Behaviors of Cavefish Offer Insight Into Developmental Evolution

MASATO YOSHIZAWA[1,2]*

[1] Department of Biology, University of Nevada, Reno, Nevada
[2] Department of Biology, University of Hawaii, Manoa, Hawaii

SUMMARY

Many developmental processes have evolved through natural selection, yet in only a few cases do we understand if and how a change of developmental process produces a benefit. For example, many studies in evolutionary biology have investigated the developmental mechanisms that lead to novel structures in an animal, but only a few have addressed if these structures actually benefit the animal at the behavioral level of prey hunting and mating. As such, this review discusses an animal's behavior as the integrated functional output of its evolved morphological and physiological traits. Specifically, we focus on recent findings about the blind Mexican cavefish, *Astyanax mexicanus*, for which clear relationships exist between its physical traits and ecosystem. This species includes two morphotypes: an eyed surface dweller versus many conspecific types of blind cave dwellers, some of which evolved independently; all of the blind subtypes derived from eyed surface dwellers. The blind cavefish evolved under clear selection pressures: food is sparse and darkness is perpetual. Simulating the major aspects of a cave ecosystem in the laboratory is relatively easy, so we can use this species to begin resolving the relationships between evolved traits and selection pressures—relationships which are more complex for other animals models. This review discusses the recent advances in cavefish research that have helped us establish some key relationships between morphological evolution and environmental shifts.

> ``Some interesting, classic evolutionary questions could be answered by surveying behavioral and morphological phenotypes in the [cavefish].''

*Corresponding author:
  Department of Biology
  University of Hawaii, Manoa
  2538 McCarthy Mall
  Edmondson Hall, Room 216
  Honolulu, HI 96822.
  E-mail: yoshizaw@hawaii.edu

INTRODUCTION

Evolutionary processes, such as genetic drift and natural selection, can cause organisms' developmental processes to evolve. The evolution of such processes can promote morphological and physiological diversity, as well as help an organism adapt to new environmental challenges and ecological niches (Hall, 1999). To understand in detail how developmental processes evolve, we first must identify the selection pressures and molecular mechanisms that elicit these changes—e.g., evolutionary development integrated with functional ecology (reviewed in Irschick et al., 2013). This multi-disciplinary approach is difficult to achieve for most organisms because the environmental conditions that originally led to their developmental changes—and thereby afforded specific selection advantages—are often ambiguous. A few recent studies have begun to reveal the advantages associated with

Abbreviations: [EO] SN, [eye-orbit] superficial neuromast; *shh*, Sonic Hedgehog; VAB, vibration-attraction behavior

developmental traits, including a feeding advantage for animals with certain jaw morphs or different tooth shapes that depend on an animals' food sources (Wainwright et al., 1995; Albertson et al., 2001; Kocher, 2004; Alfaro et al., 2005; Laffont et al., 2009; Parsons et al., 2009); increased mechanosensors to compensate for animals with poor visual cues (Yoshizawa et al., 2010); as well as conspicuous (Hert, 1989; Couldridge, 2002) and cryptic pigmentation patterns (Klingenberg, 2010; Linnen et al., 2013). Yet, these scientific accomplishments also emphasize the vast knowledge gap regarding how animals evolve adaptive traits as their genomes and developmental processes shift. If we can identify an animal that has evolved under clear and simple selection pressures, however, it will be possible to more directly test the advantages of the developmental changes they have evolved to undergo.

The cave ecosystem is characterized by perpetual darkness, the absence of primary productivity, and sparse food resources (Culver, 1982; Culver et al., 2009). These conditions exert significant pressure on cave-dwelling animals such that cave-adapted animals from most major phyla exhibit a remarkable convergence in morphological and physiological changes related to cave life, including features that are both constructive (longer appendages, novel behaviors, elaborate non-visual sensory systems) and regressive (reduced vision and pigmentation) (Culver, 1982; Culver et al., 2009). Some aquatic cave populations, including teleost, have been isolated from their surface-dwelling relatives for such a long time that they have accumulated cave-specific mutations (for examples, see Protas et al., 2006; Gross et al., 2009; Elipot et al., 2014). In contrast, it is relatively difficult to identify the original mutations and selection pressures influencing surface-dwelling animals because they frequently hybridize with other populations and live in complex fauna. In this respect, cave animals are valuable models that we can use to analyze adaptive changes that occur under clearly defined selection pressures. Behavior is a convenient window through which we can observe the selective advantages associated with morphological and physiological traits. For example, better sensitivity of a sensory organ can improve behavioral responses in predator avoidance, prey hunting, or mating, which lead to enhanced survivorship or fecundity. Natural selection then screens animals with the best combination of traits, depending on their fitness (Mayr, 1963; Bateson, 1988; Wcislo, 1989; West-Eberhard, 1989; Gittleman et al., 1996; Wimberger et al., 1996).

This review discusses recent advances in our understanding of behavioral evolution and related morphological and physiological traits in an animal that lives in a habitat with clear selection pressures: the Mexican cavefish, *Astyanax mexicanus*, an established animal model for studying evolution and development (Mitchell et al., 1977; Wilkens, 1988; Jeffery, 2001, 2008, 2009; Protas et al., 2012; Rétaux et al., 2013). Within the past few million years, at least five independent colonizations by two different migrating waves of eyed-surface-fish ancestors have established 29 geographically isolated Astyanax cavefish populations in northeastern Mexico (Ornelas-García et al., 2008; Bradic et al.,

2012, 2013; Strecker et al., 2012; Coghill et al., 2014). After an initial radiation underground, the founder cavefish populations became isolated and evolved independently. Food sources are limited in these caves, and likely consist of small soil crustaceans, micro-organisms, and organic matter present in the water that drops from the cave ceiling (Culver et al., 2009). Depending on the cave, additional nutrient sources for cavefish may come from the guano dropped by bats living at the cave ceiling and organic matter brought in by seasonal flooding (Culver et al., 2009; personal observation). When the habitat of these fish changed from the surface to the cave, the cavefish ancestor evolved regressed eyes, reduced pigmentation or albinism, enhanced oral-pharyngeal morphologies, and expanded nervous systems (Wilkens, 1988; Yamamoto et al., 2000, 2004, 2009; Jeffery, 2005; Protas et al., 2006; Alunni et al., 2007; Franz-Odendaal et al., 2007; Menuet et al., 2007; Rétaux et al., 2008; Atukorala et al., 2013; Rétaux et al., 2013). Despite their 2−6 million year separation from *A. mexicanus* surface cohorts, the cavefish and surface fish cohorts remain interfertile, allowing us to study the evolution of behavioral, morphological and physiological traits by genetic analysis.

Recent work is revealing the genetic architectures that underlie the behavioral evolution of cavefish species, including the examination of the following behaviors: vibration-attraction behavior (VAB) (Eigenmann, 1909; Hill, 1969; Parzefall, 1983; Yoshizawa et al., 2010, 2012a, 2012b); sleep loss (Duboué et al., 2011, 2012); reduced aggression (Parzefall, 2001; Elipot et al., 2013); loss of schooling and shoaling (Parzefall, 2001; Kowalko et al., 2013a); stabilized feeding posture (Schemmel, 1980; Kowalko et al., 2013b); enhanced chemosensory ability (Protas et al., 2008; Bibliowicz et al., 2013); and reduced circadian rhythms and sleep (Cavallari et al., 2011; Duboué et al., 2011, 2012; Beale et al., 2013). Among these behaviors, VAB is the most-studied, positively selected behavior regarding how it benefits cavefish in their environment, its sensory and genetic basis, and its developmental process. Here, I review the behaviors that have been evolutionarily advantageous and most informative in deciphering how *A. mexicanus* populations have adapted to their caves.

PREY DETECTION AND FORAGING BEHAVIOR
VAB: Adaptive Cave-Associated Foraging Behavior

Cavefish exhibit VAB; that is, they swim toward an oscillating object, either in a natural cave pool or in a laboratory (Fig. 1A) (Table 1). VAB represents a potential foraging behavior that has evolved repeatedly in at least three different Astyanax cavefish populations (Fig. 1B) (Parzefall, 1983; Abdel-Latif et al., 1990; Yoshizawa et al., 2010). Considering these populations likely evolved separately under similar ecological conditions (Mitchell et al., 1977; Borowsky, 2008; Ornelas-García et al., 2008; Wilkens, 2010; Bradic et al., 2012; Gross, 2012; Strecker et al., 2012; Coghill et al., 2014), the convergence
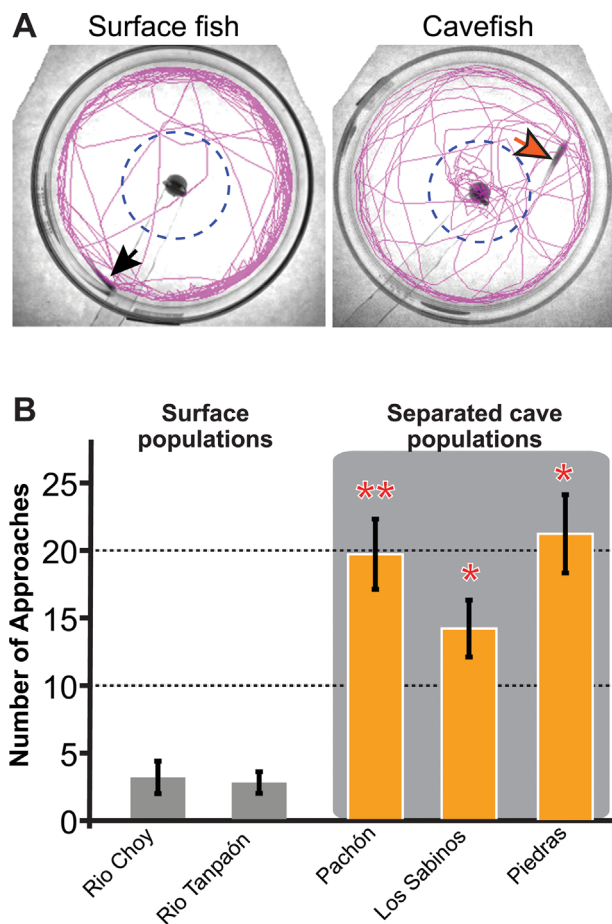
Figure 1. VAB and its repeated evolution. (A) Swimming path (purple lines) of surface fish (left) and Pachón cavefish (right) during a 3-min assay period. Dotted lines represent the 2-cm diameter quantification area surrounding the glass rod (dark spot in the center of the chamber). Arrows indicate the starting positions of the fish. (B) VAB levels measured as number of approaches in *A. mexicanus* populations. Three separated cave populations showed VAB (above the threshold level of 4 approaches), but no VAB was apparent in either surface-fish population. One-tailed Mann-Whitney tests with Bonferroni correction for multiple comparisons were performed between a group of Río Choy and Río Tampaón surface fish, and each cavefish population. *, $P < 0.05$; **, $P < 0.01$. Río Choy surface fish, $n = 4$; Río Tampaón surface fish, $n = 13$; Pachón cavefish, $n = 8$; Los Sabinos cavefish, $n = 9$; and Piedras cavefish, $n = 4$.

of these populations towards VAB suggests the distinct advantage this behavior would have in the cave environment. Yet, the extent of VAB is variable within and among populations, and some individuals of one of the oldest cavefish populations, Pachón, even lack this behavior (Fig. 2A). The converse is true for surface fish: while most surface fish lack VAB, a few individuals exhibit low levels of this behavior (Fig. 2A).

Taking advantage of this variation within populations, the adaptive significance of VAB was tested in competitive prey-capture experiments wherein pairs of fish with and without VAB were fed small amount of vibrating prey: brine

shrimp (Fig. 2B). In darkness, cavefish were significantly better at capturing the brine shrimp than surface fish (Fig. 2Ba). The key finding, however, was that surface fish with VAB had significantly more brine-shrimp captures in the dark than surface fish without VAB, a difference that disappeared in the light (Fig. 2Bb). Thus, an individual's ability to utilize VAB plays a role in foraging and is likely advantageous for survival in dark caves. In wild populations of surface fish, VAB is presumably deleterious because fish with VAB may swim toward predators, such as the nocturnal prawn, at night (Wilson et al., 2004; Yoshizawa et al., 2011). In contrast, VAB is adaptive in cavefish because it increases foraging in an environment with limited food availability, minimal light, and macroscopic predators (Yoshizawa et al., 2010, 2011). Consequently, the VAB already present as a standing variation in ancestral surface populations may have been subject to positive selection once the cavefish ancestors started colonizing caves.

Probing a little deeper reveals hints at how these cave populations have honed VAB to survive. Cavefish VAB occurs at a relatively low frequency range (about 5–50 Hz), with a peak frequency of 35 Hz (Yoshizawa et al., 2010, 2013). These values are within the range that cavefish can detect with their mechanosensory lateral line (20–80 Hz) (Coombs et al., 1989, 2014; Münz, 1989). Interestingly, none of the surface fish, even those with VAB, displayed such behavioral tuning (Yoshizawa et al., 2011). Furthermore, because many crustaceans produce water fluctuations at 30–40 Hz while swimming (Lang, 1980), the tuning of cavefish's VAB at 35 Hz is a novel trait that is likely adaptive in the cave ecosystem.

The cavefish's optimized frequency detection system suggests that the lateral-line sensory organ that cavefish use to detect water flow is likely a vibration-sensory receptor that has been enhanced in cavefish compared to surface fish (Schemmel, 1967; Münz, 1989). One type of lateral-line sensory organ is the superficial neuromast (SN), which is composed of ciliated hair cells and a gelatinous cupular matrix (Fig. 3, see below). The SNs of cavefish are larger and more numerous than those of surface fish, and are primarily responsible for VAB, as determined by SN ablation studies and genetic analysis (Schemmel, 1967; Teyke, 1990; Yoshizawa et al., 2010). Although SNs appear throughout the body and are particularly abundant on the cavefish's head, those SNs located within the orbit of the cavefish's degenerated eye seem to play a particularly important role in VAB (Yoshizawa et al., 2012b). This suggests that the extra cranial space created with the loss of the cavefish's eyes is an important event that promoted this novel behavior. As there was no difference in the number of eye-orbit SNs (EO SNs) among surface fish—in fact, no EO SN was observed in surface fish—their appearance in cavefish did not arise through the selection of a standing phenotypic variation (Yoshizawa et al., 2012b); instead, there must be a distinct evolutionary path linking VAB and EO SN evolution.

Cavefish eyes start degenerating around 36 hr post-fertilization, but no significant increases in any SNs (including EO SNs) are detectable, relative to surface fish, until 2 months

TABLE 1. Morphological/Physiological Bases and Ontogeny of Cavefish Behaviors

Behavior	Function	Morphological/physiological bases	First appearance of behavioral difference	References
Low feeding angle	Increase foraging efficiency (?)	No obvious association with morphological traits including jaw shape and taste bud number	Assayed at 4.5 mpf or older	Kowalko et al. (2013b) and Schemmel (1980)
Vibration attraction behavior (VAB)	Increase foraging efficiency	Lateral line superficial neuromast at the eye orbit	3 mpf, peak at young adult (<2 years old)	Abdel-Latif et al. (1990); Parzefall (1983) and Yoshizawa et al. (2010, 2012a, 2012b, 2014)
Loss of aggressive behavior and its shift to foraging	Increase foraging efficiency (?)	Increased serotonergic neurons at the hypothalamus	Assayed at 3 mpf	Burchards et al. (1985); Elipot et al. (2012) and Espinasa et al. (2005)
Wall-following	Spatial recognition	Lateral line (superficial/canal neuromast, pharmacological inhibition)	Assayed at adult stage. Possibly appears 3–4 mpf	Coombs et al. (2010); de Perera (2004); Hassan (1989); Hassan et al. (1992); Windsor et al. (2008); and Windsor et al. (2010)
Loss of Schooling	No need to avoid predator or group foraging (may be neutral)	Eye and one genetic factor	Assayed at 4.5 mpf or older	Kowalko et al. (2013a) and Parzefall and Fricke (1991)
Loss of male preference	No preference in mate choice (may be neutral)	Visual cue (eye)	Assayed at adult stage	Plath et al. (2006)
Chemosensory response	Increase foraging efficiency	Positively associated with naris size	Unknown: Assayed at adult stage	Bibliowicz et al. (2013) and Protas et al. (2008)
Foraging behavior (?)	Increase foraging efficiency (?)	Increased jaw width/ taste bud number	Unknown: morphological difference at 3 dpf	Varatharasan et al. (2009) and Yamamoto et al. (2009)
Reduced sleep	Increase foraging efficiency (?)	Noradrenergic system (pharmacological inhibition)	Assayed at 21 dpf	Duboué et al. (2011, 2012)
Reduced circadian rhythm	Increase DNA damage repair; Increase foraging efficiency (?); Reduced O_2 consumption	Sustained high expression level of per2 clock repressor; Note: Attenuated circadian rhythm is not enough to reduce O_2 consumption	Assayed at adult stage	Beale et al. (2013); Moran et al. (2014)

Sf, surface fish; Cf, cavefish; mpf, months post fertilization; dpf, days post fertilization; ?, no direct evidence.
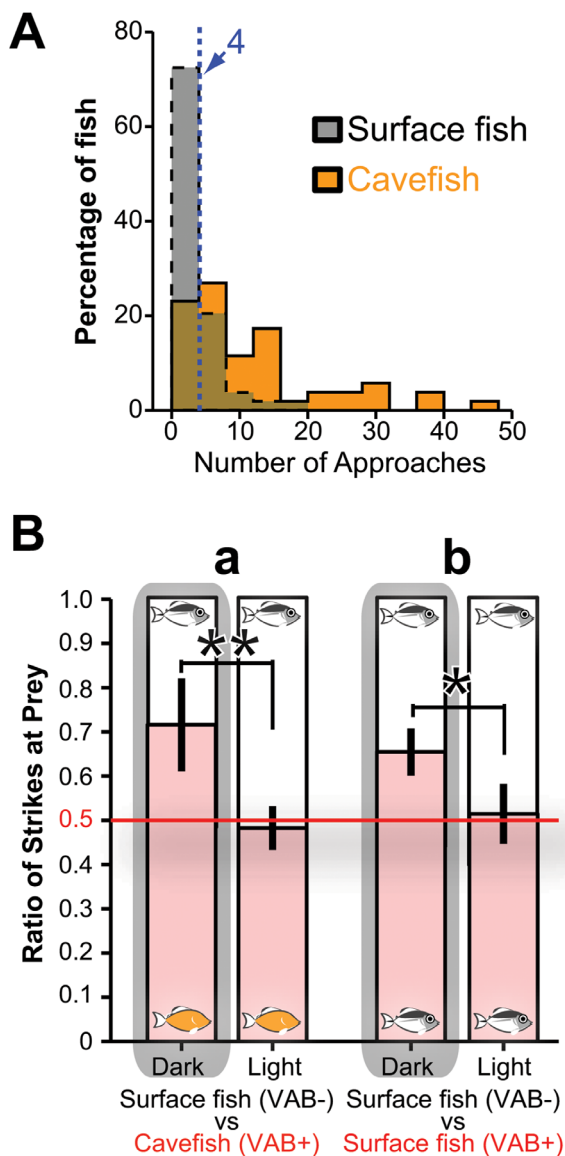
Figure 2. Significance of VAB in feeding, as determined by competitive prey-capture experiments. (A) VAB levels measured as the number of approaches. Surface fish, n = 54 (gray area); cavefish, n = 52 (orange area). Vertical dashed line represents the cut-off value of 4 approaches used to classifying fish with (>4 approaches) and without (<4 approaches) VAB using a stimulus of 50 Hz. (B) Prey-capture competition assays. Bars show the proportion of strikes at prey between pairs of surface fish (black fish cartoons) and cavefish (orange fish cartoons) with or without VAB during a 1-min assay period in darkness (left bars) and in light (right bars). A total of eight pairs of cavefish versus surface fish (Ba), and five pairs of surface fish with versus without VAB (Bb) 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. For details about the method, please see Yoshizawa et al. (2010).

post-fertilization (Yoshizawa et al., 2010). This ontogeny suggests that eye degeneration itself does not induce EO SN formation. Many aspects of eye degeneration are controlled by increased Hedgehog signaling in cavefish (Yama-

moto et al., 2004; Jeffery, 2005), so a surface fish was engineered to overexpress *shh* by mRNA injection so that its eyes would also degenerate to test the relationship between eye retention and EO SN development; these *shh*-overexpressing fish did not develop EO SN (Yoshizawa et al., 2012b). Therefore, neither eye degeneration alone nor excess Hedgehog signaling in early development induces EO SN formation or enlargement; note that tests that ask if Hedgehog signaling plays a role later in development, when EO SNs first appear at 2 months old, have not yet been conducted.

Although there is no shared developmental process between eye degeneration and EO SN formation, ontogenic analysis and a comparison using different cave populations could help establish the relationship between VAB and EO SN evolution. The first approach was to determine how the timing of VAB onset correlates with the development of EO SNs in surface fish versus Pachón cavefish by studying fish at 2, 3, and 4 months post-fertilization and in the young-adult stage (∼1-year-old). Cavefish EO SN quantity gradually increases between 2 months and the first year of age (Fig 3); this temporally correlates with the appearance and enhancement of VAB, which is first detectable between 2 and 3 months post-fertilization (Fig. 3A). Interestingly, another cavefish population, Tinaja, presents with a weak level of VAB, similar to the amount that some individual surface fish display, and does not have EO SNs even though their eyes degenerate (Fig. 3C). This difference further supports the correlation between cavefish VAB and EO SN evolution, and also reinforces that EO SNs are not directly attributable to eye degeneration.

While EO SN ontogeny corresponds with VAB cavefish, there is no correlation between VAB and the total number of SNs in an individual. While Pachón and Tinaja cavefish have significantly more SNs in their infraorbital region than surface fish do (Fig. 3C), the development of these organs is not genetically correlated with the level of VAB, and their ablation does not detectably affect VAB (Yoshizawa et al., 2012b). These observations further emphasize the model that the EO SN, as a minor group of sensory organs, plays an important role in facilitating VAB whereas other SNs in the infraorbital region may contribute to other sensations, such as hydrodynamic imaging (see below, and also Hassan, 1989; Montgomery et al., 2001; Windsor et al., 2008a, 2010a; Coombs et al., 2010). Development of the EO SN may therefore have evolved as a consequence of positive selection for the enhancement of an adaptive behavior, indicating that studying behavioral traits can help reveal distinct roles for SNs residing in different cranial areas.

Considering the importance of the EO SNs in VAB, and thus the cavefish's ability to forage, it is surprising that VAB and EO SNs emerge so late in development. Such timing may occur for two reasons: (1) the developmental process might be constrained or (2) their foraging preference might change with age. To address the first possibility, Wada et al. (2010) investigated SN development in zebrafish. The SNs first form at the edge of a developing intramembranous bone in the cranial region (the operculum, at 4−5 days post-
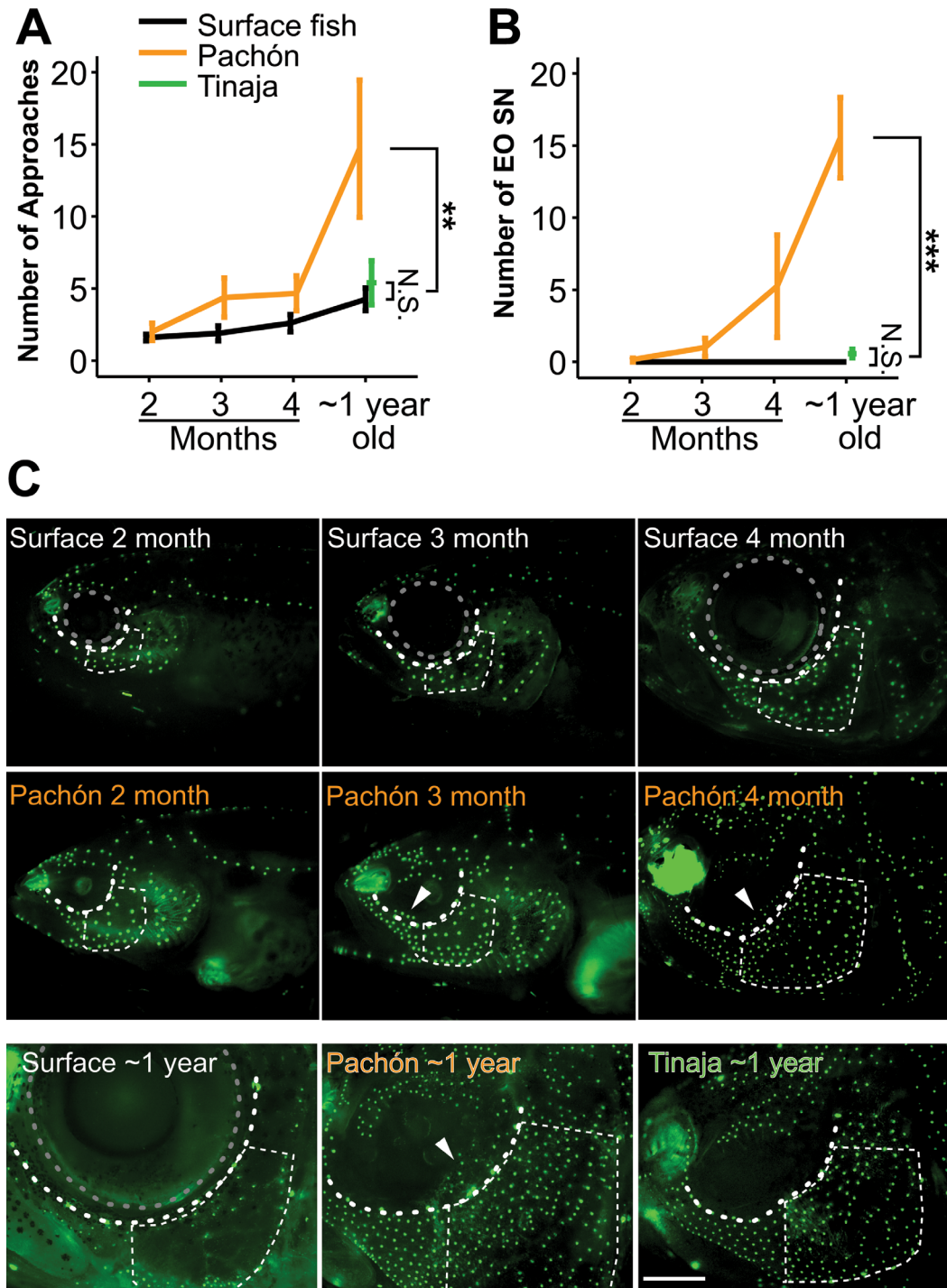
Figure 3. Ontogeny of VAB and EO SNs in surface fish versus Pachón and Tinaja cavefish. (A) VAB ontogeny measured as the number of approaches. (B) EO SN development in surface fish and cavefish. Values are means ± standard errors. Surface fish, n = 10 at 2 months post-fertilization (mpf), = 9 at 3 mpf, = 10 at 4 mpf, and = 12 at ~1-year-old; Pachón cavefish, n = 9 at 2 mpf, = 8 at 3 mpf, = 9 at 4 mpf, and = 11 at ~1-year-old; Tinaja cavefish, n = 7 at ~1-year-old. Pachón cavefish significantly increased VAB and EO SN quantity, whereas Tinaja cavefish were indistinguishable from surface fish in both VAB level and EO SN number at 1 year old (one-way ANOVA followed by planned-contrast analysis at 1 year old). **, $P < 0.01$; ***, $P < 0.001$; N.S., not significant. (C) Example fluorescence images of DASPEI-vital staining of surface fish and two cavefish. Thick, white dotted lines indicate the infraorbital canal, and white arrowheads indicate EO SNs that were counted in (B). Thin, white dotted line encloses the neuromasts in an adjacent region on the cranium, the third infraorbital bone (previously denoted as the third suborbital bone, SO-3) (Yoshizawa et al., 2010, 2012a, 2012b). Grey dotted lines indicate the eyes. Magnification is the same for all frames. Scale bar, 1.0 mm.

fertilization). In Astyanax, however, the second and third infraorbital (suborbital) bones, which underlie the infraorbital SNs and EO SNs, develop in cavefish around 2–3 months post-fertilization (Yamamoto et al., 2003). Therefore, the number of SNs in cavefish may only be able to increase after the infraorbital bone forms, meaning that the development of EO SN—and thereby VAB—is developmentally constrained until the infraorbital bone forms at 2–3 months post-fertilization. The second reason for the late development of VAB and EO SN—that cavefish foraging preferences change over time—derives from the hypothesis that cavefish larvae and adults may have different foraging preferences, possibly to reduce competition with one another. Cavefish adults quickly respond and move toward a water surface that is disturbed by falling water droplets or bat guano (likely via VAB), but they also scavenge for food, perhaps relying on chemosensory inputs originally adapted for bottom feeding. In contrast, relatively small, younger fish scavenge for food exclusively at the bottom, again possibly using chemosensation (Parzefall, 1983; personal and S. Rétaux's observation at the cave Los Sabinos). In the laboratory, however, large cavefish occasionally eat smaller cavefish, suggesting that small, younger fish cannot compete with mature adults. Therefore, the late development of EO SN and VAB may offer small, young fish a better chance to survive by avoiding the hazards of larger, hungry conspecifics that are using VAB to forage just beneath the water's surface.

While it is difficult to determine which of these possibilities—developmental constraints or an ecological advantage—is responsible for the late development of VAB and EO SNs, genetic experiments have helped. By overexpressing or knocking down the gene(s) responsible for EO SN development using available transgenic and/or genome editing methods (e.g., TALEN and CRISPR technologies) (Gaj et al., 2013; Hwang et al., 2013; Elipot et al., 2014; Ma et al., 2015), and performing gene-regulatory-network analysis (Gavin-Smyth et al., 2013), we will gain insight to the relationship between EO SN development and the timing of dermal-bone formation. Deciphering which gene(s) are responsible for VAB will—through in situ hybridization and/or immunohistochemistry techniques —help reveal if the novel sensory tuning occurs at the level of first projection, the octavolateralis nucleus, or higher, such as at the torus semicircularis area. It is unlikely that sensory tuning is achieved at the neuromast level, however, since the sensitivity estimation based on the morphometrics of neuromasts does not peak at 35 Hz (Yoshizawa et al., 2014). Nevertheless, the foraging advantage associated with VAB gives us a new way to resolve the driving forces for the evolution of other morphological traits, including cranial SN, intramembranous bone, and also neural connections in the central nervous system.

Evolution of Foraging-Related Traits Through the Pleiotropy of Hedgehog Signaling

The pleiotropic effects of enhanced Hedgehog signaling promote eye degeneration as well as increases in jaw size and number of taste buds in Astyanax cavefish (Table 1) (Yamamoto et al., 2004, 2009; Jeffery, 2005). Given the necessary redistribution of attention to the non-visual senses, selection for these other constructive traits, which are fostered by Hedgehog signaling, during cavefish evolution may have accelerated eye loss as a developmental tradeoff. No one has tested if these gustatory traits (larger jaws, more taste buds) improve cavefish's foraging abilities. Nevertheless, an enlarged jaw is likely beneficial in the darkness, possibly as an adaptation to bottom feeding, and carrying more taste buds likely improves foraging because they could help navigate to food in the dark. To understand the adaptive significance of these morphological and sensory shifts, however, we must perform behavioral tests, such as food-competition experiments. For example, behavioral analyses showing that these gustatory traits are advantageous would support the hypothesis that oral-pharyngeal shifts can promote eye reduction through pleiotropy. If having a large jaw and more taste buds does not enhance foraging efficiency, however, these traits would constitute another example of "not all evolved traits are adaptive" (see below).

Hedgehog signaling is also required for other critical developmental processes, beyond cavefish eye degeneration and a few gustatory traits. During development, enhanced Hedgehog signaling increases the number of migratory cells that enter the olfactory bulbs (Menuet et al., 2007) and serotonergic neurons at the hypothalamus (Elipot, 2013). While the former may represent a positive behavioral response linked to the chemical stimulus of food (Table 1) (Bibliowicz et al., 2013), the latter is an example of a change in behavioral "strategy." For instance, having an enhanced serotonergic system in the anterior hypothalamus and the raphe nucleus redirects attacking behavior towards foraging behavior. Attacking behavior is frequently used to establish a hierarchical relationship between individuals in surface dwelling fish (Magurran, 1993; Elipot et al., 2013), but this does not occur in cave populations, possibly because A. mexicanus cavefish lack schooling behavior (Elipot et al., 2013; Kowalko et al., 2013b; see below). Indeed, cavefish have higher serotonin levels and more serotonergic neurons at the hypothalamic anterior paraventricular nucleus and hindbrain raphe than their surface counterparts; these anatomical and endocrine changes are believed to have shifted cavefish behavior from attacking to foraging (Elipot et al., 2013). Another study reported that cavefish have higher serotonin levels in the brain because of mutations in the serotonin degradation enzyme, monoamine oxidase (mao)—which again promotes foraging behavior (Table 1) (Elipot et al., 2014). The cavefish's heightened foraging is likely adaptive to the cave environment, where there is little food and few predators: fish eat on a "first come, first served" basis. In fact, enhanced foraging behavior has converged in independently-evolved cave populations (Elipot et al., 2013).

Yet even though cavefish have higher numbers of serotonergic neurons in their hypothalamus by 1 week of age (Elipot et al., 2013), there is no obvious difference in attacking behavior between cavefish and surface fish at

that age (personal observation). This may be because fish at this young age are too immature to establish hierarchical positions, or because some unknown benefit exists for having an enhanced serotonergic system in the larval stage. Overall, if we could better understand the difference in the "aggression" neural circuits between cavefish and surface fish, and their relationship with the number of serotoninergic cells in 1-week-old juveniles, we would gain much-needed insight to the evolution of cavefish's foraging behavior. In general, this behavioral shift in cavefish from attacking to foraging provides the first example of a likely behavioral advantage underlying their enhanced Hedgehog signaling, whose trade-off is eye development (c.f. Yamamoto et al., 2009).

Evolution of Foraging Behavior Without Obvious Morphological Shifts

Many behaviors have evolved in the company of morphological changes, yet behavior can also evolve without obvious anatomical adaptations—e.g., feeding posture. When in the dark, surface fish feed at a steep angle of $\sim 90°$ relative to a substrate. In contrast, multiple cave populations feed at a much shallower angle of $\sim 45°$ (Table 1) (Schemmel, 1980). Although no one has yet determined how low-angle feeding is advantageous in the dark (though this could be demonstrated in a competition assay), three *A. mexicanus* cave populations (Pachón, Tinaja, and Los Sabinos) (Kowalko et al., 2013b and personal observation) and other benthic feeders (for example, see Ferry-Graham et al., 2002) all perform low-angle feeding, suggesting that this behavior may be advantageous for foraging at the bottom of caves. Since jaw morphology is frequently associated with feeding (Wainwright et al., 1995; Albertson et al., 2001; Kocher, 2004; Alfaro et al., 2005; Parsons et al., 2009), it was surprising when a set of studies revealed no detectable correlation between feeding angle and eight cranio-facial phenotypes in an F_2 intercross derived from a surface fish mated to a cavefish (Kowalko et al., 2013b). The genetics therefore suggests that a cavefish's low feeding angle represents an exclusively behavioral adaptation—potentially through changes in motor control—that required no accompanying morphological changes.

The convergent evolution of a cavefish's feeding posture has also been supported by genomic evidence (Kowalko et al., 2013a). Using quantitative trait loci (QTL) mapping of feeding posture (a potentially adaptive behavior) and eye degeneration in two independently evolved cavefish populations, Kowalko et al reported distinct QTL results for different genes in different populations, suggesting that many cavefish traits evolved by *de novo* mutation rather than by selection from standing genetic variation. At the single-feeding-angle QTL detected in one cavefish population (Tinaja), the "cave" allele actually worked to increase the feeding angle, which was unexpected and indicates there are still undetected QTLs (Table 1) (Kowalko et al., 2013b). Importantly, a different cave population (Pachón) showed two feeding-posture QTLs, and at both of them the

"cave" alleles worked to reduce the feeding angle, suggesting that different genes were utilized during the evolution of feeding behavior among independent cave populations (Kowalko et al., 2013a).

So how did cavefish converge onto their feeding angle? Surface fish readily stabilize their feeding angle at a $45°$ angle in lighted conditions, so cavefish may have evolved their posture control without visual cues, instead relying on or modifying the vestibular posture control centers. This is supported by observations at the larval stage, when cavefish and surface fish feed similarly. Instead of feeding at either $45°$ or $90°$, both larval types bob along the bottom of the tank and attack sunken food, trying out different feeding angles during each attempt. Since this larval feeding style is somewhat similar to adult surface fish, it would be interesting to investigate the ontogeny of neural wiring in the basal ganglia, cerebellum, pontine, and vestibular systems—which together control posture—and how this wiring is associated with feeding angle in cavefish. In addition, future competition assays should test if a lower feeding angle is advantageous in the dark, which will provide insight into the selection pressure for this behavior.

SOCIAL INTERACTION

Foraging is not the only behavior to have evolved in cavefish; they also show differences from surface fish in their social interactions, one of which is schooling. Schooling has multiple benefits, including helping fish avoid predators and foraging, but these benefits may not be valid in caves, which have limited food and few predators. Indeed, the loss of schooling depends on the loss of visual sensing and on a non-visually-related genetic factor (Kowalko et al., 2013a). Yet, the same genetic factor encoded at this allele actually promotes schooling in cavefish compared to surface fish (Table 1) (Kowalko et al., 2013a). Such a finding suggests that the absence of cavefish schooling is mainly due to eye loss, which is supported by ablation of the lateral-line sensory system. Although it was once suggested that the lateral line controls schooling behavior (Partridge et al., 1980), ablating the lateral line didn't affect schooling in either surface fish or cavefish (Kowalko et al., 2013a). Therefore loss of schooling behavior may simply be a consequence of eye loss—which could be beneficial, as opposed to deleterious, because being solitary may provide a better chance to find the rare food sources. A test of food-finding ability using eyed, schooling individuals and eyed, non-schooling individuals—e.g., those that Kowalko et al. (2013a) isolated among F_2 hybrids from a surface fish × cavefish cross—should reveal an advantage for the loss of schooling under sparse-food condition.

Mating preferences cover another social behavior. Female surface fish prefer to mate with large males; in dark environments, though, this preference disappears, suggesting that mate preference depends on visual cues (Table 1) (Plath et al., 2006). Yet even in the dark, two out of six cavefish populations evolved a preference for large males (Micos and Yerbaniz; others are Molino,

Pachón, Piedras, and Curva cavefish), suggesting the evolution of a non-visual mate preference (Plath et al., 2006). Such a preference could be a consequence of adaptations and reliance on the mechanosensory lateral-line system, which can sense the vortices produced by fish (Bleckmann et al., 1991). It would therefore be interesting to investigate if the frequency stimulus of vortices produced by large males ($\sim$100 Hz) (Bleckmann et al., 1991) attracts females and stimulates their release of gonadotropin releasing hormone (GnRH) to initiate reproductive behavior (Hofmann, 2006). Such an investigation could then explore how the reproduction process adapted after cavefish were no longer able to use visual sensory inputs.

NAVIGATION WITHOUT VISUAL INFORMATION

Another behavior cavefish exhibit is wall-following, which is thought to function in space recognition and avoiding collisions with cave walls (Table 1) (Hassan 1989; De Perera, 2004; Windsor et al., 2008, 2010a, 2010b; Coombs et al., 2010). This behavior has evolved in all Astyanax cavefish populations, and likely helps them navigate in the absence of visual cues. As mentioned, the cavefish's lateral-line system senses hydrodynamic changes in the flow field of water caused by objects in the cave (Hassan 1989, 1992; Coombs et al., 2010). The ontogeny of this behavior is not clear, although it seems to appear by 3−4 months post-fertilization (personal observations). Because numbers of infraorbital SNs significantly increase after 2 months post-fertilization (Yoshizawa et al., 2010), wall-following behavior may largely depend on this sensory organ. Furthermore, wall-following behavior may be tightly associated with the ability to recognize and navigate spaces in the absence of visual cues (De Perera, 2004; Holbrook et al., 2009). The part of the nervous system most likely involved in space recognition is at a region homologous to the hippocampus, in the lateral part of the dorsalis of the telencephalon, where spatial memories form (Lo et al., 2002). It would therefore be interesting to find out how cavefish establish spatial memory that is only based on temporal information from hydrodynamic stimuli.

ON THE EVOLUTION OF BEHAVIOR

For the most part, cave animals adeptly confront the challenges of sparse food and perpetual darkness. Consequently, many researchers have associated cave animals' traits with these selection pressures *without actually testing* the advantages of these evolved traits, thereby leading to misunderstanding or an overemphasis of the significance of the evolution of these traits. For example, the significant increase of infraorbital SN was first predicted as the receptor for adaptive VAB, but the minor sensory population of EO SNs turned out to be the major receptors for VAB (Yoshizawa et al., 2010, 2012b). This case study reminds us to consider a classic criticism of the adaptive program: not all evolved traits are adaptive (Gould et al., 1979). The

A. mexicanus cavefish system makes it easy to avoid such pitfalls by permitting laboratory studies in simulated cave environments, which are easy to replicate with dark and food-deprived conditions.

Some interesting, classic evolutionary questions could be answered by surveying behavioral and morphological phenotypes in the *A. mexicanus* system, such as: Do behavioral adaptations emerge before or after morphological changes? Since behavior can be drastically modified by a simple change—such as a hormone concentration in the central nervous system (Kobayashi et al., 1999)—the evolution of behaviors has been assumed to precede morphological and sensory evolution (reviewed in West-Eberhard, 1989). Yet, multiple studies in the cockroach, moth, silkworm, and mouse suggest that changes in the expression level of some sensory receptors is enough to trigger adaptive shifts of behaviors (Jacobs et al., 2007; Sakurai et al., 2011; Leary et al., 2012; Wada-Katsumata et al., 2013). In *A. mexicanus*, there are complex trends among cave-associated behaviors. A few behavioral traits, including VAB and stabilized feeding angle, may have preceded morphological or sensory evolution because there are standing phenotypic variations of these behaviors among the cavefish and their related surface-dwelling cohorts. Thus, during the initial steps of the adaptation process, individual cavefish ancestors that expressed these cave-associated behaviors were at a selective advantage because they did not require extreme morphological/sensory changes to adjust to the cave enviroment (Yoshizawa et al., 2010, 2011, 2012; Kowalko et al., 2013). In contrast, cavefish behaviors that require eye regression, including loss of schooling (Kowalko et al., 2013) and wall-following (personal observation of eye-ablated surface fish), may have emerged after the morphological loss of the eye. Furthermore, loss of pigment, another morphological trait, may have also induced behavioral changes, including higher locomotor activity and/or sleep loss, because a null mutation of the oculocutaneous albinism II (*oca2*) gene increases the production of dopamine and noradrenalin, two neurotransmitters that affect sleep and locomotor behaviors (Duboué et al., 2011, 2012; Bilandžija et al., 2013). Oca2 could therefore work in a pleiotropic manner at the morphological and behavioral level. In addition, well-known pleiotropic Hedgehog signaling controls both morphology/sensory system (eye regression, widening jaw, increase of taste buds) and behavior (behavioral shift from aggression to foraging via increase of serotonergic neurons in the hypothalamus) (Yamamoto et al., 2004, 2009; Elipot et al., 2013). Thus, traits controlled by pleiotropic genes could have evolved concurrently as a result of the mutation of these genes.

In summary, the cavefish serves as an excellent model to study the evolution of multiple morphological and behavioral traits because it has provided evidence that morphological and behavioral traits evolved through complex manners. The latest *A. mexicanus* behaviors studied include: enhanced prey capture ability in 25-day-old cavefish in the dark (Espinasa et al., 2014); feeding control via appetite-related hormones (Penney et al., 2014); and loss

of circadian rhythm and sleep (Duboué et al., 2011, 2012; Beale et al., 2013; Moran et al., 2014; Yoshizawa et al., 2015). The next frontier in cavefish research will be to identify more of the genes and mutations involved in the adaptation to the cave environment, thereby establishing a field where genetics, ontogeny, neuroscience, phylogeny, and ecology are integrated.

The recent development of powerful tools has led to a wealth of important information we can use to unravel evolutionary mysteries, including available genome sequences (AstMex102 from the Ensembl genome browser, at www.ensembl.org); available embryology techniques that can modify gene expression (Yamamoto et al., 2000, 2004, 2009; Gross et al., 2009; Bilandžija et al., 2013); defined embryonic and larval stages (Hinaux et al., 2011); transcriptome datasets (Gross et al., 2013; Hinaux et al., 2013); and defined evolutionary relationships among populations (Ornelas-García et al., 2008; Bradic et al., 2012; Gross, 2012; Bradic et al., 2013). Further advances in transgenic capabilities (Elipot et al., 2014) and genomic engineering methods, such as TALEN and CRISPR technologies (Gaj et al., 2013; Hwang et al., 2013), allow us to perform more directed genetic studies. Such technology is helping to fill the technical gap between the *A. mexicanus* system—with its clear selection pressures and many cave-adapted traits—and other model animal systems—with their historical knowledge base. With new information and better experimental techniques, we can further exploit *A. mexicanus* as an evolutionary vertebrate model, which will ultimately allow us to comprehensively understand the evolutionary processes through which genomic and developmental shifts produce enhanced or co-opted adaptive traits.

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