., 2014; Ma et al., 2015; McGaugh et al., 2014) may allow for the application of genetic tools to investigate neural circuits regulating prey-seeking in *A. mexicanus*. Many of the tools, including methodology for whole-brain Ca^{2+} imaging and Ca^{2+} sensors have been developed in zebrafish using technology that is readily transferable to *A. mexicanus* (Ahrens et al., 2013; Muto et al., 2013). Therefore, our findings identifying multi-modal prey capture in *A. mexicanus* and an evolutionary shift towards lateral line-dependent prey capture in cavefish provide a system for examining the integration of sensory circuits and how these circuits and behaviors evolve.

4. Materials and methods

4.1. Fish rearing and maintenance

Animal husbandry was carried out as previously described (Borowsky, 2008b) and all protocols were approved by the IACUC Florida Atlantic University. Fish were housed in the Florida Atlantic University core facilities at 21 °C ± 1 °C constant water temperature throughout rearing for behavior experiments (Borowsky, 2008b). Lights were kept on a 14:10 h light-dark cycle that remained constant throughout the animal's lifetime. Light intensity was kept between 25

and 40 lx for both rearing and behavior experiments. Adult fish were fed a diet of black worms to satiation twice daily at zeitgeber time (ZT) 2 and ZT12, (Aquatic Foods, Fresno, CA,) and standard flake fish food during periods when fish were not being used for breeding (Tetramine Pro). All fry used for experiments were reared on live *Artemia* through 33 dpf, and fed twice daily.

4.2. Artemia preparation

Approximately 24 h prior to behavioral experiments, Brine shrimp cysts (*Artemia salina,* S.K.) were prepared by adding to a plastic container with 1.2 L of water at a salinity of 25–30 ppt, pH of 7.5–8.5, temperature of 28–30 °C, and constant aeration. Immediately prior to testing, *Artemia* were rinsed with fresh water and placed into recording chambers. Only newly hatched *Artemia* nauplii, of the 1st instar stage, were used in behavioral experiments, to ensure consistency of vibrational stimuli. For experiments using dead brine shrimp, *Artemia* nauplii were flash-frozen, and then thawed by immersion in fresh water immediately prior to behavioral testing.

4.3. High speed recording of prey-seeking behavior

High speed images were acquired using a USB 3.0 camera (Grasshopper3, FLIR Systems) fitted with a zoom lens (75 mm DG Series Fixed Focal Length Lens, Edmund Optics Worldwide), and recorded with FlyCapture2 software (v2.11.3.163, FLIR Systems). All images were acquired at 100 frames per second. Recording chambers were illuminated with custom-designed infrared LED source (Infrared (IR) 850 nm 5050 LED Strip Light, Environmental Lights). All recordings were performed in 29–33 dpf fry from zeitgeber (ZT) 0 to ZT3, shortly after the onset of lights on. For dark recordings, visible light was turned off, but recordings took place at the same time of day. An IR high-pass filter (Edmund Optics Worldwide) was placed between the camera and the lens to block visible light. For larval fish recordings, individual fish were placed in 24 well tissue culture plates (Cellvis) or custom-made chambers, filled with ~3 mm of water to constrict the larvae to a single focal plane. Fish were allowed to acclimate for 2 min prior to the start of the experiment. To record feeding behavior, approximately 30 *Artemia* nauplii were added to each well and fish were imaged for 2 min.

4.4. Quantification of prey capture behavior

Recordings were analyzed using ImageJ (NIH, v.1.51). Chamber diameter was set using ImageJ's native "Set Scale" function, and strike distance and angle were measured for all successful feeding events in the two-minute recordings, using ImageJ's "Line" and "Angle" tools. Measurements of both strike distance and angle were taken in the frame prior to initiation of movement towards the prey. Strike distance was defined as the shortest distance between the edge of the fish's body and the prey (Fig. 1B). Strike angle was defined as the angle between a line extending down the fish's midline, terminating parallel with the pectoral fins, and a line extending from this point to the center of the prey (Fig. 1B). Measurements of each strike were averaged to calculate the mean strike distance and angle for that individual, and any recording with fewer than three feeding events was excluded from analysis.

4.5. Gentamicin ablation of the lateral line

To validate neuromast ablation following gentamicin treatment, fish were treated for 1 h with 0.05% DASPEI (2–4-dimethylaminostyr-yl-N-ethylpyridinium iodide) solution (Sigma Aldrich), a dye that specifically labels both superficial and canal neuromasts (Van Trump et al., 2010). Although the exact mechanism of DASPEI labeling is unknown, the dye is thought to enter cells through transduction

channels and apical endocytosis, allowing its uptake by active hair cells via transduction-dependent mechanisms and making it highly specific for labeling intact neuromasts of the lateral line (Van Trump et al., 2010). After staining, fish were anesthetized and neuromasts were observed using a microscope (Leica M205 FA) set to 40 × magnification, 5.17 mm FOV, with a GFP filter set (excitation 450–490 nm). Photographs were captured with a high-resolution CCD camera (ProgRes C14) with ImagePro software (v.9.1). All images were acquired within ~4 h of the end of baseline behavior recordings. All experimental fish were placed back in their home tanks and given approximately 24 h to recover before any further testing was done. Images represent tiled images merged in Photoshop CS6 (Adobe).

5. Statistics

Two-way ANOVA tests were carried out to test the effects of light level, lateral line ablation, and/or prey mobility among different groups and populations on behavior. Each was modeled as a function of genotype (Surface and Pachón) and genotype by condition interaction. Significance for all tests was set at $p < 0.05$. When the ANOVA test detected significance, Tukey's multiple comparison post-test was carried out to detect differences between individual groups. For comparison of two baseline groups, parametric t-tests were carried out to test for significance. Because surface fish did not strike at prey in some of the conditions (e.g. gentamicin treated fish in the dark), Two-Way ANOVAs were not possible. In these cases, an unpaired t-test was performed to detect differences in the surface fish, while a One-Way ANOVA was performed to detect differences between conditions among Pachón cavefish. All statistical analysis was carried out using InStat software (GraphPad 7.0) or SPSS software 22.0 (IBM).

Competing interests

There are no competing interests associated with this work.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2018.04.027.

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